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COVID-19

Clinical Advances and Challenges

Edited by
Shitij Arora and Leonidas Palaiodimos

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COVID-19: Clinical Advances and Challenges

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Editorial

COVID-19 Therapeutics: Improvise—Adapt—Learn

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"In the midst of chaos, there is also opportunity"—Sun Tzu, The Art of War

The protean nature of the COVID-19 pandemic has necessitated unprecedented global coordination, cooperation, and ingenuity. Eleven variants of SARS CoV-2 and a multitude of subvariants have been identified [1], over half a billion humans have been infected, and more than 6 million people have perished [2]. Mirroring the meta-morphology of the virus, the medical and scientific community has proven to be extremely versatile, pushing the bounds of translational medicine further than ever before. Therapeutics, vaccines, and an explosion of data were generated, but perhaps the most impactful sequela has been learning how to produce meaningful research in shorter and shorter spans of time.

The pressing need for results, as well as inherent public and governmental pressure during a pandemic, stressed the integrity of the scientific process and required unconventional methods to work so many lines of inquiry in parallel. Finances and timelines factor heavily into therapeutic development, and repurposing existing medications for immediate use became an attractive option, as it is both cost-effective and time-saving [3]. Many clinicians will remember the state of COVID-19 research early on in the pandemic, which evolved from individual clinicians posting their experiences on Twitter, advancing to small, preliminary, and sometimes rushed clinical trials, and finally progressing to larger, more rigorous studies. Perhaps the largest and best tool utilized to answer these pressing clinical questions while striving to maintain intellectual fidelity was the adaptive platform trial [4]. Utilized in the RECOVERY [5], SOLIDARITY [6], and TOGETHER [7] trials (among others), this study type randomly assigns patients with a single disease to a group of carefully selected therapies of interest on the basis of a decision algorithm to determine whether they confer any significant benefit.

The RECOVERY trial is a seminal study establishing the benefit of dexamethasone, tocilizumab, and monoclonal antibody combination casirivimab/imdevimab, while finding no benefit in outcomes when administering aspirin, azithromycin, colchicine, convalescent plasma, lopinavir/ritonavir, and hydroxychloroquine. The study's rapid design, enrollment, study size, and implementation are all testaments to a new phase in human research, marked by a globalist spirit and advanced logistical cooperation. However, this clinical trial is also a case study of the pitfalls of 'stressed' research. While perhaps unavoidable under the circumstances, the study used the same control group when comparing each of the intervention groups, the patients were not randomized within individual hospitals, and the data were unblinded to a data monitoring committee that performed five interim analyses.

In spite of the pressure brought to bear for immediate results, a large volume of literature was produced at the outset of the pandemic that withstood the test of time, was implemented in a timely manner, and potentially prevented a significant amount of morbidity and mortality. High-quality systematic reviews, observational studies, and

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meta-analyses were all utilized to great effect, resulting in protocols such as the one regarding steroid use implemented in our institutions near the advent of the pandemic [8], many of which were included in our very own special issue of COVID-19 Therapeutics. Additionally, open registries have proliferated with significant international buy-in, leading to collaboration that has turned COVID-19 research into a multidisciplinary venture [9–11]. These new, robust platforms have the profound potential to be applied broadly to emerging pathogens as well as other public health crises, creating an academic ecosystem where not only are data generated and shared more efficiently, but the collaborations to process and apply that information are already in place as well.

Public opinion has played an outsized role in shaping COVID-19 research and policy, as evidenced by the lopsided distribution of clinical studies dedicated to different therapeutics [12]. It is reasonable to attribute the heightened interest and investment into drugs such as hydroxychloroquine and ivermectin as a product of political and popular promotion generated by spurious results from early small and methodologically concerning studies [13,14]. Great care is required to strike a balance between heeding the needs of the general public whom we serve and shielding ourselves from the pressure they bring to bear.

An important question to ask is how our experiences have informed how we will approach the next pandemic. A vital first lesson is: do not rush. Fast science can be very bad science. We have created an unprecedented level of data sharing, and while the scale of the next pandemic may not be to the degree of COVID-19, the existing lines of communication should be maintained to more efficiently knit together small randomized-controlled trials, quality observational studies that utilize an array of statistical analyses in an effort to minimize the potential for confounding, or systematic reviews into more robust and higher-powered conclusions earlier on. The continued utilization of adaptable clinical trial models will certainly change the flexibility and scope of future inquiry.

The fruit of mankind's collective scientific labors, however, is staggering. One modeling study projected that COVID-19 vaccination may have prevented 27 million SARS-CoV-2 infections, 1.6 million COVID-19–associated hospitalizations, and 235,000 COVID-19–associated deaths through September of 2021 [15] in the US; another study puts the number of deaths prevented worldwide at a whopping 19.8 million [16]. More than 5.33 billion people worldwide [17], nearly 70% of Earth's inhabitants, are estimated to have been vaccinated to date, and despite the cycling of new, highly infectious variants, we have undoubtedly turned a corner in facing this pandemic and perhaps even closed a chapter. A generation of physicians has been tempered in the crucible of a pandemic and, in the process, have discovered the power of the individual to make a difference on a global scale. The imperative to investigate and research has been extended more solidly to the rank-and-file clinician through adaptive trial platforms, mRNA vaccines have proven their efficacy, and global registries have begun to change the way we approach data collection and processing. COVID-19 has proven to be a chameleonic adversary, but humanity has proven to be just as adaptable and, in the process, gained vital lessons to take into the future.

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Article

Ciclesonide Inhaler Treatment for Mild-to-Moderate COVID-19: A Randomized, Open-Label, Phase 2 Trial

Joon-Young Song [1], Jin-Gu Yoon [1], Yu-Bin Seo [2], Jacob Lee [2], Joong-Sik Eom [3], Jin-Soo Lee [4], Won-Suk Choi [5], Eun-Young Lee [6], Young-Ah Choi [7], Hak-Jun Hyun [1], Hye Seong [1], Ji-Yun Noh [1], Hee-Jin Cheong [1] and Woo-Joo Kim [1,*]

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Abstract: Although some intravenous drugs have been used to treat coronavirus disease 2019 (COVID-19), no effective antiviral agents are currently available in the outpatient setting. We aimed to evaluate the efficacy and adverse events of 14-day ciclesonide treatment vs. standard care for patients with mild-to-moderate COVID-19. A randomized, open-label, multicenter clinical trial of ciclesonide inhalers was conducted in patients with mild-to-moderate COVID-19. Patients were enrolled within 3 days of diagnosis or within 7 days from symptom onset and randomly assigned to receive either ciclesonide (320 μg inhalation twice per day for 14 days) or standard care. The primary endpoint was the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) eradication rate on day 14 from study enrollment. Clinical status was assessed once daily, and serial nasopharyngeal viral load was evaluated by quantitative reverse transcription polymerase chain reaction. There were 35 and 26 patients in the ciclesonide and standard care groups, respectively. The SARS-CoV-2 eradication rate at day 14 was significantly higher in the ciclesonide group ($p = 0.021$). In multivariate analysis, SARS-CoV-2 negative conversion within 14 days was 12 times more likely in the ciclesonide group (95% confidence interval, 1.187–125.240). Additionally, the clinical failure rate (high-flow nasal oxygen therapy or mechanical ventilation) was significantly lower in the ciclesonide group ($p = 0.034$). In conclusion, ciclesonide inhalation shortened SARS-CoV-2 viral shedding duration, and it may inhibit the progression to acute respiratory failure in patients with mild-to-moderate COVID-19. Clinical Trial Registration NCT04330586.

Keywords: COVID-19; SARS-CoV-2; ciclesonide; inhalation; antiviral agents

1. Introduction

Coronavirus disease 2019 (COVID-19) presents several innate challenges, including insidious symptom onset, subclinical manifestations, and highly transmissible properties during the early stage of infection [1]. Thus, despite high-level public health interventions,

COVID-19 has spread worldwide and has persisted since its first emergence in late December 2019. Cumulatively, more than 183 million people globally have been diagnosed with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, resulting in over 3.9 million deaths as of 5 July 2021 [2].

Antiviral drugs are used to improve clinical symptoms and ameliorate disease severity. Additionally, they have important clinical implications for suppressing disease transmission by reducing viral shedding duration. The development of antiviral drugs and repurposing of existing drugs are of great interest owing to limitations regarding compliance, inconvenience, and effectiveness of conventional public health measures such as wearing of mask, hand hygiene, and strengthened social distancing. Early effective antiviral therapy shortly after symptom onset may reduce viral shedding, thereby decreasing disease transmission. Hydroxychloroquine (HCQ), ritonavir-boosted lopinavir (lopinavir/r), and remdesivir have been investigated as drugs repurposed for the treatment of COVID-19 [3]. In the early stages of the COVID-19 pandemic, HCQ and lopinavir/r were expected to be effective repurposed drugs [4]. HCQ blocks endosomal acidification and inhibits viral uncoating, thereby inhibiting viral proliferation, while lopinavir/r is a protease inhibitor that inhibits enzymes that process 16 nonstructural proteins (NSPs) required for viral replication [4]. However, clinical trials of both these drugs (HCQ and lopinavir/r) have yielded disappointing results [5–7]. In a randomized clinical trial, intravenous administration of remdesivir, a polymerase inhibitor, significantly shortened the time to clinical recovery by 5 days but did not decrease the mortality rate [8]. Moreover, the currently available therapies for COVID-19 are injectables; thus, it is difficult to use them for patients with mild COVID-19 in outpatient clinics.

In comparison, ciclesonide (Alvesco®) is an inhaled steroid agent, which has been used to treat asthma. Although the mechanism is not yet clear, ciclesonide is presumed to exert antiviral effects by acting on the NSPs of SARS-CoV-2 [9]. Thus, ciclesonide is expected to have a dual effect (antiviral and anti-inflammatory effects) in the treatment of COVID-19. In case series reports from Japan, clinical symptoms and oxygen saturation improved when ciclesonide was administered to patients with COVID-19 pneumonia [10,11]. Based on favorable results from retrospective studies, randomized clinical trials have been conducted to evaluate the clinical efficacy of ciclesonide treatment for COVID-19 [12,13].

To evaluate the efficacy and adverse events of 14-day ciclesonide treatment vs. standard care for patients with mild-to-moderate COVID-19, we conducted a phase 2 randomized, open-label, multicenter study.

2. Materials and Methods

2.1. Study Design

This randomized, open-label, multicenter clinical trial was conducted in six hospitals in South Korea from 8 May 2020 to 31 March 2021 (Clinical Trial Number—NCT04330586). Clade GH SARS-CoV-2 circulated dominantly (>90%) in South Korea during study periods. Patients (aged $\geq$19 years) with mild-to-moderate COVID-19, confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR), were enrolled in the study within 3 days of diagnosis or within 7 days from symptom onset. Patients were eligible for the trial if they had a low National Early Warning Score (NEWS) ranging from 0 to 4. NEWS is a scoring system based on routine physiological parameters (respiratory rate, oxygen saturation, supplemental oxygen, body temperature, systolic blood pressure, heart rate, and level of consciousness), which can be obtained easily at the bedside. For each parameter, a score of zero is considered normal, and simple addition allows a total score from 0 to 20. A score of $\geq$5 represents the key threshold for urgent response, and patients with a score of $\geq$7 would be deemed to have a high-risk clinical condition requiring emergency response. Exclusion criteria included oxygen saturation <95% breathing room air, pregnancy or breastfeeding, renal impairment (estimated creatinine clearance <30 mL/min), hepatic dysfunction (alanine aminotransferase or aspartate aminotransferase levels more than five times the upper limit of normal), immunocompromising conditions, severe uncontrolled

comorbidities, chronic airway diseases (asthma and chronic obstructive lung disease), and contraindications for use of ciclesonide inhaler.

Eligible patients were randomly assigned in a 1:1:1 ratio to receive ciclesonide (320 μg inhalation twice per day for 14 days), ciclesonide-HCQ (320 μg inhalation twice per day for 14 days/400 mg daily for 10 days), or standard care. Expecting the synergistic or additive effect of ciclesonide and HCQ, the ciclesonide-HCQ combination was included in the comparison group. However, as data indicating that HCQ is not effective were published, the study design was altered to randomly assign patients to either ciclesonide or standard care groups. Thus, in the analyses, the ciclesonide-HCQ combination group was included in the ciclesonide group. Standard care comprised intravenous fluid, supplementary oxygen, and antibiotics, as necessary. The randomization was performed by computer-generated variable blocks ranging from 4 to 8 patients per each center, and the code numbers for eligible patients were assigned in ascending sequential order. Investigators of each hospital directly trained patients about the inhalation technique, providing educational materials to the patients. Even if symptoms improved, ciclesonide inhalation was maintained for 14 days.

The study was approved by the Institutional Review Board (IRB) of each participating hospital: Korea University Guro Hospital (2020GR0145), Hallym University Kangnam Sacred Heart Hospital (HKS2020-04-012), Gachon Gil Medical Center (GCIRB2020-152), Inha University Hospital (2020-04-023), Korea University Ansan Hospital (2020AS0085), Korea Cancer Center Hospital (KIRAMS 2020-04-002-002), and Seoul Metropolitan Seobuk Hospital (2020GR0145). In Seoul Metropolitan Seobuk Hospital, the study was conducted under the supervision of Korea University Guro Hospital IRB. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice, and all participants provided written informed consent prior to enrollment.

2.2. Clinical and Laboratory Monitoring

Enrolled patients were assessed once daily by the study investigators regarding symptoms and drug-related adverse events. Oxygen saturation was measured daily, and chest X-ray was taken weekly on day 1, 7 and 14. Serial nasopharyngeal samples were obtained on day 1 (before ciclesonide inhalation) and on days 4, 7, 10, and 14 for qRT-PCR until discharge. In addition, on day 3 (after inhalation of 320 μg ciclesonide four times) and day 4 (after inhalation of 320 μg ciclesonide six times) of study enrollment, saliva samples were collected from three study centers. The viral load (cyclic threshold (Ct) value) of SARS-CoV-2 from saliva was evaluated by qRT-PCR, and these were compared with the standard care control group. In each hospital, qRT-PCR tests for SARS-CoV-2 were conducted using test kits approved by the Korean Ministry of Food and Drug Safety, including Allplex™ 2019-nCoV Assay kit (Seegene, Seoul, Korea) and PowerCheck™ 2019-nCoV RT-PCR kit (KogeneBiotech, Seoul, Korea). Ct values of the RNA-dependent RNA polymerase (RdRp) gene were used for the assessment of viral load change. Clinical data were recorded in an electronic database and validated by the trial staff.

2.3. Outcome Measures

The primary endpoint was the SARS-CoV-2 eradication rate based on qRT-PCR on day 14 of study enrollment. SARS-CoV-2 eradication was defined as negative conversion of two consecutive negative results of qRT-PCR. Secondary endpoints were as follows: SARS-CoV-2 eradication rate based on qRT-PCR at days 7 and 10 from study enrollment; rate of clinical improvement (resolution of all systemic and respiratory symptoms) at days 7, 10, and 14 from study enrollment; rate of clinical failure within 28 days; safety/tolerability of ciclesonide. Clinical failure was defined as the case of clinical deterioration requiring high-flow nasal oxygen or mechanical ventilation, resulting in salvage treatment with dexamethasone and remdesivir.

2.4. Statistical Analysis

The original study sample size was estimated at 60, assuming that the virus eradication rate on day 14 after study enrollment would be 75% for the ciclesonide treatment group and 40% for the standard care group based on our previous clinical experience. This size sample would provide at least 80% power to detect a between-group difference at a two-sided significance level of $\alpha = 0.05$. Considering the 10% dropout rate, 68 patients (34 per group) would be required.

Outcome analyses were conducted on an intention-to-treat basis, which included all patients who had undergone randomization. All ciclesonide-treated patients included in the analysis completed treatment by day 14 after enrollment. However, we excluded patients who withdrew consent, transferred to other hospitals within 7 days, and violated eligibility criteria. All statistical analyses were performed using SPSS (version 20.0; IBM Corp., Armonk, NY, USA). For categorical variables, univariate analysis was performed using either the chi-square test or Fisher's exact test. Student's *t*-test was used to compare continuous variables between the two groups and was expressed as median (interquartile range, IQR) or mean (standard deviation, SD). Statistical significance was set at $p < 0.05$. Multivariate analysis was performed to assess the independent contribution (odds ratio) of ciclesonide treatment for each clinical outcome using a logistic regression model; age, sex, underlying medical conditions, accompanying pneumonia, and Ct value at enrollment were adjusted.

3. Results

Among 68 patients who underwent randomization, seven patients were excluded from the analyses because of issues with eligibility criteria (two patients), withdrawal of consent (three patients), or transfer to other hospitals within 3 days after study enrollment (two patients) (Figure 1). Among 61 patients in the analysis set, 35 patients were assigned to the ciclesonide group and 26 patients to the standard care group; eight patients in the ciclesonide group received oral HCQ treatment concomitantly for 10 days.

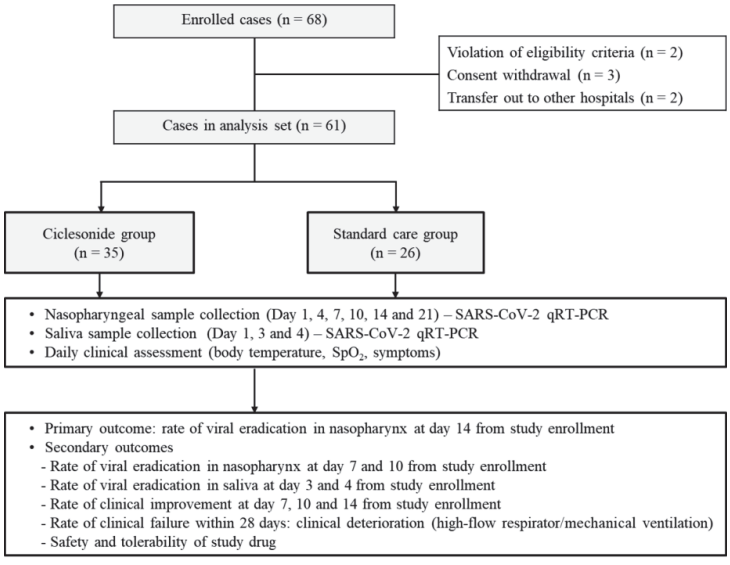

Figure 1. Study flowchart: randomization and treatment assignment.

Patients' median age was 53 (IQR, 35–61) years, and 47% were men (Table 1). The median interval from symptom onset to enrollment was 3 (IQR, 2–7) days, and the mean Ct value of the qRT-PCR for SARS-CoV-2 was 21.9 (standard deviation, 6.4) at study enrollment. At

enrollment, no significant differences were found in demographics, underlying medical conditions, clinical manifestations, interval from symptom onset, Ct value, and NEWS between the two study groups (Table 1). Laboratory findings indicated that white blood cell counts were lower in the ciclesonide group than in the standard care group (3.262 vs. 4.493 cells/μL, $p = 0.043$), but all other lab tests were similar between the two groups.

Table 1. Baseline demographic and clinical characteristics of study participants.

	Ciclesonide Group ($n = 35$)	Standard Care Group ($n = 26$)	p-Value
Age, mean days ± SD	44.9 ± 17.9	49.0 ± 16.8	0.362
Male sex, No. (%)	11 (31.4)	9 (34.6)	0.503
Underlying conditions (%)			
Diabetes	4 (11.4)	5 (19.2)	0.477
Hypertension	7 (20.0)	10 (38.5)	0.151
Cerebrovascular diseases	0 (0)	2 (7.7)	0.095
Clinical manifestations (%)			
Fever	17 (48.6)	12 (46.2)	0.852
Myalgia	16 (45.7)	12 (46.2)	0.973
Fatigue	11 (31.4)	7 (26.9)	0.781
Cough	20 (57.1)	10 (38.5)	0.198
Sputum	12 (34.3)	7 (26.9)	0.587
Sore throat	11 (31.4)	7 (26.9)	0.781
Rhinorrhea	7 (20.0)	4 (15.4)	0.745
Pneumonia (%)	8 (22.9)	9 (34.6)	0.391
Interval from symptom onset to enrollment, median days (IQR)	4 (2–7)	3 (1.8–5.5)	0.540
Ct value at enrollment, mean ± SD	21.7 ± 6.7	22.3 ± 6.1	0.731
NEWS at enrollment median (IQR)	0 (0)	0 (0–1)	0.519
Arterial oxygen saturation (%)	97.3 ± 1.5	97.5 ± 1.0	0.743
White cell count (cells/μL), mean ± SD	3262 ± 1934	4493 ± 2343	0.043
Hemoglobin (mg/dL)	13.9 ± 1.1	13.7 ± 1.4	0.617
Platelet count (cells/μL), mean ± SD	217 ± 63	206 ± 58	0.549
AST (IU/L), mean ± SD	26.0 ± 10.1	27.5 ± 18.4	0.677
ALT (IU/L), mean ± SD	23.7 ± 15.2	21.5 ± 18.3	0.610
BUN (mg/dL), mean ± SD	11.9 ± 3.6	13.6 ± 8.0	0.272
Serum creatinine (mg/dL), mean ± SD	0.8 ± 0.2	0.8 ± 0.2	0.964

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; Ct, cyclic threshold; IQR, interquartile range; NEWS, National Early Warning Score; SD, standard deviation.

Regarding the primary outcome, the SARS-CoV-2 eradication rate at day 14 was significantly higher in the ciclesonide group than in the standard care group (32.3% vs. 5.0%, $p = 0.021$) (Table 2). In the ciclesonide inhaler group, SARS-CoV-2 was negative-converted in 10 patients on the 14th day of treatment, and three of them received HCQ concurrently. Multivariate analysis revealed that SARS-CoV-2 was 12 times more likely to be eradicated at day 14 in the ciclesonide group than in the standard care group. Although not significant, SARS-CoV-2 eradication rates at days 7 and 10 were also higher in the ciclesonide group than in the standard care group. No significant between-group difference was observed in symptom-based clinical improvement rates at days 7, 10, and 14. However, the clinical failure rate was significantly lower in the ciclesonide group than in the standard care group (2.9% vs. 19.2%, $p = 0.034$). In the multivariate analysis, ciclesonide lowered the clinical failure rate by 97.4% (odds ratio 0.026; 95% confidence interval 0.001–0.845) compared with the standard care. No fatal cases were recorded in this study. Among non-pneumonic cases at study enrollment, pneumonia developed in 11.1% (3 of 27 cases) of ciclesonide group and 23.5% (4 of 17 cases) of standard care group, respectively ($p = 0.273$).

When comparing the Ct values of nasopharyngeal specimens (Figure 2), no significant difference was found between the ciclesonide group and the standard care group at day 1 (21.7 ± 6.7 vs. 22.3 ± 6.1, $p = 0.731$), day 4 (26.0 ± 7.2 vs. 24.1 ± 5.5, $p = 0.295$), day 7 (29.4 ± 5.7 vs. 27.9 ± 5.5, $p = 0.345$), and day 10 (31.7 ± 5.1 vs. 29.9 ± 4.9, $p = 0.226$) from study

enrollment, but the Ct value of the ciclesonide group on day 14 was marginally higher than that of the standard care group (35.3 ± 4.9 vs. 32.6 ± 4.2, $p = 0.051$). The change of the Ct value from day 1 to 14 was significantly larger in the ciclesonide group than in the standard care group (13.2 ± 5.8 vs. 9.1 ± 6.2, $p = 0.021$). If the qRT-PCR result was negative, the Ct value was assigned as 40.

Table 2. Comparison of clinical outcomes between ciclesonide and standard care groups.

	Ciclesonide Group ($n = 35$)	Standard Care Group ($n = 26$)	p-Value	Adjusted OR (95% CI) of Ciclesonide Treatment
Clinical failure rate, No. (%)	1 (2.9)	5 (19.2)	0.034	0.026 (0.001–0.845)
Clinical improvement rate at day 7, No. (%)	19 (54.3)	15 (57.7)	0.793	-
Clinical improvement rate at day 10, No. (%)	21 (60.0)	14 (53.8)	0.794	-
Clinical improvement rate at day 14, No. (%)	26 (74.3)	14 (53.8)	0.111	-
Virologic eradication rate at day 7, No. (%)	2/34 (5.9) [a]	0/22 (0) [b]	0.247	-
Virologic eradication rate at day 10, No. (%)	4/33 (12.1) [a]	0/22 (0) [b]	0.090	-
Virologic eradication rate at day 14, No. (%)	10/31 (32.3) [a]	1/20 (5.0) [b]	0.021	12.194 (1.187–125.240)
Duration of hospitalization, mean days ± SD	19.1 ± 7.7	19.5 ± 7.4	0.839	-

SD, standard deviation; OR, odds ratio; CI, confidence interval. [a] One patient was excluded at days 7, 10, and 14 because of clinical failure. In addition, one patient was excluded at day 10, and two more patients were excluded at day 14 because of early discharge with clinical improvement; respiratory specimens were not available. [b] Four patients were excluded at days 7 and 10, while five patients were excluded at day 14 because of clinical failure. In addition, one more patient was excluded at day 14 because of early discharge with clinical improvement; respiratory specimens were not available.

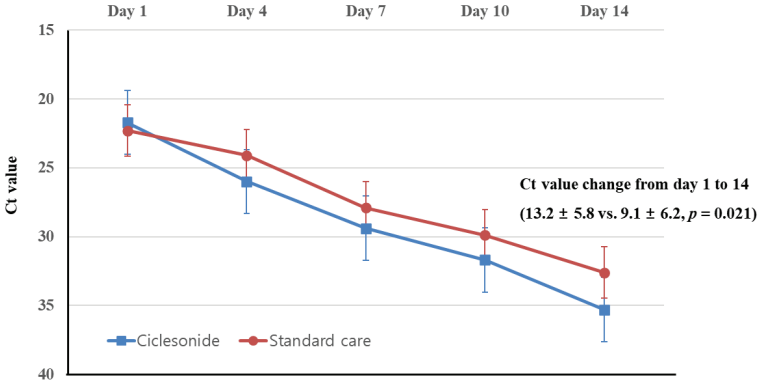

Figure 2. Comparison of serial cyclic threshold (Ct) values based on quantitative reverse transcription polymerase chain reaction targeting RdRp gene between ciclesonide and standard care groups. Four patients of ciclesonide group and six patients of standard care group were excluded in the analysis because of clinical failure or early discharge with clinical improvement, respectively.

For 22 patients, qRT-PCR was performed serially with saliva samples. When Ct values (mean ± SD) were compared between the ciclesonide group ($n = 13$) and standard care group ($n = 9$), no significant difference was observed at day 1 (28.5 ± 6.2 vs. 27.5 ± 6.8, $p = 0.715$), day 3 (31.4 ± 4.5 vs. 28.7 ± 5.5, $p = 0.215$), or day 4 (28.7 ± 4.1 vs. 29.8 vs. 6.1, $p = 0.605$).

Among the 35 patients who received ciclesonide, three complained of nausea, odynophagia, or headache after inhalation. These ciclesonide-related symptoms were tolerable, so treatment was continued for 14 days. The patient who had headaches received HCQ concomitantly. No serious adverse event was reported in any patients.

4. Discussion

This prospective, multicenter, randomized, open-label, phase 2 trial demonstrated that ciclesonide eradicated SARS-CoV-2 earlier and prevented the progression to severe COVID-19 among patients with mild-to-moderate COVID-19. Ciclesonide treatment increased the probability of SARS-CoV-2 negative conversion within 14 days by more than 12 times compared with standard care. Additionally, reduced risk of clinical failure (progression to hypoxia requiring respiratory management) by 97.4% was observed among patients who received ciclesonide compared with those who received standard care. However, in this study, we could not observe a significant shortening of symptom duration in the ciclesonide treatment group compared to the standard care group. The discrepancy may be due to the limitation of this study conducted in mild patients. Most mild symptoms other than fever are subjective, and self-limiting. Furthermore, because of individual variation, it is difficult to evaluate clinical improvement in mild patients. In order to obtain meaningful results, it would be necessary to evaluate patients with objective indicator (fever) in the acute stage within 48 h from symptom onset, as taken in the influenza study.

Inhaled ciclesonide can be safely delivered to lung tissues in high concentrations because it is essentially not absorbed into the bloodstream [14]. The antiviral mechanism of ciclesonide remains unclear. However, some studies have suggested that ciclesonide might suppress viral replication by inhibiting viral endoribonuclease (NSP15), p21 activated kinase-1, or viral RNA replication-transcription complex [9,12,15]. Ciclesonide is a prodrug that is converted to the active metabolite desisobutyryl-ciclesonide (des-CIC) by tissue esterases in the lung [15,16]. Although both ciclesonide and des-CIC are capable of interacting with NSP15, des-CIC has larger binding energy [9,15]. According to an in vitro study comparing diverse cell lines, the 90% effective concentration (EC90) of ciclesonide against SARS-CoV-2 was 10-fold lower (EC90 = 0.55 μM) in differentiated human bronchial tracheal epithelial cells than in VeroE6/TMPRSS2 or Calu-3 cells [9]. Considering that normal extravascular lung water may be <10 mL/kg, but increase with pulmonary edema, the EC90 for ciclesonide supports the administration of 640 μg/day (320 μg inhalation twice per day) in this study; 0.55 μM is equivalent to 1200 μg of ciclesonide dissolved in 4 L of exudate fluid [9,17].

During the early stage of infection, most cases of COVID-19 are mild, but 30–40% of patients experience pneumonia, and some rapidly worsen at approximately days 7–10. Thus, 14% require intensive care treatment and 5% become critical [18]. Therefore, even if the initial symptoms are mild, older and chronically ill patients should be closely monitored for possible worsening during treatment. Pathophysiologically, COVID-19 begins in the viral phase, passes through the immune (inflammation) phase, and then reaches the recovery phase. Some patients display acute exacerbation at 7–10 days of symptom onset, progressing to respiratory failure because of excessive inflammatory reactions. Given this, the corticosteroid dexamethasone appears to have a beneficial effect in patients with acute exacerbation of COVID-19 [19]. Ciclesonide is an inhaled corticosteroid used to treat bronchial asthma. Thus, in addition to its antiviral effect, the anti-inflammatory effects of ciclesonide may be useful in the treatment of lung injury, preventing progression to severe pneumonia and acute respiratory distress syndrome. Actually, favorable results have been reported in Japan when COVID-19 pneumonia cases were treated with ciclesonide inhalers [10,11]. Of note, in our study, the clinical failure rate due to acute respiratory failure was significantly lower in the ciclesonide treatment group than in the standard care group. Similar to our results, another inhaled glucocorticoid budesonide reduced clinical deterioration of mild COVID-19 by 91% in a randomized clinical trial [20].

Antiviral treatment for patients with mild COVID-19 requires consideration of two aspects: symptom relief and inhibition of viral transmission. A high SARS-CoV-2 viral load in saliva may contribute to efficient disease transmission in patients with mild COVID-19. Considering that ciclesonide is an inhalant, we expected a viral inhibitory effect in saliva during the early stage of infection, but contrary to expectations, the ciclesonide group did not display any difference in salivary SARS-CoV-2 suppression compared with the

standard care group. Since ciclesonide is inhaled into the lower airways, the exposure time in the oral cavity is short, and the active metabolites generated in the lung tissue mainly exert antiviral effects [9,16]. Thus, ciclesonide inhalation may not sufficiently suppress salivary SARS-CoV-2. Given the antiviral effect of chlorhexidine, it may be effective to use a chlorhexidine gargle with ciclesonide to inhibit the excretion of SARS-CoV-2 from saliva during the early stage of infection [21]. Thus, it is necessary to evaluate whether this combined strategy is effective in blocking the transmission of SARS-CoV-2.

This study has some limitations. First, the trial was not blinded and was limited to a small sample size. Nevertheless, we recruited patients with COVID-19 during the early stage of infection within a mean of 3–4 days from symptom onset, and the baseline characteristics were comparable between the two study groups. Thus, the findings suggest the clinical usefulness of ciclesonide, but a larger, well-designed study is warranted to confirm our results. Second, viral culture tests were not conducted in this study, so the inhibitory effect of ciclesonide on viral viability could not be evaluated. Third, we evaluated viral shedding duration using two different Korean MFDS (Ministry of Food and Drug Safety)-approved qRT-PCR kits. Therefore, to minimize the effect of using two different kits, only one kit was used for each study participating institution, and block randomization was performed for each institution. Finally, data on the occurrence of secondary bacterial pneumonia and specific antibiotic treatment were not collected in this study.

In conclusion, our results indicate that ciclesonide shortened SARS-CoV-2 viral shedding duration. Ciclesonide may inhibit the progression to acute respiratory failure in patients with mild-to-moderate COVID-19. Ciclesonide inhalation could be a useful therapeutic option for mild-to-moderate COVID-19 in an outpatient setting.

Author Contributions: J.-Y.S. and W.-J.K. contributed to the conception and design of the study. J.-Y.S. and W.-J.K. analyzed the data with responsibility for its integrity and prepared the manuscript. All authors contributed to acquisition of clinical and laboratory data. J.-Y.S., J.-G.Y., Y.-B.S., J.L., J.-S.E., J.-S.L., W.-S.C., E.-Y.L., Y.-A.C., H.-J.H., H.S., J.-Y.N., H.-J.C., and W.-J.K. contributed to the interpretation of data. J.-Y.S. and W.-J.K. contributed to the statistical analysis. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was approved by the Institutional Review Board (IRB) of each participating hospital: Korea University Guro Hospital (2020GR0145), Hallym University Kangnam Sacred Heart Hospital (HKS2020-04-012), Gachon Gil Medical Center (GCIRB2020-152), Inha University Hospital (2020-04-023), Korea University Ansan Hospital (2020AS0085), Korea Cancer Center Hospital (KIRAMS 2020-04-002-002), and Seoul Metropolitan Seobuk Hospital (2020GR0145). In Seoul Metropolitan Seobuk Hospital, the study was conducted under the supervision of Korea University Guro Hospital IRB. The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice.

Informed Consent Statement: All participants provided written informed consent prior to study enrollment.

Data Availability Statement: The data are not publicly available because of ethical and regulatory restrictions on participant privacy. However, a pseudonymized individual study dataset will be made available on request directed to the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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Efficacy of Remdesivir-Containing Therapy in Hospitalized COVID-19 Patients: A Prospective Clinical Experience

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Abstract: Objectives: Remdesivir is currently approved for the treatment of COVID-19. The recommendation for using remdesivir in patients with COVID-19 was based on the in vitro and in vivo activity of this drug against SARS-CoV-2. Methods: This was a prospective observational study conducted on a population of patients hospitalized for COVID-19. The primary endpoint of this study was the impact of remdesivir-containing therapy on 30-day mortality; the secondary endpoint was the impact of remdesivir-containing therapy on the need for high-flow oxygen therapy (HFNC), non-invasive ventilation (NIV), or mechanical ventilation. The data were analyzed after propensity score matching. Results: A total of 407 patients with SARS-CoV-2 pneumonia were consecutively enrolled. Out of these, 294 (72.2%) were treated with remdesivir and 113 (27.8%) were not. Overall, 61 patients (14.9%) were treated during hospitalization with HFNC, NIV, or mechanical ventilation, while 30-day mortality was observed in 21 patients (5.2%). Univariate analysis of patients treated with remdesivir or not showed no differences in 30-day mortality (4% vs. 6%, $p = 0.411$) in the two study groups. Cox regression analysis, after propensity score matching, showed that therapies, including remdesivir-containing therapy, were not statistically associated with 30-day survival or mortality. The Kaplan–Meier curves of 30-day survival in patients treated with remdesivir or not before ($p = 0.24$) and after ($p = 0.88$) propensity score matching showed no differences between the two study groups. Finally, patients treated with remdesivir or not showed the same need for HFNC/NIV or mechanical ventilation. Conclusions: This real-life experience of remdesivir use in hospitalized patients with COVID-19 was not associated with significant increases in rates of survival or reduced use of HFNC/NIV or mechanical ventilation compared with patients treated with other therapies not including remdesivir.

Keywords: remdesivir; COVID-19; pneumonia; non-invasive ventilation; mechanical ventilation

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1. Introduction

Corticosteroids, particularly dexamethasone, have been the standard of care in patients with severe coronavirus disease (COVID-19) since the publication of the results of the RECOVERY trial [1,2]. Other anti-inflammatory and immunomodulatory therapies, such as tocilizumab and baricitinib, can be considered in patients with severe COVID-19 [2]. Remdesivir has been the only antiviral medication suggested for the treatment of hospitalized patients with severe COVID-19 [2].

Remdesivir is an inhibitor of viral RNA polymerase that was initially studied on Ebola virus [3]. Remdesivir was found to have in vitro and in vivo activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and has an acceptable safety

profile [4–6]. Therefore, remdesivir was studied in large clinical trials that were initiated in the early phase of the pandemic [1–7]. In particular, a large trial conducted by the ACTT-1 study group revealed that remdesivir was superior to placebo in shortening the time to recovery in hospitalized patients with COVID-19 [4]. The results of this study led to approval by [8,9] for the treatment of patients with COVID-19. A subsequent meta-analysis of randomized trials conducted by Kaka et al. demonstrated that remdesivir may reduce mortality in patients that require supplemental oxygen but are not on mechanical ventilation [10].

Real-world data on the efficacy of remdesivir in the treatment of hospitalized patients with COVID-19 are needed. Therefore, we performed this prospective observational study aiming to investigate the impact of remdesivir on 30-day mortality and the need for invasive and non-invasive ventilation in a large Italian institution.

2. Materials and Methods

2.1. Study Design and Data Collection

This prospective observational study included patients admitted to Policlinico Umberto I of the University Hospital of Rome, Italy, from October 2020 to February 2021. Inclusion criteria were (1) positive SARS-CoV-2 real-time polymerase chain reaction test or antigenic test on nasopharyngeal swab, (2) pneumonia diagnosed either by thorax CT or chest x-ray, and (3) need for hospitalization. Patients who required high-flow oxygen therapy (HFNC) or non-invasive ventilation (NIV) or mechanical ventilation at the time of hospitalization were excluded from this analysis.

All patients were evaluated in a dedicated emergency department by dedicated infectious diseases specialists who identified patients with SARS-CoV-2 pneumonia, followed the patients during hospitalization, and collected all data prospectively without interfering with patient management. This observational study was conducted according to the principles stated in the Declaration of Helsinki, and it conforms to standards currently applied in our country. This study was approved by the local EC. Informed consent was obtained from the patients.

Data were extracted from the hospital's computerized databases and the patients' medical records. The following data were collected: demographics, clinical and laboratory findings, comorbidities, Charlson comorbidity index, microbiologic data, date of COVID-19 diagnosis, radiological characteristics of the pneumonia, therapies used, concomitant infections, duration of mechanical ventilation, time of negative nasopharyngeal swab, need for oxygen or ventilation support during the hospital stay, length of ICU stay, and length of hospital stay. Development of moderate to severe ARDS was defined as the acute onset of hypoxemia, manifestations of pneumonia of noncardiac origin on chest computed tomography imaging, and a PaO_2/FiO^2 ratio of less than 200 mmHg according to the Berlin definition [11].

Remdesivir was administered, after written informed consent was obtained, to patients according to the following criteria: presence of pneumonia, need for low-flow oxygen therapy, less than 10 days from the onset of symptoms, no need for HFNC or NIV or mechanical ventilation, alanine aminotransferase no more than 5-fold the upper limit of the reference range, and estimated glomerular filtration rate (eGFR) greater than 30 mL/min. A 5 day regimen was prescribed in all cases. Patients without these criteria were not eligible for remdesivir treatment.

All patients were followed until discharge or death. All discharged patients were followed for 30-days to assess outcomes.

2.2. Endpoints and Statistical Analysis

The primary endpoint of this study was the impact of remdesivir-containing therapy on 30-day mortality in hospitalized patients with SARS-CoV2 pneumonia. The secondary endpoint was the impact of remdesivir-containing therapy on the need for NIV or mechanical ventilation.

To reduce the impact of treatment selection bias in the estimation of treatment effects, propensity score matching was conducted with the nearest neighbor matching procedure without replacement [12]. Variables were selected for inclusion in the propensity score based on the potential impact on receipt of remdesivir and the association with mortality [13]. The variables included were steroids, antibiotics (excluding macrolides), age, gender, oxygen, comorbidities, CRP concentrations and the use of LMWH during hospital admission. A propensity score density plot and a Love plot were generated to examine the balance of propensity score and covariate distribution between the two groups (see Supplementary Figure S1).

To evaluate the demographic factors, Welch's t-test assuming unequal variances was used for continuous independent variables, while Pearson's Chi-square or Fisher's exact test was used, where appropriate, for categorical variables. Welch's analysis of variance (ANOVA) was used to assess group differences for continuous outcomes. Welch's t-test assuming unequal variances was used for post hoc comparisons.

All tests were two tailed, and a p-value < 0.05 was considered statistically significant. Results are expressed as the mean with standard deviation (±SD) for continuous normally distributed variables and as a count (n) and percentage (%) for categorical variables. Multivariate analysis was used to identify independent predictors of 30-day mortality and the need for NIV or mechanical ventilation. Matched bivariate analysis was conducted using a conditional logistic regression model, incorporating all variables found to be significant in the univariate analysis ($p < 0.05$) with a stepwise method. Matched multivariate models were constructed using Cox proportional hazard (HR) regression if appropriate, accounting for clustering of matched pairs. The final selected model was tested for confounding. In addition, a 95% confidence interval was calculated for HR. Survival was analyzed by Kaplan–Meier curves. All data were analyzed using a commercially available statistical software package (SPSS Statistics for Mac, 22.0; IBM Corp., Armonk, NY, USA).

3. Results

During the study period, 407 patients with SARS-CoV-2 pneumonia were consecutively enrolled. Out of these, 294 (72.2%) were treated with remdesivir and or 113 (27.8%) were not (control group). The mean time for remdesivir administration was 5.2 days (±2.9) from the onset of symptoms. Overall, 61 patients (14.9%) were treated during hospitalization with HFNC, NIV, or mechanical ventilation, and the 30-day mortality rate was 5.2% (21 patients).

Table 1 reports the univariate analysis of demographics and clinical characteristics of COVID-19 patients treated with remdesivir or not. Statistically significant differences were observed in the remdesivir group with regard to male sex (80% vs. 62%, $p < 0.001$), fever (79% vs. 50%, $p < 0.001$), cough (50% vs. 29%, $p < 0.001$), and dyspnea (57% vs. 37%, $p < 0.001$) compared to patients in the control group. No statistically significant differences were observed in the remdesivir group with regard to age (63.2 vs. 62.5 years, $p = 0.717$), and days to negative nasopharyngeal swab (22.07 vs. 24.77 days, $p = 0.378$).

Table 1. Univariate analysis regarding demographics and clinical characteristics of COVID-19 patients treated with remdesivir or not.

Variable	Control Group n = 113 (%)	Remdesivir n = 294 (%)	p-Value
Male sex	70 (62%)	250 (80%)	<0.001
Age, years, mean (± SD)	62.5 (±20)	63.2 (±15.3)	0.717
Days from symptoms/positive nasopharyngeal swab to admission, mean (± SD)	4.5 (±4.3)	5.3 (±3.8)	0.084
Charlson comorbidity index (± SD)	2.5 (±2.1)	2.6 (±1.9)	0.719
Cardiovascular disease	17 (15%)	33 (11%)	0.203
COPD	19 (17%)	31 (10%)	0.051
Chronic kidney disease	10 (9%)	18 (6%)	0.256
Liver cirrhosis	2 (2%)	12 (4%)	0.293
Diabetes mellitus	21 (19%)	53 (17%)	0.672

Table 1. *Cont.*

Variable	Control Group n = 113 (%)	Remdesivir n = 294 (%)	p-Value
Solid lung cancer (primary or metastasis)	1 (1%)	3 (1%)	1
Fever	56 (50%)	246 (79%)	<0.001
Cough	33 (29%)	157 (50%)	<0.001
Dyspnea	42 (37%)	178 (57%)	<0.001
Gastrointestinal symptoms (diarrhea, abdominal discomfort, nausea, vomiting)	16 (14%)	58 (18%)	0.271
Fatigue	21 (19%)	57 (19%)	1
Arthralgia/myalgia	13 (12%)	45 (14%)	0.46
Anosmia	3 (3%)	9 (3%)	1
Conjunctivitis	0 (0%)	2 (1%)	0.397
Chest pain	5 (4%)	11 (3%)	0.655
Parenchymal thickening	72 (64%)	232 (74%)	0.046
Interstitial lung disease	16 (14%)	15 (5%)	<0.001
Pleural effusion	20 (18%)	26 (9%)	0.012
Bronchiectasis/emphysema	27 (24%)	50 (15%)	0.032
White blood cells $\times 10^3$/uL, mean (±SD)	7.38 (±3.59)	8.06 (±5.99)	0.287
Neutrophils $\times 10^3$/uL, mean (±SD)	5.60 (±3.61)	6.31 (±5.31)	0.211
Lymphocytes $\times 10^3$/uL, mean (±SD)	1.18 (±0.65)	1.12 (±2.28)	0.769
Platelets $\times 10^3$/uL, mean (±SD)	247.66 (±100.85)	218.47 (±81.73)	0.004
D-dimer ng/mL, mean (±SD)	1365.71 (±1456.18)	814.91 (±766.45)	<0.001
Ferritin ng/mL, mean (±SD)	692.35 (±942.17)	645.02 (±489.52)	0.591
Procalcitonin ng/mL, mean (±SD)	1.20 (±6.4)	0.61 (±3.73)	0.334
LDH mU/mL, mean (±SD)	288.46 (±163.73)	302.37 (±119.57)	0.3
CPK U/L, mean (±SD)	278.90 (±1430.63)	149.47 (±161.35)	0.17
Lactates mmol/L, mean (±SD)	1.57 (±0.36)	1.33 (±0.83)	0.328
C-reactive protein mg/dL, mean (±SD)	4.91 (±6.61)	8.23 (±21.13)	0.116
PaO_2/FiO_2, mean (±SD)	315.67 (±117.14)	329.94 (±98.36)	0.411
Aspartate transaminase U/L, mean (±SD)	34.16 (±47.76)	35.24 (±27.41)	0.784
Alanine transaminase U/L, mean (±SD)	32.20 (±28.42)	33.24 (±28.84)	0.755

Legend. SD, standard deviation; COPD, chronic obstructive pulmonary disease; LDH, lactate dehydrogenase; CPK, creatine phosphokinase.

In-hospital treatments for COVID-19 patients are reported in Table 2. A comparison between patients treated with remdesivir or not shows that steroids (93% vs. 81%, $p < 0.001$) and LMWH (93% vs. 52%, $p < 0.001$) were more frequently prescribed in the remdesivir group; antibiotic therapy (58% vs. 27%, $p < 0.001$) was more frequently prescribed for patients in the control group; and no differences were reported regarding the use of HFNC/NIV or mechanical ventilation in the two study groups.

Table 2. In-hospital treatments for COVID-19 patients treated with remdesivir or not.

Variable	No Remdesivir n = 113 (%)	Remdesivir n = 294 (%)	p-Value
Steroids	92 (81%)	289 (93%)	<0.001
Antibiotics (excluding macrolides)	65 (58%)	83 (27%)	<0.001
Macrolides	74 (65%)	146 (46%)	<0.001
Low-molecular-weight heparin	59 (52%)	280 (93%)	<0.001
No need for oxygen therapy	24 (21.2%)	-	<0.001
Low-flow oxygen therapy	65 (57.5%)	275 (87%)	<0.001
HFNC/NIV	17 (15%)	33 (10%)	0.155
Mechanical ventilation	1 (1%)	10 (3%)	0.2

Legend. HFNC, high-flow nasal cannula; NIV, non-invasive ventilation.

In Table 3 are reported outcomes of hospitalized patients in the two study groups. No statistically significant differences were observed about length of hospital stay (15.02 vs.

16.06 days, $p = 0.487$), bacterial co-infection (20% vs. 21%, $p = 0.928$), and 30-day mortality (4% vs. 6%, $p = 0.411$).

Table 3. Outcomes of COVID-19 patients treated with remdesivir or not.

Variable	Control Group $n = 113$ (%)	Remdesivir $n = 294$ (%)	p-Value
Bacterial co-infection	24 (21%)	65 (20%)	0.928
Days of hospitalization, mean (±SD)	16.06 (±17.62)	15.02 (±9.98)	0.487
Days to negative nasopharyngeal swab, mean (±SD)	24.77 (±17.1)	22.07 (±13.77)	0.378
30-day mortality	4 (4%)	17 (6%)	0.411

Supplementary Tables S1 and S2 reports the results of univariate analysis before and after propensity score matching to evaluate the impact of the remdesivir-containing regimen on the study population. Figure 1 shows Kaplan–Meier curves for 30-day survival of patients treated with remdesivir or not before ($p = 0.24$) and after ($p = 0.88$) propensity score matching, showing no differences between the 2 study groups. Standardized differences before and after propensity score matching are reported in Supplementary Figure S1.

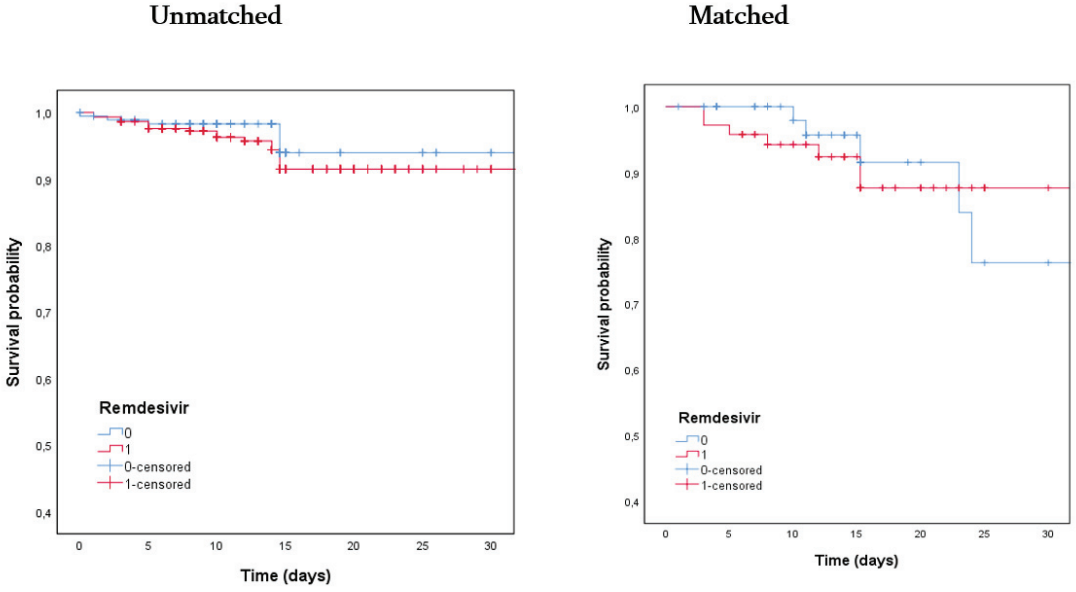

Figure 1. Kaplan–Meier curves for 30-day survival of patients treated with remdesivir (red line) or not (blue line) before ($p = 0.24$) and after ($p = 0.88$) propensity score matching.

Multivariate Cox regression analysis of 30-day mortality after propensity score matching is reported in Table 4. Therapies, including remdesivir-containing therapy, were not statistically associated with 30-day survival or mortality. However, mechanical ventilation (HR 4.22, 95% CI 5.4–16.2, $p = 0.003$) was independently associated with 30-day mortality.

Table 4. Multivariate Cox regression analysis of 30-day mortality after propensity score matching.

Variable	HR	95% CI	*p*-Value
Charlson comorbidity index < 2 points	0.2	0.1–2.0	0.012
Chronic kidney disease	1.8	0.1–140.2	0.812
COPD	3	0.08–95	0.523
Bacterial co-infection	0.88	0.1–7.81	0.772
Low-molecular-weight heparin	0.2	0.013–2.2	0.174
Macrolides	23.3	0.273–20.8	0.17
Antibiotics (excluding macrolides)	1.54	0.18–13	0.822
Steroids	0.12	0.0–1.24	0.892
No need for oxygen therapy	2.12	0.234–8.6	0.782
Low-flow oxygen therapy	10.7	0.434–176.4	0.122
Remdesivir	0.87	0.12–1.2	0.184
HFNC/NIV	182	0.954–336.7	0.054
Mechanical ventilation	4.22	5.4–16.2	0.003

Legend. COPD, chronic obstructive pulmonary disease; HFNC, high-flow nasal cannula; NIV, non-invasive ventilation.

Finally, multivariate Cox regression was used to analyze the need for non-invasive or invasive ventilation after propensity score matching (see Table 5). The data show that comorbidities and therapies, including the remdesivir-containing regimen, were not independently associated with a lower or higher risk of needing HFNC/NIV or mechanical ventilation.

Table 5. Multivariate Cox regression analysis of need for non-invasive or invasive ventilation after propensity score matching.

Variable	HR	IC	*p*-Value
Charlson comorbidity index <2 points	0.2	0.1–1.4	0.156
Chronic kidney disease	1.8	0.24–4.2	0.788
COPD	1.03	0.068–15.64	0.548
Bacterial co-infection	0.7	0.1–1.8	0.768
Low-molecular-weight heparin	0.18	0.01–2.2	0.174
Macrolides	0.4	0.01–8.8	0.494
Antibiotics (excluding macrolides)	1.6	0.18–12.67	0.722
Steroids	3.6	0.8–137	0.892
Remdesivir	1.03	0.8–15.6	0.802

Legend. COPD, chronic obstructive pulmonary disease.

4. Discussion

This prospective clinical study reports a real-life experience with the use of remdesivir in a large population of consecutively hospitalized patients with COVID-19. Our data, also after propensity score matching, show that the remdesivir-containing regimen was not associated with 30-day patient survival compared to treatment with other therapies not including remdesivir. Moreover, the remdesivir-containing regimen was not independently related to the need for HFNC/NIV or mechanical ventilation.

In Italy, remdesivir was specifically licensed for the treatment of COVID-19 in hospitalized patients with pneumonia who require oxygen therapy but not HFNC/NIV or mechanical ventilation at the time of remdesivir prescription [14].

Different data were reported around the world regarding the efficacy of remdesivir, taking into account different outcomes. In patients with severe COVID-19, treatment with remdesivir was significantly associated with higher recovery rates and lower mortality compared to standard-of-care treatment without remdesivir [15]. In this study, the mortality rate was significantly lower for patients treated with remdesivir (7.6%) compared with control groups (12.5%). Conversely, data from the Solidarity trial, conducted in 30 countries [16], showed no decrease in in-hospital mortality in patients treated with remdesivir, with the important limitation that other outcomes (clinical improvement and adverse events) were not carefully evaluated.

Recent real-word studies reporting data on the use of remdesivir [17] also compared it with lopinavir/ritonavir [18]. Some important meta-analysis showed that COVID-19 patients receiving remdesivir showed significantly higher rates of recovery and hospital discharge with lower rates of serious adverse events when compared to patients receiving other treatments [19,20]. However, these analyses also noted that there were no significant differences in clinical improvement and rate of mortality during hospitalization. Specifically, mortality was the main outcome reported in all analyzed studies, which showed no significant decrease in mortality if they were not adequately powered for this outcome [12].

Wang et al. [21] reported the first double-blind randomized clinical trial evaluating patients with a mean interval from symptom onset to enrollment of 12 days. No differences in mortality were recorded in the two arms, and the authors highlighted a possible trend of clinical benefit in patients treated with remdesivir. Of importance, a large number of patients in this study were also treated with steroids (65% in the remdesivir arm and 68% in the placebo arm), which may have confounded the results and conclusions. A strength of our study, with the limitation of the non-randomized cohort, was weighting all possible therapeutic confounders, including the use of steroids and LMWH [22,23].

Beigel et al. [7] randomized 1062 patients hospitalized with COVID-19 and evidence of pneumonia to remdesivir or placebo. This study demonstrated that remdesivir was superior to placebo in shortening the time to recovery in COVID-19 patients, with a trend toward survival benefit at day 29, without statistically significant differences. Of interest, the authors reported a beneficial effect of remdesivir in severe COVID-19 patients who did not require mechanical ventilation at enrollment; they suggested to start remdesivir early in the disease course.

Finally, in another randomized clinical trial [24] of patients with low to moderate COVID-19 (no oxygen requirement, but about 15% of patients required oxygen at the time of enrollment), the authors randomized 596 patients in a 1:1:1 ratio to receive a 5 day or 10 day course of remdesivir or standard-of-care therapy. The 5 day, but not the 10 day treatment showed a statistically significant difference with regard to the main clinical outcome. In the analysis, excluding patients who required oxygen at baseline, statistically significant differences favoring remdesivir over standard care were reported.

Our study has some limitations. First, considering the monocentric design, these results might be affected by local practice in the management of COVID-19. Second, although the criteria for HFNC/NIV and mechanical ventilation were based on the degree of respiratory impairment, critically ill elderly patients with ultimately fatal diseases were probably excluded from non-invasive/invasive ventilation, modifying the interpretation of some interventions; moreover, the small sample size did not permit definitive conclusions, including about HFNC/NIV and mechanical ventilation (only 61 patients were analyzed) and some important variables (like body mass index [BMI]) were not available for all study population. Third, this analysis evaluated consecutively hospitalized patients independently from COVID-19 severity, as demonstrated by the low mortality rate (6% of remdesivir group vs. 4% of those not treated with remdesivir). Finally, the analysis of the beneficial effects of treatments should be interpreted cautiously because it was not conducted with randomized groups and might therefore be affected by several measured and unmeasured confounding factors. However, the comparison of patients treated and not treated with remdesivir was based on a robust statistical methodology appropriate for non-randomized cohort studies about therapy.

5. Conclusions

In conclusion, in this real-life experience, the use of remdesivir in hospitalized patients with COVID-19 was not associated with significantly increased rates of survival or reduced use of HFNC/NIV or mechanical ventilation compared to treatment with other therapies not including remdesivir. These results suggest the need to conduct other RCTs to evaluate the impact of remdesivir in hospitalized COVID-19 patients at different stages of the disease or in combination with other drugs [5]. However, considering its safety profile and

the lack of alternative drugs, remdesivir should continue to be administered for patients with COVID-19.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10173784/s1, Figure S1: Standardized differences before and after propensity score matching; Table S1: Univariate analysis before propensity score matching; Table S2: Univariate analysis after propensity score matching.

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Data Availability Statement: The data are available upon request by email to a.russo@unicz.it.

Conflicts of Interest: The authors declare no conflict of interest.

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An Immunogenicity Report for the Comparison between Heterologous and Homologous Prime-Boost Schedules with ChAdOx1-S and BNT162b2 Vaccines

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Abstract: Background: There is a small amount of immunological data on COVID-19 heterologous vaccination schedules in humans. We assessed the immunogenicity of BNT162b2 (Pfizer/BioNTech) administered as a second dose in healthcare workers primed with ChAdOx1-S (Vaxzevria, AstraZeneca). Methods: 197 healthcare workers were included in a monocentric observational study in Foch hospital, France, between June and July 2021. The main outcome was the immunogenicity measured by serum SARS-CoV-2 IgG antibodies. Results: 130 participants received the ChAdOx1-S/BNT vaccine and 67 received the BNT/BNT vaccine. The geometric mean of IgG antibodies was significantly higher in the BNT/BNT vaccine group compared to the ChAdOx1-S/BNT vaccine group, namely 10,734.9, 95% CI (9141.1–12,589.3) vs. 7268.6, 95% CI (6501.3–8128.3), respectively ($p < 0.001$). However, after adjustment for time duration between the prime and second vaccinations, no significant difference was observed ($p = 0.181$). A negative correlation between antibody levels and time duration between second dose and serology test was observed for the BNT/BNT vaccine ($p < 0.001$), which remained significant after adjustment for all covariates ($p < 0.001$), but not for the ChAdOx1-S/BNT vaccine ($p = 0.467$). Conclusions: Heterologous and homologous schedules of ChAdOx1-S and BNT vaccines present robust immune responses after the second vaccination. The results observed were equivalent after adjustment for covariates and emphasize the importance of flexibility in deploying mRNA and viral vectored vaccines. Nevertheless, applying the ChAdOx1-S schedule vaccination for the heterologous second dose of BNT was associated with decreased IgG antibody levels compared to the homologous BNT/BNT vaccination.

Keywords: COVID-19; COVID-19 vaccine; ChAdOx1-S; BNT162b2; immunogenicity

1. Introduction

As of June 2021, SARS-CoV-2 infection has caused more than 185 million infections worldwide with a total death toll of more than 4.0 million. The COVID-19 pandemic has impacted the world in economic, social and health terms. Herd immunity remains the fundamental way to reduce the burden of the viral pandemic [1]. A massive vaccine

campaign has been started in several countries with different vaccines (Moderna (mRNA-1273), Pfizer/BionTech (BNT162b2), Sputnik V, AstraZeneca (ChAdOx1-S)). Epidemiologic studies have observed that COVID-19 vaccines should reduce the rates of infection, which will eventually yield to herd immunity when around 70% of the populations become fully vaccinated [2]. Nevertheless, on 15 March 2021, numerous European countries stopped ChAdOx1-S vaccine use as a precaution to investigate the death of a few dozen patients developing blood clots associated with deep vein thrombosis (DVT) [3]. In Europe, on 15 March 2021, only 30 suspect cases of DVT had been observed [4]. However, on 22 March 2021, the ChAdOx1-S vaccine campaign resumed in many countries, including France [5].

On 19 March 2021, the French High Authority for Health (HAS) announced that it was recommending the ChAdOx1-S vaccine only for people aged over 55 years. This decision was taken based on the rare cases of DVT occuring only in people aged under 55 years. The European Medicine Agency (EMA) asked not to ignore rare events, namely serious incidents that occurred among the 20 million vaccinations in Europe and the United Kingdom, which are 18 occurrences of cerebral venous thrombosis and seven disseminated intravascular coagulations [4].

In France, these changes in vaccine strategy induced as alternative the possibility of sequentially administering different COVID-19 vaccines, known as heterologous schedules. Thus, the French government advised administering a second dose with an mRNA (BNT or Moderna) vaccine in people primed with the ChAdOx1-S vaccine, even without supporting data regarding the immunogenicity of this schedule.

Heterologous strategies were not novel as they have been used in multiple HIV vaccines [6], Ebola vaccines [7] and in influenza vaccines [8]. However, few efficacy data using heterologous schedules incorporating COVID-19 vaccine are available in the world [9]. Previous studies have shown that a second vaccination with BNT was associated with increased anti-spike IgG levels for ChAdOx1-S-primed peopled compared to those having only one ChAdOx1-S dose [10–12]. However, contradictory anti-spike IgG levels were observed between participants who received homologous BNT/BNT vaccines or heterologous ChAdOx1-S/BNT vaccines with similar rates [10,13] and higher rates for heterologous ChAdOx1-S/BNT vaccines [9,14,15]. Nevertheless, no evidence of immune response outcomes with heterologous vaccine strategies is clearly available to date for the COVID-19 pandemic [16].

Thus, to answer this fundamental question, we designed in the Foch hospital, Suresnes, France, the retrospective ASTERMIX Foch COVID-19 study, in real-life practice according to the French recommendations, to evaluate the immune responses to heterologous schedules deploying ChAdOx1-S/BNT vaccines to the homologous BNT/BNT vaccines.

2. Methods Design

The ASTERMIX Foch COVID-19 study is a retrospective, cross-sectional and mono-center study. Participants were healthcare workers, adults (aged over 18 years) who had no previous COVID-19 infection. In France, the prime dose with ChAdOx1-S vaccine was not recommended for people younger than 55 years for vaccination since 19 March 2021. In our study, participants aged over 55 years were excluded from the study due to, after prime ChAdOx1-S vaccination, second BNT vaccination was not being recommended for people over 55 years. Exclusion criteria were the presence of clinically significant acute illness or temperature over 38 °C, clinical manifestations compatible with COVID-19 and any condition contraindicating or discouraging BNT administration for a second dose, including pregnancy, according to the French recommendations in March 2021.

The study was approved by the Foch IRB: IRB00012437 (approval number: 21-06-03) on 4 June 2021. A non-opposed consent was obtained from all participants.

2.1. Procedures

Healthcare workers received online and/or telephone screening to be invited for a serology test between June and July 2021. Two COVID-19 vaccines were used in our study. ChAdOx1-S is a replication-deficient chimpanzee adenovirus vectored vaccine, expressing the SARS-CoV-2 spike surface glycoprotein with a leading tissue plasminogen activator signal sequence. Administration was via 0.5 mL intramuscular injection into the upper arm. BNT is a lipid nanoparticle-formulated, nucleoside-modified mRNA vaccine encoding a trimerized SARS-CoV-2 spike glycoprotein. Administration is via 0.3 mL intramuscular injection into the upper arm.

Participants of the study either received two vaccinations for BNT four weeks apart or an initial dose of ChAdOx1-S followed by a heterologous boost with BNT 12 weeks later (on the scheme of homologous vaccination with a second dose of ChAdOx1-S), in accordance with the French recommendations on COVID-19 vaccines [17]. Vaccines were administrated by the occupational medical team and vaccination unit of the Foch hospital, Suresnes, France.

2.2. Covariates

Age and gender of healthcare workers, date of prime and second vaccination and date of serology test were reported. Different time durations were calculated as "time between second vaccination and serology test", "time between prime and second vaccination" and "time between prime vaccination and serology test". IgG antibody levels were reported for each participant between 30 and 60 days after the second vaccination.

2.3. Outcomes

The primary outcome is the serum SARS-CoV-2 IgG antibody level 30 to 60 days after the second vaccination.

2.4. Laboratory Method

SARS-CoV-2 IgG II Quant assays were performed on the Abbott Alinity i platform in accordance with the manufacturer's package insert [18,19]. In this antibody CMIA test, the SARS-CoV-2 antigen-coated paramagnetic microparticles bind to the IgG antibodies that attach to the virus's spike protein in the serum sample. The resulting chemiluminescence in relative light units (RLU) following the addition of anti-human IgG (mouse, monoclonal) acridinium labeled conjugate in comparison with the IgG II calibrator/standard indicates the strength of response, which reflects the quantity of IgG_{SP} present.

2.5. Statistical Analysis

Data for antibodies were presented as the geometric mean and 95% confidence interval (95% CI), as median and interquartile ranges for continuous variables and as number and percentage for categorical variables. Qualitative variables were compared using Fisher's exact test, while a T-test or Mann–Whitney's test was used for continuous variables. Linear correlations were performed for the relationship between each vaccine group and all covariates. Significance was defined by a p value < 0.05. For each model, multivariate analyses were performed with adjustment for covariates (age, gender, and the different time durations reported between vaccination and serology test). Statistical analyses were performed using SAS software (version 9.4; SAS Institute, Carry, NC, USA).

3. Results

Between June and July 2021, 197 participants were tested in Foch hospital, Suresnes, France. Demographic characteristics are shown in Table 1. A total of 130 participants received the ChAdOx1-S/BNT vaccination and 67 received the BNT/BNT vaccination.

Table 1. Characteristics of the study population.

	ChAdOx1-S/BNT Vaccination		BNT/BNT Vaccination		*p* Value	*p* Value **
	n = 130		*n* = 67			
Age	37	(13)	32	(11)	<0.001	
Gender (Female)	104	80.0%	59	88.1%	0.156	
T1	38	(7)	42	(9)	<0.001	
T2	84	(3)	27	(6)	0.008	
T1 + T2	120	(8)	70	(10)	<0.001	
GM Antibodies *	7268.6	(6501.3–8128.3)	10,734.9	(9141.1–12,589.3)	<0.001	0.181

T1: time between second vaccination and serology test. T2: time between prime and second vaccination. T1 + T2: time between prime vaccination and serology test. * GM: geometric mean (mean with 95% confidence interval). ** *p* value for antibody levels after adjustment for T2 (time between prime and second vaccination). IQR: interquartile range. Age, T1, T2, T1 + T2 are expressed in median + (IQR). Gender is expressed in number and percentage.

A significant difference was observed between the two groups for age (respectively, median of 37 (13) vs. 32 (11), $p < 0.001$) but not for gender (respectively, female, 104 (80.0%) vs. 59 (88.1%), $p = 0.156$).

In univariate analysis, the geometric mean of antibodies was significantly higher in the BNT/BNT vaccination group compared to the ChAdOx1-S/BNT vaccination group (respectively, 10,734.9, 95% CI (9141.1–12,589.3) vs. 7268.6, 95% CI (6501.3–8128.3) $p < 0.001$). The inclusion period was comprised between 30 and 60 days after the second dose, but a significant difference was observed among the two groups (respectively, 38 (7) days vs. 42 (9) days, $p < 0.001$). As expected, time duration between the first and the second vaccination was higher in the ChAdOx1-S/BNT vaccination group compared to the BNT/BNT vaccination group (respectively, 84 (3) days vs. 27 (6) days, $p = 0.008$). The total time duration between serology testing and prime vaccination was higher among the ChAdOx1-S/BNT vaccination group compared to the BNT/BNT vaccination group (respectively, 120 (8) days vs. 70 (10) days, $p < 0.001$) (Figure 1A).

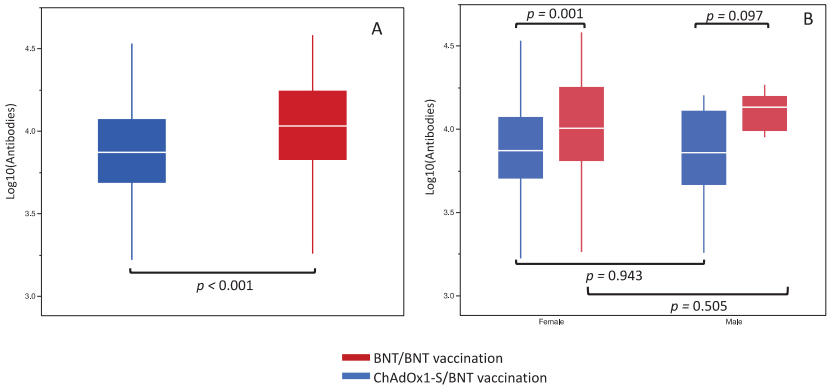

Figure 1. IgG antibody levels in overall population according to BNT/BNT and ChAdOx1-S/BNT (**A**) and according to gender (**B**).

In multivariate analysis, antibody levels for BNT/BNT remained significantly higher compared to ChAdOx1-S/BNT after adjustment for the time duration between the second vaccination and serology test ($p < 0.001$) and after adjustment for age ($p = 0.007$), but not after adjustment for the time duration between the prime and second vaccinations ($p = 0.181$) (Table 1).

In each vaccination group, no significant differences were observed for antibody levels between males and females (males vs. females for ChAdOx1-S/BNT, $p = 0.943$, and males vs. females for BNT/BNT, $p = 0.505$). However, antibodies in the BNT/BNT group

were higher than for ChAdOx1-S/BNT in females ($p = 0.001$) but not in males ($p = 0.097$) (Figure 1B).

A negative relationship between antibody levels and time duration between the second dose and the serology test was observed for the antibody levels of BNT/BNT ($p < 0.001$), which remained significant after adjustment for all covariates ($p < 0.001$), but not for ChAdOx1-S/BNT ($p = 0.467$) (Figure 2A).

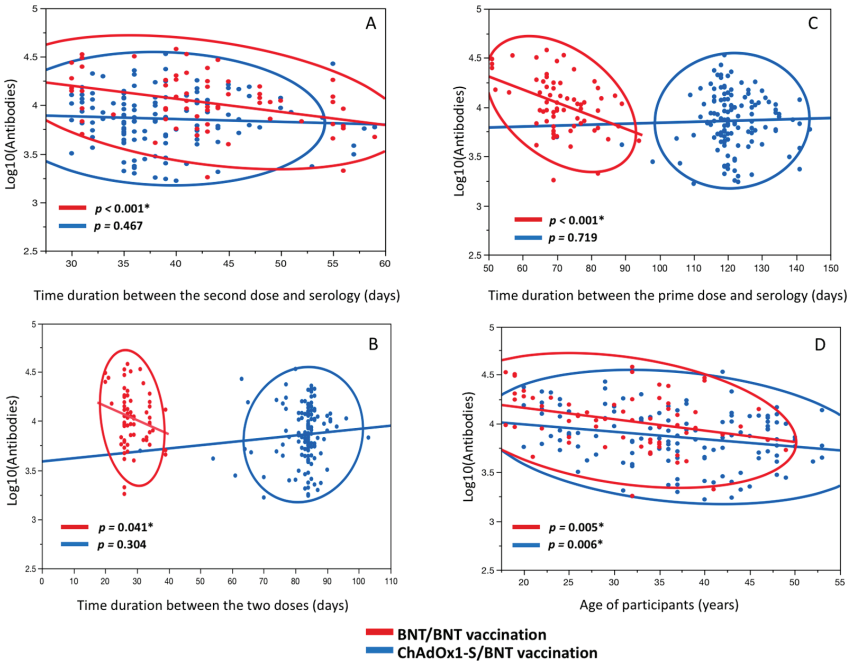

Figure 2. Association between IgG antibody levels and time duration aspects and age. (**A**) Association between IgG antibody levels and time duration between second vaccination and serology test for BNT/BNT and ChAdOx1-S/BNT. (**B**) Association between IgG antibody levels and time duration between prime and second vaccinations for BNT/BNT and ChAdOx1-S/BNT. (**C**) Association between IgG antibody levels and time duration between prime vaccination and serology test for BNT/BNT and ChAdOx1-S/BNT. (**D**) Association between IgG antibody levels and age of healthcare workers for BNT/BNT and ChAdOx1-S/BNT. * Significant models after adjustment for all covariates.

No significant correlation was observed between antibody levels and time duration between the prime and second dose for ChAdOx1-S/BNT ($p = 0.304$). No significant correlation was observed for the BNT/BNT ($p = 0.089$), but it became significant after adjustment for all covariates ($p = 0.041$) (Figure 2B). When considering the total time duration between the prime vaccination and the serology test with antibody levels, a negative correlation was observed for BNT/BNT ($p < 0.001$), which remained significant after adjustment for all covariates ($p = 0.001$), but not for ChAdOx1-S/BNT ($p = 0.719$) (Figure 2C). A negative relationship was observed for antibody levels and age for the ChAdOx1-S/BNT group ($p = 0.007$), which remained significant after adjustment for all covariates ($p = 0.006$) and for BNT/BNT ($p = 0.008$) which remained significant after adjustment for all covariates ($p = 0.005$) (Figure 2D).

4. Discussion

Our study showed a significant difference for antibody levels between BNT/BNT and ChAdOx1-S/BNT; however, this became non-significant after adjustment for the time duration between the prime and second doses of vaccination (Figure 1A). The change in

vaccine strategy was associated with a prolonged duration between the prime and second doses for the ChAdOx1-S group, leading to a lower level of antibodies after the second dose (Figure 2C).

Recent phase 1/2 studies have shown robust immunogenicity of homologous BNT and ChAdOx1-S immunizations [20,21]. By contrast, immunogenicity of heterologous ChAdOx1-S/BNT immunization has been rarely reported [10,13,14] with preliminary results. Our results appear to be consistent with this literature, showing no significant difference in concentrations of antibodies between heterologous and homologous vaccination.

In contrast to other studies that determined the time duration between the prime and second vaccinations, we report the real-life duration after a change in vaccine strategy for people in France. Very few studies have focused on this topic, and thus the comparison of our results appears difficult. Nevertheless, previous studies have shown that immunogenicity was impacted by the time between the doses. These studies showed that the longer the interval between the prime and the second vaccination of ChAdOx1-S, the higher is the IgG spike protein-specific response [10,14].

Our results showed that there was no difference between homologous and heterologous vaccination schedules. Previous studies have suggested that cellular responses are maintained regardless of age and gender after two-vaccination schedules with homologous ChAdOx1-S and with heterologous ChAdOx1-S/BNT [10,14]. Studies reported time between first and second vaccinations as 28 days in a Com-COV study [13] and 71 days for a German study focused on healthcare workers [14], showing a similar rate of immune responses. In our study this time was different for the two groups of vaccination, with 27 days for the BNT/BNT group and 84 days for ChAdOx1-S/BNT. These delays were the clinical recommendations for these vaccines in France. Thus, we can question the clinical relevance of having retained the homologous schedule for the second vaccination in the case of applying a heterologous vaccination. In the case of heterologous vaccination, it would be more effective for the second dose to use the schedule corresponding to the additional vaccine used (i.e., BNT with three months), rather than to respect the schedule of homologous vaccination (i.e., ChAdOx1-S with three weeks).

Here, we hypothesize that the lengthening of the vaccine interval between first dose with ChAdOx1-S and second dose with BNT could be associated with a low rate of immunogenicity. The change in the French vaccine campaign strategy may be associated with a lower rate of immune response for people who received a heterologous vaccine (ChAdOx1-S/BNT) due to a delay between the prime and second vaccination.

However, our results show a significant decrease in antibodies between the second vaccination and the serology test for the homologous BNT/BNT vaccine but not for the heterologous ChAdOx1-S/BNT vaccine (Figure 2A). Thus, a possible decrease may be observed between these two vaccine strategies. It could be of interest to extend the clinical study of these healthcare workers to compare the evolution of antibodies in future prospective studies. To our knowledge, no other study has observed this result and the comparison with the literature remains difficult.

Limitations

Our study presents potential limitations, as it is not a randomized controlled trial. Due to the current recommendations for heterologous ChAdOx1-S/BNT vaccination in people under 55 years, we could not recruit a matched cohort of homologous ChAdOx1-S/ChAdOx1-S vaccinated healthcare workers, since most of the healthcare workers have chosen the recommended heterologous booster. The majority of our healthcare workers were female, and this proportion could affect the interpretation of the results focused on gender differences. Hence, we could not define the exact action of the heterologous BNT booster vaccine compared to ChAdOx1-S homologous boosting alone. In our study, we compared the immunogenicity of homologous BNT/BNT and heterologous ChAdOx1-S/BNT vaccination. In addition to the different combinations of prime and boost vaccines, the time between first and second vaccines was significantly different in the homologous

(27 days) and heterologous (84 days) groups (Table 1). Moreover, the short duration of the study after the second vaccination (i.e., 30 to 60 days) could be a limitation for interpretation of the results and future study with a longer duration of follow-up after the second vaccination should be performed to compare with our actual results. No SARS-CoV-2 anti-Spike (or anti-NC) antibody levels were collected before inclusion of participants due to the French legislation. In our study, we cannot exclude bias from participants who had previous asymptomatic COVID-19 infection, which could influence the data. No antibody serum was collected after the first dose in our study, and we cannot clearly conclude that differences in the antibody levels observed can be attributed to the different time durations between the first dose and the serology test for homologous and heterologous vaccination.

5. Conclusions

In conclusion, our study observed that heterologous and homologous schedules with ChAdOx1-S and BNT vaccinations present robust immune responses 30 days to 60 days after the second dose. Moreover, the results observed were equivalent after adjustment for covariates and emphasize the importance of flexibility in deploying mRNA and viral vectored vaccines. Nevertheless, applying the ChAdOx1-S vaccination schedule for the second vaccination when the BNT vaccine was administered did not seem appropriate in light of a decrease in IgG antibody levels in the heterologous vaccination compared to the homologous vaccination. The second vaccination with BNT after a ChAdOx1-S prime may be more efficient with a schedule strategy of BNT rather than with a ChAdOx1-S vaccination schedule. However, these results should be confirmed by applying prospective clinical trials.

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Anakinra versus Baricitinib: Different Strategies for Patients Hospitalized with COVID-19 †

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Abstract: Background: Immunomodulatory drugs have been used in patients with severe COVID-19. The objective of this study was to evaluate the effects of two different strategies, based either on an interleukin-1 inhibitor, anakinra, or on a JAK inhibitor, such as baricitinib, on the survival of patients hospitalized with COVID-19 pneumonia. Methods: Individuals admitted to two hospitals because of COVID-19 were included if they fulfilled the clinical, radiological, and laboratory criteria for moderate-to-severe disease. Patients were classified according to the first immunomodulatory drug prescribed: anakinra or baricitinib. All subjects were concomitantly treated with corticosteroids, in addition to standard care. The main outcomes were the need for invasive mechanical ventilation (IMV) and in-hospital death. Statistical analysis included propensity score matching and Cox regression model. Results: The study subjects included 125 and 217 individuals in the anakinra and baricitinib groups, respectively. IMV was required in 13 (10.4%) and 10 (4.6%) patients, respectively ($p = 0.039$). During this period, 22 (17.6%) and 36 (16.6%) individuals died in both groups ($p = 0.811$). Older age, low functional status, high comorbidity, need for IMV, elevated lactate dehydrogenase, and use of a high flow of oxygen at initially were found to be associated with worse clinical outcomes. No differences according to the immunomodulatory therapy used were observed. For most of the deceased individuals, early interruption of anakinra or baricitinib had occurred at the time of their admission to the intensive care unit. Conclusions: Similar mortality is observed in patients treated with anakinra or baricitinib plus corticosteroids.

Keywords: COVID-19; anakinra; baricitinib; corticosteroids; mortality

1. Introduction

Two processes may occur in SARS-CoV-2 infection that causes COVID-19. First, viral replication predominates. Subsequently, a subset of patients may develop hyperinflamma-

tion, leading to moderate or severe COVID-19 pneumonia [1,2]. A cytokine storm is caused by excessive immune reactions and has been recognized as a pathophysiologic mechanism in severe COVID-19 [3]. Therefore, blocking the hyperimmune response and the secondary cytokine storm is critical for the treatment of severe COVID-19. Corticosteroids have been used to control the hyperimmune state. In fact, dexamethasone, at a dose of 6 mg once daily, has been shown to reduce the mortality of patients with severe COVID-19 pneumonia [4]. Moreover, pulses of methylprednisolone have also been demonstrated to be effective in treating cases of COVID-19 characterized by a strong inflammatory profile and severe respiratory symptoms [5,6].

Several immunomodulatory drugs have also been considered in the treatment of COVID-19, including recombinant human interleukin (IL) inhibitors [7], such as tocilizumab and anakinra, or the Janus kinase (JAK) inhibitor baricitinib [8]. An agent blocking the IL-6 receptor, tocilizumab, was one of the first immunomodulatory therapies to be proposed, given the fact that higher IL-6 concentrations have been associated with worse outcomes in patients with COVID-19 [9–11]. However, different studies with tocilizumab, including clinical trials, have revealed mixed results [12–16]. A central role of IL-1 in the inflammatory response has also been described [17]. In this sense, anakinra, a recombinant IL-1-receptor antagonist (IL-1ra), has been proposed as a potential therapeutic in severe COVID-19. It is well tolerated, has only mild immunosuppressive effects, and can be easily administered subcutaneously [18]. In addition, anakinra decreases IL-6 production because IL-1 is a potent inducer of IL-6 [17]. Therefore, the suggested beneficial effects of tocilizumab are also expected to be observed with anakinra. The published data for anakinra are based on a few observational studies with different designs, regimens, and concomitant or non-concomitant corticosteroid therapy [19–23]. However, further validation through ongoing randomized clinical trials is needed. In the hyperinflammatory syndrome, JAK–signal transducer and activator of transcription (STAT) signaling plays an important role in the pro-inflammatory cytokine-mediated signaling process [24]. Baricitinib is a potent and selective JAK inhibitor, requiring once-daily oral dosing and having an acceptable side-effect profile [25]. Baricitinib has a double effect against severe COVID-19. It inhibits the entry of SARS-CoV-2 into the target cells and blocks the induction of cytokine storms by suppressing JAK1/JAK2 [25]. In recent observational studies, baricitinib was associated with greater improvement in pulmonary function [26] and a reduction in mortality rate and intensive care unit (ICU) admissions in patients with moderate-to-severe COVID-19 [27]. In a randomized trial, the use of baricitinib plus remdesivir was superior to remdesivir alone in reducing recovery time and accelerating improvements in clinical status among patients with COVID-19 [28]. However, no consistent data have published about the relationship of baricitinib with hard clinical outcomes to date. Based on the above pathophysiological hypothesis, baricitinib could be used early in COVID-19 patients to inhibit SARS-CoV-2 entry into target cells. However, anakinra does not appear to be effective in non-severe infections. In fact, a randomized controlled trial was stopped early because anakinra did not improve outcomes in patients with mild COVID-19 pneumonia [29].

The aim of this study was to compare the efficacy in terms of the need for IMV and the mortality of two different strategies, based either on anakinra or on baricitinib therapies, applied to patients hospitalized with COVID-19 pneumonia.

2. Materials and Methods

2.1. Design and Patients

Our retrospective cohort included all patients who were admitted to internal medicine units in two tertiary healthcare centers in Seville (southern Spain) because of moderate-to-severe COVID-19 from the beginning of September to the end of November 2020. The inclusion criteria were as follows: (i) age over 18 years with SARS-CoV-2 infection indicated by PCR or the presence of antigen in nasopharyngeal swab; (ii) the use of immunomodulatory drugs; (iii) one of the following criteria suggestive of lower respiratory tract infection at the time of enrolment—lung infiltrates on a chest X-ray and/or

computed tomography scan or hypoxemia, defined as requiring any oxygen (O_2) support to achieve O_2 saturation of >93%; and (iv) at least one of the following laboratory criteria—C-reactive protein (CRP) > 50 mg/L, ferritin > 500 ng/mL, D-dimer > 500 ng/mL, or lactate dehydrogenase (LDH) > 250 U/L.

Clinical data were recorded daily from all consecutive patients admitted to the hospital for COVID-19 in their electronic records. The same physicians collected this information from patients' records, and they were manually entered by clinicians in a specific database.

A different strategy was implemented in each hospital according to its units' protocols based on anakinra or baricitinib as the first immunomodulatory drug recommended in that hospital. Both protocols included corticosteroid and anticoagulant therapies (Figure 1). Thus, patients were classified in each arm according to the first immunomodulatory drug used.

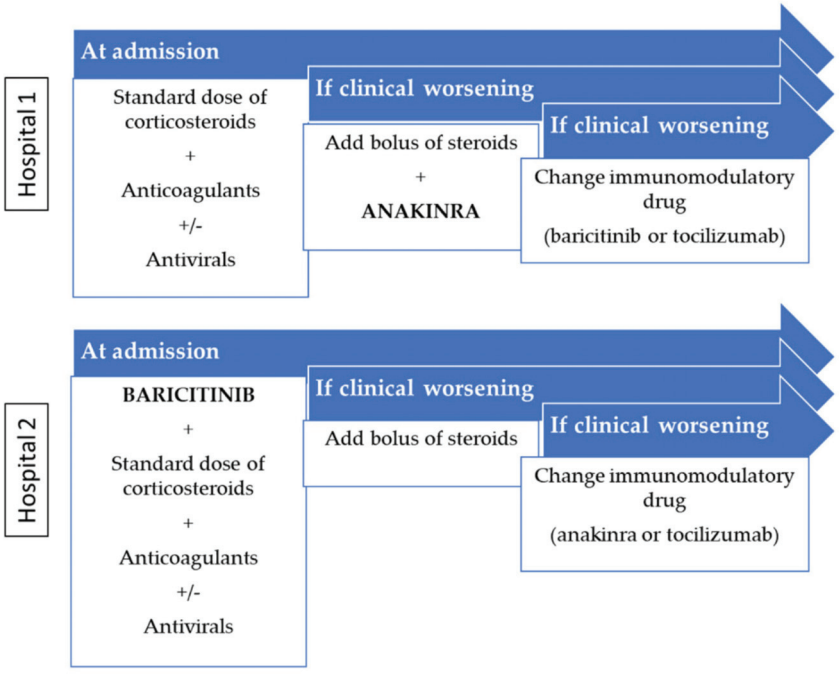

Figure 1. Internal medicine protocols in hospitalized patients with COVID-19.

Patients were excluded if a major clinical event (IMV or death) or a change in the immunomodulatory drug occurred before two consecutive doses of baricitinib (48 h) or anakinra (24 h) had been administered.

2.2. Variables and Follow-Up

The primary endpoint was in-hospital mortality, assessed by time-to-event analysis. The secondary outcomes were the need for invasive mechanical ventilation and the change in category based on the ordinal score of a modified WHO progression scale from baseline to the censored data according to supplemental oxygen.

All patients were censored at discharge from the hospital or on the date of death if this occurred first. Therefore, data included hospitalization in conventional and intensive units. Demographic information (sex, age, residence), baseline comorbidities measured by the Charlson index and a performance measure of activities of daily living by the Barthel scale, laboratory tests at the beginning of immunomodulatory therapy (CRP, ferritin, D-dimer, LDH, liver aminotransferases, platelet and lymphocyte counts), and COVID-19 treatment

(antivirals, corticosteroids, immunomodulatory and anticoagulant drugs) were collected. Details regarding the need to change to another immunomodulatory drug, if applicable, and the cause of this were also included.

The World Health Organization working group on the clinical characterization and management of COVID-19 developed a minimum set of common outcome measures for studies of COVID-19. This set included a measure of clinical progression based on the WHO clinical progression scale [30]. However, hospitalized patients requiring supplemental oxygen without intubation are not classified properly, because categories 5 and 6 include a wide range of non-ICU individuals, ranging from those with mild disease requiring low-flow oxygen to severe cases with non-invasive ventilation. Thus, we recategorized our patients into specific subsets (Table 1). Based on this classification, the oxygen therapy requirements at the initiation of immunomodulatory therapy and the maximum supplemental oxygen used during follow-up were also recorded.

Table 1. Modified WHO clinical progression scale. (5a) Supplemental oxygen (O_2) by nasal cannula requiring low-flow oxygen ($\leq$4 lpm); (5b) supplemental oxygen by nasal cannula requiring $\geq$5 lpm oxygen flow; (5c) supplemental oxygen by mask using FiO_2 between 35% and 50%; (6a) supplemental oxygen by mask with a reservoir bag; (6b) supplemental oxygen by high-flow nasal cannula (HFNC); and (6c) non-invasive mechanical ventilation.

Patient State	Descriptor
5. Hospitalized moderate disease	(5a) Supplemental O_2 by nasal cannula requiring $\leq$4 lpm flow
	(5b) Supplemental O_2 by nasal cannula requiring $\geq$5 lpm flow
	(5c) Supplemental O_2 by mask using FiO_2 between 35% and 50%
6. Hospitalized severe disease	(6a) Supplemental O_2 by mask with reservoir bag
	(6b) Supplemental O_2 by high-flow nasal cannula (HFNC)
	(6c) Non-invasive mechanical ventilation

Comorbidities were calculated using the Charlson index [31]. The Barthel scale was used to measure performance for 10 items about activities of daily living [32]. To interpret the Barthel scale values, they were categorized into 5 groups: total dependency (0–20 points), severe (21–35), moderate (40–55), slight (60–85), and no dependency (90–100). The laboratory tests included determination of lymphocyte and platelet counts; LDH, serum ferritin, alanine aminotransferase (ALT), CRP, and D-dimer levels; and the erythrocyte sedimentation rate.

2.3. Treatments

Anakinra was administrated subcutaneously at a standard dose of 200 mg twice on the first day, followed by 100 mg twice daily until a course of 10 days had been completed. The dose of this drug was adjusted to half if the renal glomerular filtration rate was under 30 mL/min/1.73 m^2.

Baricitinib was administrated orally at a standard dose of 4 mg once a day for up to 10 days. In the same way, the dose was adjusted to half if the renal filtration rate was under 60 mL/min/1.73 m^2.

Other therapies were administered to both groups as concomitant treatments based on the physician's criteria, including antiviral drugs, corticosteroids, and anticoagulant therapy to prevent coagulopathic complications. Methylprednisolone or dexamethasone at a once-daily dose equal to or higher than 125 or 20 mg was considered as pulses of steroids and was administered for 3 or more days.

2.4. Statistical Analysis

Continuous variables were presented as medians and interquartile ranges (IQRs) and categorical variables as absolute (n) or relative (%) frequencies. We applied the chi^2 test and

Student's *t*-test (or the Mann–Whitney test if the variables had non-normal distributions) to assess the differences in the clinical outcomes according to the type of variables.

Propensity score matching was used to adjust for some baseline characteristics with differences between them. A standardized difference of <0.2 as the upper limit of acceptable imbalance in baseline covariates was calculated.

We calculated the rates of intubation and death in the anakinra and control groups by a time-to-event analysis. The association of the main variables with time-related endpoints was analyzed using Kaplan–Meier curves and Cox regression analysis. Statistically significant differences were considered when $p < 0.05$. However, any variable with $p < 0.1$ in the univariate model was included in multivariate analysis.

Statistical analyses were performed using IBM SPSS software version 25 (SPSS, Chicago, IL, USA).

3. Results

During the period of the study, 291 and 323 patients were admitted in the internal medicine ward of two different hospitals. Of them, 129 (44.3%) and 219 (67.8%) patients were treated with anakinra and baricitinib, respectively. Six patients were excluded because early major clinical events occurred: four in the anakinra group due to intubation before 24 h at the initiation of anakinra and two subjects in the baricitinib group because of death before the first 48 h under treatment (Figure 2).

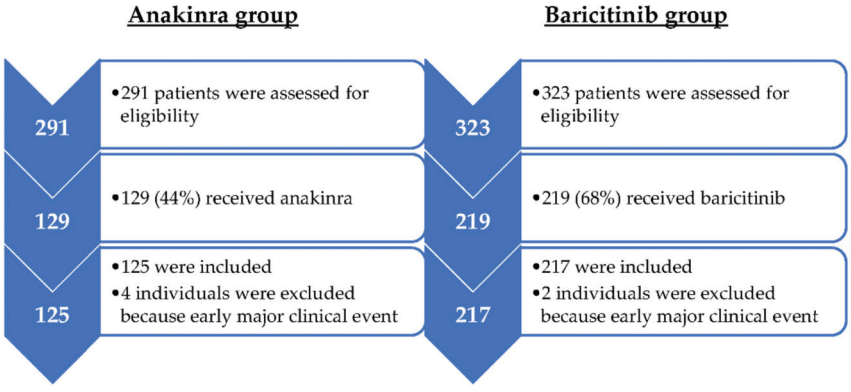

Figure 2. Flowchart of patients included.

Propensity scores were calculated based on the patients' following baseline characteristics: comorbidity index, Barthel scale, high-flow oxygen on presentation, dexamethasone at admission, and baseline CRP. After matching, there was a total of 93 subjects within each group.

3.1. Baseline Characteristics

The mean age of the total cohort was 69.4 years, and 57.6% were male. Total, severe, or moderate grade of dependency (Barthel scale < 60 points) was observed in 12.3% of patients, while more than two comorbidities were present in 54.7% of patients. Demographic, laboratory, and clinical data of both groups are shown in Table 2. A higher rate of individuals with comorbidities and dependency in the baricitinib group was observed. By contrast, the subjects in the anakinra group showed a significant elevation in several biomarkers of inflammation at the beginning of therapy, such as CRP, ALT, and LDH (Table 2). Statistically relevant values are highlighted in bold in Table 2.

Table 2. Demographic, laboratory, and clinical data of patients in anakinra and baricitinib groups.

Variables	Anakinra Group (n = 125)	Baricitinib Group (n = 217)	*p* Univariate
Demographic data			
Age (median, IQR), in years	73 (59–78)	71 (59–82)	0.528
Male sex, *n* (%)	70 (56)	127 (58)	0.649
Charlson index > 2, *n* (%)	60 (48)	127 (58)	0.060
Barthel scale ≥ 60, *n* (%)	116 (93)	184 (85)	**0.030**
Cardiopulmonary resuscitation candidate, *n* (%)	97 (78)	157 (72)	0.285
Living in a nursing home, *n* (%)	3 (2)	11 (5)	0.230
Laboratory values (median, IQR)			
Ferritin, in ng/mL	746 (324–1329)	579 (299–1312)	0.577
D-dimers, in μg/mL	900 (550–1640)	1055 (595–2163)	0.936
C-reactive protein, in mg/L	103 (58–168)	98 (44–143)	**0.044**
Procalcitonin, in ng/mL	0.14 (0.08–0.23)	0.11 (0.07–0.20)	0.770
Erythrocyte sedimentation rate, in mm/h	42 (14–77)	51 (25–83)	0.057
Alanine aminotransferase, in U/L	30 (20–53)	26 (17–43)	**0.013**
Lactate dehydrogenase, in U/L	326 (254–414)	302 (230–387)	0.078
Platelets × 10^3/μL	233 (174–305)	236 (157–320)	0.831
Lymphocytes/μL	880 (620–1265)	880 (620–1280)	0.390
Interleukin-6, in pg/mL *	16 (8–22)	20 (5–49)	**<0.001**
Time to event (median, IQR), in days			
Time of symptoms before admission	7 (5–10)	7 (5–10)	0.505
Time from admission to censored date	11 (8–15)	10 (7–16)	0.898
Time under first ID	10 (8–10)	8 (5–10)	0.239
Time from ID to combination event	7 (5–10)	9 (6–15)	**0.005**
Mean time from admission to ID	2.41	0.93	**<0.001**
Treatments, *n* (%)			
Remdesivir	2 (2)	35 (16)	**<0.001**
Lopinavir/ritonavir	0	77 (35)	**<0.001**
Dexamethasone at admission	77 (62)	172 (79)	**<0.001**
Pulses of corticosteroids at any time	125 (100)	99 (46)	**<0.001**
Changes in immunomodulatory therapy	5 (4)	31 (14)	**0.001**
Tocilizumab	1 (1)	13 (6)	**0.020**
Intermediate or high doses of LMWH [1]	67 (57)	82 (38)	**<0.001**
Mask with reservoir bag at admission	61 (49)	22 (10)	**<0.001**

[1] LMWH: low-molecular-weight heparin. *Available for 15 and 93 individuals.

3.2. Treatments

Antiviral therapy was less common in the anakinra arm than in the baricitinib arm, including remdesivir or lopinavir/ritonavir (2% vs. 51%, $p < 0.001$), respectively. All the patients were treated with corticosteroids in both groups. However, dexamethasone at admission was used more frequently among individuals under baricitinib treatment (62% vs. 79%, $p < 0.001$) as the first corticosteroid used. By contrast, all the patients in the

anakinra group received high doses of corticosteroids as a concomitant therapy if clinical worsening was observed.

Immunomodulatory drugs were switched in 5 (4%) and 31 (14.3%) subjects in the anakinra and baricitinib groups, respectively, because they were considered non-effective. In the anakinra group, baricitinib was used in four individuals and tocilizumab in the remaining individuals. In the baricitinib group, anakinra and tocilizumab were prescribed for 18 and 13 subjects when clinical conditions worsened, respectively. Time from the start to switch the first immunomodulatory drug was 8 (5–9) days and 5 (2–7) days in anakinra and baricitinib groups, respectively ($p < 0.001$). More details about the treatment used are shown in Table 2.

Patients in the anakinra arm needed higher levels of oxygen support at day 0 than those in the baricitinib group (Figure 3). Supplemental oxygen with high-flow oxygen, ≥5 lpm (category 5b or more), was required at baseline in 70.5% and 34.4% of the individuals in the anakinra and baricitinib groups, respectively. However, among these patients with severe infection, 36.3% and 45.3% (difference 9.0%, $p < 0.001$) of the subjects worsened by one or more steps during hospitalization based on the modified ordinal scale.

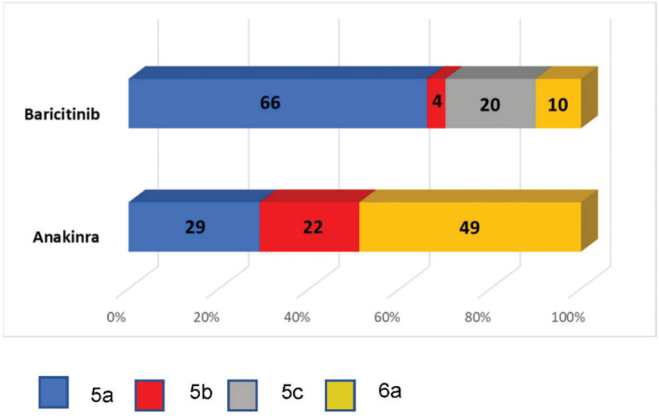

Figure 3. Bar plots at the beginning of immunomodulatory drug treatment according to the modified WHO clinical progression scale.

3.3. Outcome Events

In the anakinra and baricitinib original groups without matching, 13 (10.4%) and 10 (4.6%) patients required IMV, respectively ($p = 0.039$). Meanwhile, 22 (17.6%) and 36 (16.6%) patients died during this period, respectively ($p = 0.811$). When both events were analyzed together, 25 (20%) vs. 39 (18%) subjects needed IMV or died during follow-up ($p = 0.643$). According to the propensity score, 7 (7.5%) and 7 (7.5%) patients required IMV, respectively ($p = 1$). In terms of mortality, 15 (16.1%) and 21 (22.6%) patients died during this period, respectively ($p = 0.811$).

Depending on the need for intubation, 10 (77%) of 13 and 6 (60%) of 10 individuals who required IMV died in the anakinra and baricitinib groups, respectively ($p = 0.382$). By contrast, among the subjects who did not need intubation, 12 (10.7%) of 112 and 30 (14.5%) of 207 died, respectively ($p = 0.341$) (Figure 4). The median times receiving immunomodulatory drugs before needing intubation were 3 (2–6) and 4 (3–7) days among the anakinra and baricitinib patients, respectively. All eight individuals died when anakinra was discontinued in the first 3 days after intubation. However, three (60%) of five subjects in the ICU with more than 3 days of anakinra treatment survived. By contrast, five (83%) of six intubated and deceased individuals in the baricitinib group were treated for less than 3 days with this drug in the ICU.

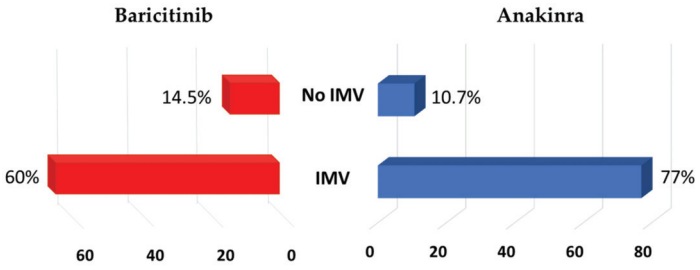

Figure 4. Mortality according to requiring invasive mechanical ventilation (IMV).

Mortality was higher in patients who were in higher categories according to the ordinal modified scale when treatment with the immunomodulatory drug began. The mortality of the patients classified at the beginning in category 5a or 5b ($FiO_2 < 35\%$) vs. category 5c or more ($FiO_2 \geq 35\%$) was 6.2% vs. 29.5% ($p < 0.001$) in the anakinra group and 13.3% vs. 23.9% ($p = 0.048$) in the baricitinib group, respectively.

3.3.1. Immunomodulatory Drug

The Kaplan–Meier curves for the primary endpoints according to the immunomodulatory drugs are shown in Figure 5. The estimated median intubation-free periods (95% confidence interval (CI)) (Figure 5a) were 66.3 (61.7–70.8) and 83.5 (78.4–88.7) days in the anakinra and baricitinib groups, respectively ($p = 0.044$). The median survival periods (95% CI) were 42.3 (30.1–54.5) vs. 74.5 (51.7–97.3) days ($p = 0.675$) in the anakinra and baricitinib groups, respectively (Figure 5b). Among matching individuals, no differences were found in the frequency of IMV or deaths (Figure 5c,d).

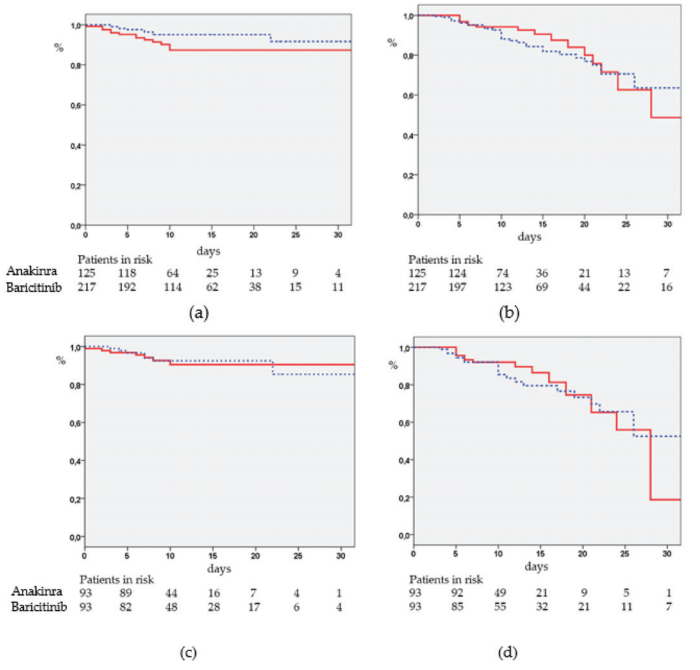

Figure 5. Probability of remaining free of invasive mechanical ventilation (**a**) and death (**b**) in the anakinra (continuous line) and baricitinib (dashed line) groups. Kaplan–Meier curves for IMV (**c**) and mortality (**d**) according to the immunomodulatory drugs and matching populations.

Based on changes in the immunomodulatory drug when it was considered non-effective, only one (25%) of the four patients who switched from anakinra to baricitinib survived. The only subject in whom anakinra was changed to tocilizumab also died. In the baricitinib group, 12 (66%) and 7 (54%) individuals survived after switched from baricitinib to anakinra or tocilizumab, respectively.

3.3.2. Multivariate Analysis

High levels of LDH ($p = 0.027$) and the need for oxygen supplementation with masks with reservoir bags at the beginning of immunomodulatory drug treatment ($p = 0.036$) were associated with intubation. Dexamethasone at baseline is a protective factor in intubation (Table 3).

Table 3. Multivariate Cox proportional analysis for the outcome of invasive mechanical ventilation.

Variables	Intubation	*p* Univariate	*p* Multivariate	Hazard Ratio (95% CI)
Lactate dehydrogenase				
≥350 U/L	15 (12.3)	0.002	0.015	2.907 (1.227–6.827)
<350 U/L	8 (3.6)			
Mask with reservoir bag				
Yes	13 (15.7)	<0.001	0.033	4.983 (1.141–21.770)
No	10 (3.9)			
Pulses of corticosteroids				
Yes	22 (9.9)	0.002	0.176	–
No	1 (0.8)			
C-reactive protein				
≥100	17 (10	0.017	0.241	–
<100	6 (3.5)			
Antiviral therapy				
Yes	4 (3.6)	0.110	0.228	–
No	19 (8.2)			
First immunomodulatory drug				
Anakinra	13 (10.4)	0.039	0.594	–
Baricitinib	10 (4.6)			
Dexamethasone at baseline				
Yes	8 (3.2)	<0.001	0.002	0.256 (0.108–0.611)
No	15 (16.1)			

By contrast, the need for intubation ($p < 0.001$), age older than 70 years ($p < 0.001$), dependency as indicated by a Barthel index value less than 60 ($p = 0.037$), and the use of pulses of corticosteroids ($p < 0.001$) were associated with a higher proportion of mortality (Table 4). Survival was not related to the use of dexamethasone at baseline ($p = 0.532$). No clinical events were related to the choice of the first immunomodulatory drug.

The multivariate analysis for 186 matching patients showed similar results. The Barthel score, Charlson index, IMV, older age, and high-flow oxygen at admission were associated with mortality (Table 5). Survival was not related to the use of pulses of corticosteroids.

Table 4. Multivariate Cox proportional analysis for the outcome of mortality.

Variables	Mortality	*p* Univariate	*p* Multivariate	Hazard Ratio (95% CI)
Lactate dehydrogenase				
≥350 U/L	26 (21.3)	0.110	0.199	–
<350 U/L	32 (14.5)			
Mask with reservoir bag				
Yes	25 (30.1)	<0.001	0.118	–
No	33 (12.7)			
Pulses of corticosteroids				
Yes	49 (22)	0.001	<0.001	1.668 (1.308–2.127)
No	9 (7.6)			
Age				
≥70	50 (25.6)	<0.001	<0.001	1.634 (1.279–2.087)
<70	8 (5.4)			
Dose of LMHW				
Prophylaxis	22 (12)	0.008	0.072	–
Intermediate or high dose	36 (22.8)			
Invasive ventilation				
Yes	16 (69.6)	<0.001	<0.001	12.576 (5.113–30.932)
No	42 (13.2)			
Barthel index				
<60	19 (45.2)	<0.001	0.037	1.604 (1.030–2.497)
≥60	39 (13)			
Charlson index				
<3	12 (7.7)	<0.001	0.076	–
≥3	46 (24.6)			

3.3.3. Adverse Events

In terms of related symptomatic adverse events, bowel perforation was observed in a patient treated in the anakinra group, but invasive diagnostic and therapeutic procedures were rejected because of the basal functional status. In terms of infections, 15 (12%) and 36 (16.6%) cases of bacterial pneumonia infection were suspected during the hospital stays in the anakinra and baricitinib groups, respectively ($p = 0.351$). Bacteriemia was diagnosed in six (4.8%) and seven (3.2%) subjects, respectively. Delirium was observed more frequently in the anakinra group compared to the baricitinib group(15 (12%) vs. 10 (4.6%), $p = 0.011$). Finally, 5 (4%) and 11 (5.1%) individuals in the anakinra and baricitinib groups developed heart complications, respectively (4 and 10 new arrhythmias, respectively, and one myocardial infarction in each group).

4. Discussion

This was a retrospective observational study investigating two different therapeutic strategies based on two immunomodulatory drugs: anakinra and baricitinib. Our findings can be summarized as follows: (i) Similar mortality was observed in both populations, and (ii) older age, high-flow oxygen at baseline, low functional status, high comorbidity, and the need for IMV were found to be associated with reduced survival.

Table 5. Multivariate Cox proportional analysis for the outcome of mortality after propensity score matching.

Variables	Mortality	*p* Univariate	*p* Multivariate	Hazard Ratio (95% CI)
Lactate dehydrogenase				
≥350 U/L	18 (26.5)	0.062	0.188	–
<350 U/L	18 (15.3)			
Mask with reservoir bag				
Yes	18 (35.3)	0.001	0.003	2.949 (1.463–5.947)
No	18 (13.3)			
Pulses of corticosteroids				
Yes	31 (21.8)	0.125	0.289	–
No	5 (11.4)			
Age				
≥70	30 (27)	0.001	0.040	1.222 (1.024–3.932)
<70	6 (8)			
Dose of LMHW				
Prophylaxis	12 (13)	0.031	0.747	–
Intermediate or high dose	24 (25.5)			
Invasive ventilation				
Yes	10 (71.4)	<0.001	0.047	2.276 (1.011–6.360)
No	26 (15.1)			
Barthel index				
<60	12 (44.4)	<0.001	0.002	3.338 (1.559–7.150)
≥60	24 (15.1)			
Charlson index				
<3	5 (6.1)	<0.001	0.003	3.544 (1.330–9.441)
≥3	31 (29.8)			
First immunomodulatory drug				
Anakinra	15 (16.1)	0.265	0.631	–
Baricitinib	21 (22.6)			
Dexamethasone at baseline				
Yes	21 (16.7)	0.179	0.396	–
No	15 (25)			
C-reactive protein				
≥100	22 (23.7)	0.138	0.764	–
<100	14 (15.1)			

To date, the experience with anakinra in patients with COVID-19 is limited and it is based on mainly small observational studies [19–23]. To the best of our knowledge, this study reports on the largest number of patients with COVID-19 treated with anakinra to date. Mixed results have previously been reported. Admission to the ICU for invasive mechanical ventilation support occurred for more than 27% of patients treated with anakinra in three previous studies [19,21,22]. Meanwhile, mortality rates between 10% and 14% have been published [19–21,23]. Despite the populations not being comparable, the rate

of IMV was lower in this study, but the mortality rate was slightly higher compared to prior studies.

In the same way, few studies on the use of baricitinib for patients hospitalized with COVID-19 pneumonia have been published to date. In a retrospective study, no death was reported and only 1 patient was admitted to the ICU among 113 individuals treated with baricitinib [27]. However, included patients seemed to have moderate disease at the time of initiation of baricitinib based on oxygen saturation at presentation [27]. In a randomized trial, the incidence of progression to death or intubation in the first 28 days from admission was lower in the baricitinib plus remdesivir group vs. the remdesivir group (12.2% vs. 17.2%) [28]. However, almost 14% of included patients did not require supplemental oxygen (no death occurred on either arm in the baseline ordinal score 4 subgroup) [28]. In our study, baricitinib was combined with an antiviral in around 57% of patients. Less than 5% of the patients treated with baricitinib required invasive mechanical ventilation in our population, but the mortality rate was almost 17%, higher than that previously reported.

These differences in our survival results can be explained because individuals were censored at discharge from the hospital or on the date of death. So, data included complete hospitalization in conventional and intensive units' periods. Several studies have censored patients with COVID-19 at the time of invasive mechanical ventilation or at 3 or 4 weeks after hospital admission. Therefore, their results cannot reflect the true mortality for COVID-19, because many of these patients may develop late complications and final outcomes were not collected. In fact, at day 21 after hospital admission, only 45% had been discharged from the hospital at the censored date in one of the anakinra studies [19]. However, in our study, 16 (69.6%) of the 23 individuals who needed invasive mechanical ventilation died after intubation. However, only 42 (13.2%) of the 319 subjects treated under immunomodulatory therapy in conventional hospitalization died. The overall ICU mortality rate of patients with COVID-19 in a systematic review was 30.6%, but when only mechanically ventilated subjects or acute respiratory distress syndrome subjects were considered, the mortality was 59% or up to 93%, respectively [33]. In our study, early interruption of anakinra or baricitinib occurred in most of the deceased individuals at the time of admission to the ICU. Therefore, we cannot rule out the possibility that the use of immunomodulatory drugs in critically ill patients would also be of benefit. In this setting, lower mortality was observed with the use of intravenous anakinra and concomitant corticosteroids in mechanically ventilated patients with COVID-19 in the ICU, but it was not statistically significant [22]. Moreover, in contrast to other studies, all included patients required supplemental oxygen support at admission. However, there were some differences among anakinra and baricitinib populations. Most patients in the anakinra group required a higher flow of oxygen support (category 5b or more) at the time of starting the immunomodulatory drug compared to the baricitinib group (70% vs. 34%, $p < 0.001$). These findings are in line with those of a recent clinical trial that showed anakinra to be inefficacious in mild COVID-19 patients [29]. By contrast, baricitinib, alone or in combination with antiviral drugs, could have early clinical benefits in the first days of infection or at the initial stages of the inflammatory phase [25]. Moreover, because venturi masks show a theoretically higher dispersion distance for aerosol particles [34], these delivery devices were not used in the anakinra group. However, during hospitalization, individuals treated with anakinra required less change in oxygen flow than those treated with baricitinib, with a difference of 9%. In both populations, we observed that the faster the drug is initiated in the management of patients with hypoxemic respiratory failure, the better the survival among patients with moderate or severe COVID-19. Prospective randomized studies will be necessary to dilucidated the best time to start this drug among hypoxemic patients with COVID-19.

Beneficial effects of corticosteroids have generally been found in patients with severe COVID-19 [4–6,35]. However, there is little information about the combination of corticosteroids with anakinra or baricitinib [22,26,28]. The clinical benefits of steroids might be related to the indication (severity of illness), timing of the intervention, and dose and dura-

tion of corticosteroid therapy [36]. In our study, all patients in both groups were treated with corticosteroids. Dexamethasone was extensively used in both groups at admission based on actual recommendations [4]. However, dexamethasone was changed to pulses of corticosteroids when worsening of clinical status occurred according to the physician's criteria. In this sense, all patients were started on pulses of methylprednisolone, previous or concomitant to the initiation of anakinra. By contrast, high doses of corticosteroids were only used in 46% of subjects in the baricitinib group. We cannot rule out a deleterious effect when dexamethasone is dropped out and higher doses of corticosteroids are started. However, after propensity score matching, there were no significant differences between the use or no use of pulses of corticosteroids. A benefit of steroids, including high doses of corticosteroids, has been observed in the inflammatory phase of COVID-19 [4–6,35,36]. Moreover, we are concerned that the potential risk factor of higher doses of steroids in our global population could be associated to selection bias because they were used when clinical worsening was suspected.

Our study has several limitations. The most important is the retrospective design, with dynamic therapy recommendations over time. To reduce the bias due to confounding variables, propensity score matching was performed to adjust for baseline characteristics between cohorts. However, not many differences were found before and after matching. At the time of writing this paper, there was no significant evidence from clinical trials for the efficacy of anakinra or baricitinib in COVID-19 patients. The different strategies used in two close hospitals reflect the absence of global recommendations and the heterogeneous management during the pandemic. However, both therapies are based on drugs with short durations of action and effect, acceptable side-effect profiles, and ease of administration. Another important limitation is the lack of a concomitant control group. All the severe COVID-19 patients in the internal medicine ward were included for regimens based on immunomodulatory drugs. Only corticosteroids have been reported to have some clinical benefits [4–6], but this therapy was also used in all patients in both groups. Therefore, we cannot rule out a potential benefit of anakinra or baricitinib added to steroids based on the pathophysiology described in patients with COVID-19 [3]. Finally, complement system inhibition is also a potential therapeutic target for COVID-19 [8]. In this study, immunomodulatory drugs were used independently, but it will be interesting to investigate the potential role of the combination or sequential use of IL and JAK inhibitors in COVID-19.

In our experience, clinical, laboratory, and radiographic items should be considered when deciding on the use of immunomodulatory drugs in real life among patients with moderate or severe COVID-19. The exact time to start them may be related to their efficacy.

5. Conclusions

Similar mortality was observed in real life with a different strategy based on anakinra or baricitinib. Older age, low functional status, high comorbidity, a need for IMV, elevated LDH, and the use of a high flow of oxygen at admission were found to be related to the occurrence of major clinical events.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

The Effect of Anakinra in Hospitalized Patients with COVID-19: An Updated Systematic Review and Meta-Analysis

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Abstract: The role of immunomodulatory agents in the treatment of hospitalized patients with COVID-19 has been of increasing interest. Anakinra, an interleukin-1 inhibitor, has been shown to offer significant clinical benefits in patients with COVID-19 and hyperinflammation. An updated systematic review and meta-analysis regarding the impact of anakinra on the outcomes of hospitalized patients with COVID-19 was conducted. Studies, randomized or non-randomized with adjustment for confounders, reporting on the adjusted risk of death in patients treated with anakinra versus those not treated with anakinra were deemed eligible. A search was performed in PubMed/EMBASE databases, as well as in relevant websites, until 1 August 2021. The meta-analysis of six studies that fulfilled the inclusion criteria (n = 1553 patients with moderate to severe pneumonia, weighted age 64 years, men 66%, treated with anakinra 50%, intubated 3%) showed a pooled hazard ratio for death in patients treated with anakinra at 0.47 (95% confidence intervals 0.34, 0.65). A meta-regression analysis did not reveal any significant associations between the mean age, percentage of males, mean baseline C-reactive protein levels, mean time of administration since symptoms onset among the included studies and the hazard ratios for death. All studies were considered as low risk of bias. The current evidence, although derived mainly from observational studies, supports a beneficial role of anakinra in the treatment of selected patients with COVID-19.

Keywords: anakinra; COVID-19; COVID-19 therapeutics; immunomodulatory treatment; meta-analysis; mortality; updated

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1. Introduction

The course of coronavirus disease 2019 (COVID-19) is divided in two main phases: the viral and the host inflammatory response phases [1–3]. During the second phase, a dysregulation of the immune system might occur in a subset of patients leading to a cytokine storm and immune hyperactivation cascade [1]. In these cases, antiviral treatment has little to offer, and thus the role of immunomodulatory agents has been of increasing interest [4,5].

Anakinra is an interleukin-1 inhibitor that has been shown to offer benefits alone or in combination with other agents for the treatment of diseases characterized by a cytokine storm (e.g., pediatric secondary hemophagocytic lymphohistiocytosis, and macrophage activation syndrome) [6,7]. It plays an important role in the inhibition of the cytokine storm cascade and can offer benefits to selected patients with COVID-19 [1]. Four recently published meta-analyses indicated that anakinra administration in hospitalized patients with COVID-19 and moderate to severe disease offered significant benefits in terms of mortality and the risk of intubation [8–11]. However, these analyses included mainly unadjusted effect estimates [8–11]. Unadjusted analyses might be significantly affected by

several confounding factors since treatment options in COVID-19 may differ according to patient characteristics and the severity of the disease. Interestingly, the most recent study included an individual patient-level meta-analysis in a subgroup of 895 patients, which allowed a multivariate analysis and showed a significant adjusted risk reduction with the use of anakinra [11].

The aim of the present study was to conduct an updated systematic review and meta-analysis on the impact of anakinra on the survival of hospitalized patients with COVID-19. To compensate for the nature of derived evidence, this analysis included randomized studies and observational ones presenting adjusted hazard ratios for several confounders.

2. Materials and Methods

2.1. Search Strategy

An updated systematic review and meta-analysis was performed according to the preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines [12]. A systematic search of PubMed and EMBASE databases was performed until 1 August 2021, using the following search algorithm: ("coronavirus 2019" OR "2019-nCoV" OR "SARS-CoV-2" OR "COVID-19" OR COVID OR COVID19) AND anakinra. Articles were also identified from reference lists of previously conducted relevant systematic reviews and meta-analyses and relevant papers and websites through the snowball procedure. The study selection was performed independently by two investigators (K.G.K. and I.G.K.). Disagreements were resolved by consensus with a senior author (A.K.).

2.2. Study Selection

Eligible studies were full-text articles in English language including $\geq$15 patients (not case series) that had a randomized design or were observational but reported exclusively adjusted hazard ratios for mortality between patients treated with anakinra versus those who did not receive anakinra. More precisely, eligible studies were: (i) randomized studies, (ii) observational studies with propensity matched controls, and (iii) observational studies with multivariate analysis models (including several potential confounders such as demographics, comorbidities, laboratory indices and background treatment with other therapeutic agents).

2.3. Data Extraction

Two investigators (K.G.K. and I.G.K.) independently extracted and tabulated data regarding study design, the main characteristics of included populations (age, sex, number of patients treated with anakinra, number of patients that required invasive mechanical ventilation, comorbidities, symptoms duration before anakinra administration, and severity indices at baseline, such as C-reactive protein) and data regarding the outcome of interest (adjusted hazard ratio for mortality).

2.4. Risk of Bias Assessment

The risk of bias was assessed in terms of the selection of patients, exposure measurement, confounding factors identification, outcome measurement, methodology and analysis independently by two investigators (K.G.K. and I.G.K.). A checklist for cohort studies from the Joanna Briggs Institute Critical Appraisal Tools was used [13]. Studies fulfilling $\geq$8 of the quality domains were deemed as low risk of bias.

2.5. Certainty (Confidence) of the Outcome

The certainty of the body of evidence for the outcome of death was independently assessed by two investigators (K.G.K. and A.K.) using the grading of recommendations assessment, development and evaluation (GRADE) approach described in Chapter 14 of the Cochrane handbook for systematic reviews of interventions [14]. The certainty of evidence was deemed as high, moderate, low, or very low, depending on factors that either decrease the confidence of the outcome such as the risk of bias, the publication bias, the

inconsistency, the indirectness and the imprecision of results, or factors that increase the certainty such as the large effect size, the dose response, and the effect of plausible residual confounding [15].

2.6. Statistical Analysis

Meta-analysis was performed using the Stata/SE 11 (Texas) software. The logarithms of adjusted hazard ratios and corresponding standard errors were used for the analysis (fixed-effects meta-analysis when I^2 statistic value < 50%). The hazard ratio was used as the effect measure of the outcome of interest as it was reported in all included studies. Results were graphically displayed as forest plots. A meta-regression analysis was performed for assessing associations of the logarithms of the hazard ratios for mortality with the mean age, percentage of males, mean baseline C-reactive protein levels, and mean time of administration since symptoms onset. The mean values of the subgroups were combined where feasible [16]. Median (interquartile range) values were converted to mean values (standard deviation) using the appropriate formulas [17]. Heterogeneity was tested using I^2 statistics. Publication bias was assessed by inspecting funnel plots, as well as Egger's test (linear regression method) and Begg's test (rank correlation method) [18,19]. Two-sided p values < 0.05 were considered statistically significant. Missing information was retrieved after communication with the corresponding authors.

3. Results

3.1. Literature Search and Inclusion of Studies

Four relevant meta-analyses on the impact of anakinra on the outcomes of hospitalized COVID-19 patients were identified [8–11]. Among the 28 studies included in these analyses (with significant overlap), four studies that reported adjusted hazard ratio for mortality were identified and included in our synthesis [20–23].

Regarding the updated literature search, among 1018 initially retrieved articles, one study was additionally identified to fulfill the inclusion criteria and was included in our analysis [24]. This study provided two hazard ratios for early and delayed administration of anakinra versus standard of care, respectively [24].

Finally, after a website search, the first placebo-controlled randomized trial on the effect of anakinra in hospitalized COVID-19 patients was identified, at a preprint version at the time of the search [25].

The main characteristics of the six included studies are shown in Table 1. The PRISMA 2020 checklist for the present meta-analysis is presented in the Supplementary File, Table S1. The PRISMA 2020 abstracts checklist is presented in the Supplementary File, Table S2. The PRISMA 2020 flow diagram for updated systematic reviews and meta-analyses study selection is presented in the Supplementary File, Figure S1.

Table 1. Main characteristics and findings of included studies.

Study	Design	*n*	Treated with Anakinra (%)	Intubated (%)	Male Sex (%)	Age (mean)	Symptoms Duration before Anakinra Administration (days; mean)	Baseline CRP (mg/L)	Oxygen Requirements (%)	HR for Death (95% CI) (Treated with Anakinra vs. Not)
Kyriazopoulou et al. [25]	Double-blind RCT	594	68	0	58	62	9	51	• No oxygen: 8% • Low-flow oxygen: 92%	0.45 (0.21, 0.98)
Kyriazopoulou et al. [23]	NR	260	50	0	63	64	7	47	• No oxygen: 45% • Oxygen: 55%	0.49 (0.25, 0.97)
Cavalli et al. [22]	NR	337	18	0	75	67	11	143	• Oxygen 82% • NIMV: 18%	0.45 (0.20, 0.99)
Pontali et al. (early) [24]	NR	107	59	7	69	63	9	87	• Not reported: 67% • NIMV: 24%	0.33 (0.10, 1.12)
Pontali et al. (late) [24]	NR	65	32	8	69	68	15	67	• IMV: 9%	0.82 (0.30, 2.27)
CORIMUNO-19 Collaborative group [21]	R	114	52	0	70	67	10	121	• Low-flow Oxygen: 100%	0.77 (0.33, 1.77)
Bozzi et al. [20]	NR	120	54	33	80	62	12	148	• Oxygen: 67% • IMV: 33%	0.18 (0.07, 0.50)

CI: confidence intervals; CRP: c-reactive protein; HR: hazard ratio; IMV: invasive mechanical ventilation; NIMV: non-invasive mechanical ventilation; NR: non-randomized; R: randomized non-controlled; RCT: randomized-controlled trial.

3.2. Data Synthesis

The meta-analysis of the six included studies (n = 1553, weighted age 64 years, male sex 66%, treated with anakinra 50%, intubated 3%) showed a pooled hazard ratio for death in patients treated with anakinra versus those who did not receive anakinra at 0.47 (95% confidence intervals [CI] 0.34, 0.65) (Figure 1). A 28-day mortality was the endpoint of interest in the majority of studies [20–22,25]. Most patients had moderate to severe COVID-19 (Table 1).

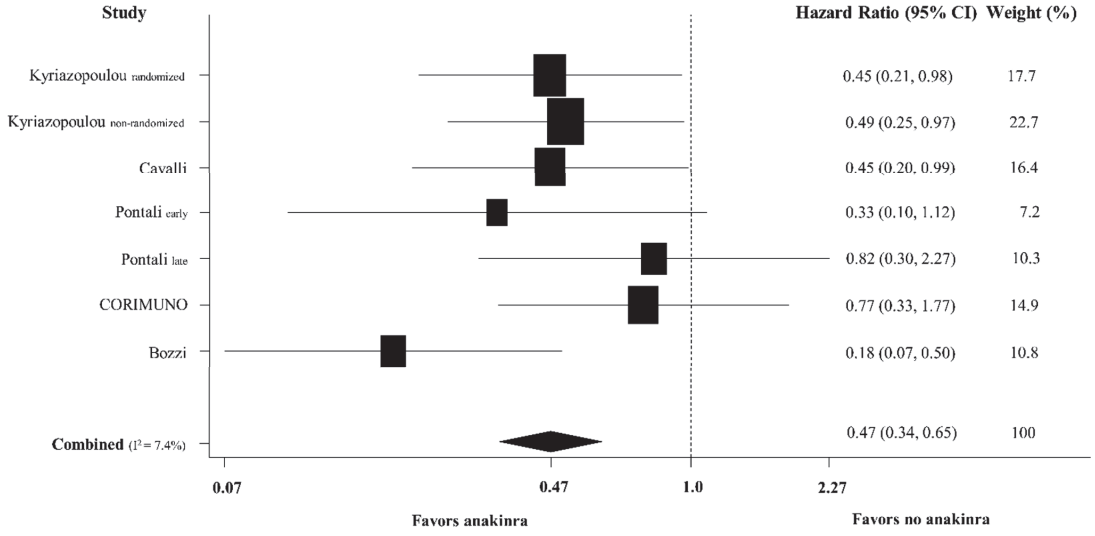

Figure 1. Forest plot of adjusted hazard ratios for death for patients treated with anakinra versus those who did not receive anakinra among hospitalized patients with COVID-19.

3.3. Sensitivity and Meta-Regression Analyses

Three sensitivity analyses were performed for robustness: (1) after excluding the only randomized placebo-controlled trial [25], the pooled adjusted hazard ratio remained the same at 0.47 (95% CI 0.33, 0.68); (2) after excluding the two randomized studies (a placebo-controlled and one standard of care-controlled study) [21,25], the pooled adjusted hazard ratio was similar at 0.42 (95% CI 0.28, 0.63); (3) after excluding a study that seemed to differ significantly from the others both in terms of percentage of intubated patients (33%) and in terms of the hazard ratio for mortality (0.18) [20], the pooled adjusted hazard ratio was 0.53 (95% CI 0.37, 0.74). A multivariate meta-regression analysis did not reveal any significant associations between the mean age (regression coefficient [RC] 0.17, 95% CI −0.19, 0.53), percentage of males (RC −0.05, 95% CI −0.33, 0.23), mean baseline C-reactive protein levels of the patients receiving anakinra (RC 0.001, 95% CI −0.04, 0.04), and mean time of administration since symptoms onset (RC 0.01, 95% CI −0.40, 0.42) among the included studies and the hazard ratios for death (all $p > 0.10$). In addition, there was no association between the daily dose of anakinra during the first three days of administration and the hazard ratios (RC −0.001, 95% CI −0.005, 0.002, $p = 0.45$) (the variable of the daily dose was not included in the multivariate meta-regression analysis due to insufficient observations).

3.4. Risk of Bias, Publication Bias and Certainty of the Evidence Assessment

All studies were deemed as having a low risk of bias. The assessment of the risk of bias of the included studies is presented in the Supplementary File, Table S3.

Egger's test and Begg's funnel plots did not reveal any small study effect ($p > 0.10$ for both) (Supplementary File, Figure S2).

The certainty of the evidence on the outcome of death was high and in favor of a beneficial effect of anakinra administration in hospitalized patients with COVID-19 (Supplementary File, Table S4).

4. Discussion

This updated meta-analysis showed about a 50% decrease in the adjusted risk of death in hospitalized patients with moderate-to-severe COVID-19 treated with anakinra compared with patients that did not receive anakinra.

Four meta-analyses have been previously conducted investigating the impact of anakinra treatment on the outcomes of hospitalized patients with COVID-19 [8–11]. These studies confirmed the safety profile of anakinra and further demonstrated a beneficial impact of this treatment in patients with mainly moderate to severe COVID-19 pneumonia along with increased inflammatory indices [8–11]. However, these meta-analyses included mainly observational studies and used unadjusted ratios for calculating pooled estimates [8–11]. A major methodological limitation inevitably accompanying observational studies is the fact that their results are influenced by the lack of randomization and the subsequent indication bias for each arm of treatment. Specifically, it seems that earlier or more aggressive and combination treatment or higher doses have been selectively administered to patients with critical COVID-19. However, the effectiveness of such interventions might be muffled by the adverse outcome in cases with irreversible establishment of severe complications [26]. In addition, the selection of candidate patients and the optimal time of each intervention might also play a major role in preventing adverse events [27]. The meta-analysis by Kyriazopoulou et al. had the advantage of individual data meta-analysis (and thus of adjusted analyses) in a subsample and confirmed the findings of the unadjusted analyses [11].

In our updated meta-analysis only high-quality studies providing adjusted ratios were included. Most studies were non-randomized observational studies designed to compare the standard of care treatment plus anakinra versus the standard of care treatment alone [20,22–24]. One study was randomized but not placebo controlled [21] and only one study was a placebo controlled double-blind trial [25]. Interestingly, in one observational study both early and late anakinra administration were investigated [24]. In the early administration group, anakinra was administered after a mean of 9 days of symptoms initiation, while in the late administration group anakinra was administered after 15 days of symptoms initiation. Early administration tended to have a greater beneficial effect compared with late administration; however, both hazard ratios were not at the level of statistical significance (0.33 (95% CI 0.10, 1.12) and 0.82 (95% CI 0.30, 2.27), respectively). Although the sample size was limited and robust conclusions cannot be drawn, it appears that the proper time of anakinra administration might play an important role.

Another important point regarding COVID-19 therapeutics is the proper patient selection for each therapeutic regimen. Selection criteria in most studies included increased inflammation indices and/or severe COVID-19. Indeed, the baseline characteristics of the included studies indicated that most patients needed any type of oxygen supply, and their admission CRP levels were increased. Thus, in most cases a moderate to severe pneumonia accompanied by a hyperinflammation syndrome had already been established. Anakinra, an interleukin-1 inhibitor, plays an important role in the inhibition of the cytokine storm cascade and can apparently offer benefits to this group of patients. Interestingly, in the studies by the research group of Giamarellos-Bourboulis [23,25] a biomarker indicating a high probability of future hyperinflammation syndrome (soluble urokinase plasminogen activator receptor (suPAR)) was used to guide therapeutic decisions, possibly allowing the administration of anakinra earlier in the course of COVID-19 before clinical establishment of severe disease. In the meta-analysis by Kyriazopoulou et al. subgroup sensitivity analyses were performed and showed that anakinra was more effective in mortality reduction in patients with CRP higher than 100 mg/L [11]. In our meta-regression analysis, there was no association between baseline CRP levels in patients receiving anakinra and hazard

ratios, but it should be highlighted that in general, a meta-regression analysis examines the associations between the outcome and several characteristics which are aggregate and summarized at the level of the study which in turn introduces ecological bias. A tailored and individualized approach to indicate (i) the optimal time of administration and (ii) the group of patients that will benefit the most, appears to be of paramount importance.

One of the main limitations of the current analysis is the paucity of randomized controlled trials on the role of anakinra on the outcomes of patients with COVID-19. However, the inclusion of studies that provided adjusted hazard ratios might at least partially compensate for this limitation. Furthermore, the findings were consistent in several sensitivity analyses.

5. Conclusions

Anakinra seems to have a beneficial role as a therapeutic agent for selected patients with COVID-19, especially those with moderate or severe pneumonia accompanied by increased levels of inflammatory indices. Findings of previous observational studies and meta-analyses of unadjusted ratios were confirmed by the current analysis of adjusted hazard ratios derived from high quality (low risk of bias) studies. Additional placebo-controlled randomized trials are needed to further evaluate the efficacy of this intervention.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10194462/s1, Figure S1: The PRISMA 2020 flow diagram for updated systematic reviews and meta-analyses study selection, Figure S2: The assessment of the risk of bias of the included studies, Table S1: The PRISMA 2020 Checklist for the present meta-analysis, Table S2: The PRISMA 2020 for Abstracts Checklist for the present meta-analysis, Table S3: The assessment of the risk of bias of the included studies for the present meta-analysis using a checklist from Joanna Briggs Institute Critical Appraisal Checklists for Cohort Studies, Table S4: Certainty of the evidence on the outcome of death for the present meta-analysis using the GRADE approach.

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Early Administration of Bamlanivimab in Combination with Etesevimab Increases the Benefits of COVID-19 Treatment: Real-World Experience from the Liguria Region

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Abstract: Monoclonal antibodies, such as bamlanivimab and etesevimab combination (BEC), have been proposed for patients with mild or moderate coronavirus disease 2019 (COVID-19). However, few studies have assessed the factors associated with the early administration of BEC or the impact of early BEC treatment on the clinical evolution of the patients. We conducted a retrospective cohort study of all adults with COVID-19 who received BEC at three institutions in the Liguria region. The primary endpoint was to investigate the clinical variables associated with early BEC infusion. Secondary endpoints were 30-day overall mortality and the composite endpoint of requirement of hospital admission or need for supplemental oxygen during the 30-day follow-up period. A total of 127 patients (median age 70 years; 56.7% males) received BEC. Of those, 93 (73.2%) received BEC within 5 days from symptoms onset (early BEC). Patients with a higher Charlson comorbidity index were more likely to receive early treatment (odds ratio (OR) 1.60, 95% confidence interval (CI) 1.04–2.45; $p = 0.03$) in contrast to those reporting fever at presentation (OR 0.26, 0.08–0.82; $p = 0.02$). Early BEC was associated with lower likelihood of hospital admission or need for supplemental oxygen (OR 0.19, 0.06–0.65; $p = 0.008$). Five patients who received early BEC died during the follow-up period, but only one of them due to COVID-19-related causes. Early bamlanivimab and etesevimab combination was more frequently administered to patients with a high Charlson comorbidity index. Despite this, early BEC was associated with a lower rate of hospital admission or need for any supplementary oxygen compared to late administration. These results suggest that efforts should focus on encouraging early BEC use in patients with mild–moderate COVID-19 at risk for complications.

Keywords: COVID-19; bamlanivimab; etesevimab; hospital admission

1. Introduction

Coronavirus disease 2019 (COVID-19) has resulted in a massive strain on healthcare infrastructures [1–4], with more than 350,000 patients requiring hospital admissions in the United States alone according to Center for Disease Control and Prevention (CDC) reports [5]. Older age [4,6], obesity [6,7] and certain medical conditions, such as hypertension, chronic obstructive pulmonary disease, chronic kidney disease or immunological disease, have been associated with higher disease severity and need for hospital admission [2–4,8,9].

Accordingly, there is need for effective and well-tolerated treatments that can halt the progression of COVID-19 at this early phase. Bamlanivimab and etesevimab are potent anti-spike neutralizing monoclonal antibodies that were derived from two separate patients who recovered from America and China, respectively [10,11]. Recent studies demonstrated that administering bamlanivimab and etesevimab combination (BEC) for mild-to-moderate COVID-19 reduces the viral load and duration of symptoms as well as possibly preventing hospitalizations [12,13].

The pathophysiology of COVID-19 suggests that inhibiting viral replication as early as possible after infection onset could possibly reduce the intensity of clinical symptoms [14,15]. Thus, our main objectives were to investigate (1) the clinical variables associated with the receipt of early BEC administration and (2) whether timely BEC administration resulted in any differences in hospital admission or other important outcomes among adults with COVID-19.

2. Materials and Methods

2.1. Study Design and Setting

This multicenter retrospective cohort study was performed across the three major tertiary hospitals of the Liguria region (San Martino Policlinico hospital, Sant 'Andrea hospital and Sanremo hospital) between 18 March 2021 and 18 April 2021. These institutions serve approximately 700,000 inhabitants altogether, offer readily available infectious disease consultation services and are referral centers for COVID-19.

2.2. Inclusion Criteria

Non-hospitalized adults (aged $\geq$18 years) were eligible for inclusion if they (i) had a confirmed SARS-CoV-2 infection by polymerase chain reaction; (ii) had signs/symptoms attributable to COVID-19 for $\leq$10 days prior to the day of BEC infusion; (iii) received BEC for mild or moderate disease and (iv) had at least one characteristic (body mass index, BMI > 35 kg/m^2) or underlying medical condition (renal failure requiring hemodialysis treatment; poorly controlled diabetes mellitus II, with glycated hemoglobin $\geq$75 mmol/mol or diabetes-related organ damage; primary or acquired immunodeficiency; cardiovascular disease, cereberovascular disease, including hypertension with secondary organ damage; chronic obstructive pulmonary disease or other chronic pulmonary diseases) associated with an increased risk of severe COVID-19 [16]. Hospitalized patients were excluded from the present study unless they had been hospitalized for reasons other than COVID-19 (e.g., elective surgical procedure) and otherwise met all inclusion criteria.

2.3. Data Collection and Study Definitions

The following data were collected from the patients' medical records at the baseline (i.e., at the time of BEC treatment): age in years; gender; underlying disease (both separately and summarized by means of the Charlson comorbidity index [17]); date of illness onset; signs and symptoms (fever, cough, shortness of breath, arthralgia–myalgia, asthenia, headache, diarrhea and ageusia or anosmia). As for clinical evolution, the following

variables were assessed during a 30-day follow-up period starting from BEC infusion: need for hospital admission, need for supplementary oxygen and survival status or death.

The date of illness onset was defined as the date when signs or symptoms related to disease were first noticed. Patients were considered to have mild COVID-19 if there was evidence of mild symptoms (e.g., fever, cough) without dyspnea [18]. Moderate illness was defined as clinical or radiological evidence of lower respiratory tract infection with oxygen saturation $\geq$94% [18]. According to the Authorization for Emergency Use (EUA) of monoclonal antibodies by the Food and Drug Administration (FDA), the following characteristics or underlying medical conditions were considered to be associated with an increased risk for severe illness from COVID-19: BMI $\geq$35 kg/m^2, chronic kidney disease, diabetes, $\geq$65 years of age, immunosuppressed, $\geq$55 years of age with cardiovascular disease (CVD), hypertension or chronic obstructive pulmonary disease (COPD)/chronic respiratory disease [16]. Supplemental oxygen use was defined as the delivery of oxygen by any modality, including nasal cannula, mask, non-invasive positive pressure ventilation or mechanical ventilation, and was recorded if sustained for >4 hours. Supplemental oxygen was administered per standardized clinical protocols at all centers only if patients presented PaO_2 <60 mmHg at rest in ambient air.

Secondary immunodeficiency was defined by the presence of an active solid or hematological cancer, solid organ or stem cell transplantation, HIV infection or autoimmune disease requiring immunosuppressive therapy.

Death was considered to be related to COVID-19 when (i) it resulted from a clinically compatible severe/critical COVID-19 illness, (ii) there was no clear alternative cause of death and (iii) there was no period of complete recovery between the illness and death.

2.4. Main Outcomes Measures

For all patients, follow-up ended 30 days after BEC infusion. Outcome data were obtained from the hospital medical charts or by a virtual visit performed by telephoning participants at their home. For the purpose of this study, the infusion of BEC within 5 days from illness onset was a priori (before starting the data analysis) considered an early treatment. The primary endpoint was to investigate the association between the receipt of an early BEC infusion and the clinical variables of the patients collected at the time of COVID-19 diagnosis. Secondary endpoints were 30-day overall mortality and the composite endpoint of requirement of hospital admission or need for supplemental oxygen during the 30-day follow-up period.

2.5. Infusion of Bamlanivimab and Etesevimab Combination

According to the manufacturer's instruction, bamlanivimab and etesevimab were administered together as a single intravenous infusion; the authorized dose of 700 mg of bamlanivimab and 1400 mg of etesevimab was ensured for all patients [16]. No dose adjustment was required for patients with renal or hepatic impairment.

2.6. Statistical Analysis

Quantitative variables are expressed as median and interquartile range (IQR), and qualitative variables as number and percentage. Qualitative variables were compared using the χ^2 and Fisher's exact tests, as appropriate. Quantitative variables were compared using the Wilcoxon rank sum test. Missing data for each variable were excluded from the denominator. Logistic regression was used (i) to determine the independent baseline patient-level factors (i.e., variables collected at the time of BEC infusion) associated with receipt of early BEC and (ii) to determine the association between early BEC and poor outcome. For all regression analyses, we first performed a univariate analysis in order to identify the association with each outcome of interest. Gender and sex were forced into each model. Variables with a p-value of $\leq$0.20 in each univariable analysis were retained in the final models if they remained significantly associated with the outcome at a p-value <0.05. Odds ratio (ORs) were estimated for logistic regressions, and 95% confidence

intervals (CIs) were estimated to evaluate the strengths of the association. Analyses were conducted using SPSS for Windows, version 20.0 (SPSS Inc., Chicago, IL, USA).

2.7. Ethical Considerations

The study protocol was approved by the Ethics Committee of Liguria Region (N.CER Liguria 114/2020-ID10420). Written informed consent was provided by all participants in the study.

3. Results

During the study period, 127 patients with mild or moderate COVID-19 received treatment with bamlanivimab and etesevimab combination and were included in the present analysis. The clinical characteristics of the study population are shown in Table 1. The median (IQR) age was 70 (59–78), and 56.7% were males. The majority of patients had multiple comorbidities with a median (IQR) Charlson comorbidity index of 1 (0–2). Of these, 50.4% had a history of cardiovascular disease and 22.0% had chronic obstructive lung disease. Thirty-three patients (26.0%) had a BMI equal to or higher than 35 kg/m2. The most reported symptoms of COVID-19 illness at initial presentation were fever (59.8%), cough (50.4%) and asthenia (27.6%).

Table 1. Comparison of demographics and baseline clinical data between patients receiving early (n = 93) or late (n = 34) bamlavinimab and etesevimab combination therapy (univariate and multivariate analysis).

		Univariate Analysis			Multivariate Analysis	
Characteristics	**Overall n = 127 (%)**	**Late Group n = 34 (%)**	**Early Group n = 93(%)**	**p Value**	**OR (95% CI)**	**p Value**
Age, years	70 (59–78)	69 (51–76)	71 (62–78)	0.18	1.21 (0.47–3.31)	0.68
Sex, male	72 (56.7)	18 (52.9)	54 (58.1)	0.68	1.00 (0.97–1.04)	0.65
Comorbidities						
Cardiovascular disease	64 (50.4)	14 (41.2)	50 (53.8)	0.23	-	
Obesity (BMI > 35)	33 (26.0)	8 (23.5)	25 (26.9)	0.82	-	
Chronic obstructive lung disease	28 (22.0)	4 (11.8)	24 (25.8)	0.14	2.75 (0.76–9.93)	0.12
Diabetes mellitus	22 (17.3)	5 (14.7)	17 (18.3)	0.79	-	
Cerebrovascular disease	24 (18.9)	9 (26.5)	15 (16.1)	0.21	-	
Secondary immunodeficiency	19 (15.0)	5 (14.7)	14 (15.1)	1.00	-	
Chronic kidney disease	11 (8.7)	4 (11.8)	7 (7.5)	0.48		
Charlson comorbidity index	1 (0–2)	0 (0–1)	1 (1–3)	0.003	1.60 (1.04–2.45)	0.03
McCabe Scale					-	-
Non-fatal	109 (85.4)	34 (100)	75 (80.6)	0.003	-	
Ultimately fatal	14 (11.0)	0	14 (15.1)	0.02		
Rapidly fatal	4 (3.1)	0	4 (4.3)	0.57		

BMI: Body Mass Index.

3.1. Comparison of Early Versus Late BEC

Almost three-quarters (75.6%) of all patients receiving BEC presented initially to the outpatient clinic, whereas the remaining patients received the monoclonal antibodies as inpatients because the diagnosis of COVID-19 was made during a hospitalization required for other medical reasons. Overall, the median time from symptoms onset to the BEC therapy was 4 days (IQR 2–6 days), with 93 patients (73.2%) receiving BEC within 5 days of symptoms onset. Of the remaining patients, 34 (26.8%) received treatment >5 days after symptoms onset. Univariate and multivariate analyses of factors associated with early BEC administration are outlined in Table 1. In the multivariate analysis, higher Charlson comorbidity index was the only factor associated with early BEC administration (OR 1.60, 95% CI 1.04–2.45; p = 0.03). Conversely, fever at presentation was inversely associated with early BEC (OR 0.26, 95% CI 0.08–0.82; p = 0.02).

3.2. Secondary Endpoints

In total, 19 out of 127 patients (15.0%) required hospital admission or supplemental oxygen during the 30-day follow-up period (Table 2). Factors associated with the need for hospital admission or supplemental oxygen in the univariate analysis (Table 3) were older age ($p = 0.02$), cerebrovascular disease ($p = 0.01$) and late BEC administration ($p = 0.01$). In the multivariate analysis (Table 3), early BEC remained the only factor associated with lower likelihood for hospital admission or need for supplemental oxygen (OR 0.19; 95% CI 0.06–0.65; $p = 0.008$). In contrast, shortness of breath at presentation was significantly associated with higher likelihood for hospital admission or need for supplemental oxygen (OR 5.58; 95% CI 1.03–30.45; $p = 0.04$).

Table 2. Comparison of care setting and clinical outcomes of patients receiving early ($n = 93$) or late ($n = 34$) bamlavinimab and etesevimab combination therapy.

Characteristics	Overall $n = 127$ (%)	Late Group $n = 34$ (%)	Early Group $n = 93$(%)	p Value
Care setting				
Outpatient clinic	96 (75.6)	31 (91.2)	65 (69.9)	0.18
Hospital ward	31 (24.4)	3 (8.8)	28 (30.1)	
Need for hospital admission	10/97 (10.3)	7/31 (22.6)	3/66 (4.5)	0.01
Need for supplemental oxygen				
Any supplemental oxygen	17 (13.4)	9 (26.5)	8 (8.6)	0.02
Non-invasive positive pressure ventilation	4 (3.1)	3 (8.8)	1 (1.1)	0.06
Mechanical ventilation	0	0	0	-
Poor clinical outcome *	19 (15.0)	10 (24.9)	9 (9.7)	0.01
30-day overall mortality	5 (3.9)	0	5 (5.4)	0.32

* For patients receiving BEC in an outpatient clinic, poor clinical outcome was observed in 7 out of 31 in the late group (22.6%) versus 4 out of 65 in the early group (6.2%, $p = 0.03$). For patients receiving BEC in the hospital ward poor clinical outcome was observed in 3 out of 3 (100%) patients in the late group versus 5 out of 28 patients in the early group (17.9%, $p = 0.01$).

Table 3. Univariate and multivariate analysis of factors associated with poor clinical outcome.

	Univariate Analysis			Multivariate Analysis	
Characteristics	Improvement $n = 108$ (%)	Poor Clinical Outcome $n = 19$ (%)	p Value	OR (95% CI)	p Value
Age, years (median, IQR)	70 (57–76)	76 (68–83)	0.02	1.03 (0.98–1.09)	0.20
Sex, male	60 (55.6)	12 (63.2)	0.62	2.35 (0.71–7.77)	0.16
Underlying disease					
Cardiovascular disease	53 (49.1)	11 (57.9)	0.62		
Obesity (BMI > 35)	31 (28.7)	2 (10.5)	0.15	0.45 (0.06–3.02)	0.41
Diabetes mellitus	18 (16.7)	4 (21.1)	0.74		
Chronic obstructive lung disease	22 (20.4)	6 (31.6)	0.36		
Cerebrovascular disease	16 (14.8)	8 (42.1)	0.01	2.84 (0.84–9.63)	0.09
Chronic kidney disease	9 (8.3)	2 (10.5)	0.67		
Secondary immunodeficiency	17 (15.7)	2 (10.5)	0.73		
Charlson comorbidity index (median, IQR)	1 (0–2)	1 (1–3)	0.27		
Mc Cabe Scale					
Non-fatal	94 (87.0)	15 (78.9)	0.47		
Ultimately fatal	12 (11.1)	2 (10.5)	1		
Rapidly fatal	2 (1.9)	2 (10.5)	0.10	4.38 (0.42–45.06)	0.21

Table 3. *Cont.*

Characteristics	Univariate Analysis			Multivariate Analysis	
	Improvement n = 108 (%)	Poor Clinical Outcome n = 19 (%)	p Value	OR (95% CI)	p Value
Signs and symptoms					
Fever (Temperature > 37.3)	64 (59.3)	12 (63.2)	0.80		
Cough	53 (49.1)	11 (57.9)	0.62		
Asthenia	30 (27.8)	5 (26.3)	1		
Headache	19 (17.6)	3 (15.8)	1		
Arthralgia-myalgia	18 (16.7)	1 (5.3)	0.30		
Diarrhea	8 (7.4)	3 (15.8)	0.21		
Ageusia and anosmia	9 (8.3)	1 (5.3)	1		
Dyspnoea	6 (5.6)	3 (15.8)	0.13	5.58 (1.03–30.45)	0.04
Early BEC ($\leq$ 5 days)	84 (77.8)	9 (47.4)	0.01	0.19 (0.06–0.65)	0.008

BEC: Bamlanivimab and Etesevimab Combination.

After 30 days from BEC infusion, the overall mortality rate of the study population was 3.9% (5/127). All deceased patients acquired COVID-19 during their hospital stay because of other medical reasons. All of them received early BEC infusion, but only one death was considered to be COVID-19-related (Supplementary Table S1).

4. Discussion

These results suggest that bamlanivimab and etesevimab combination is effective for the treatment of patients with mild and moderate COVID-19 who are at high risk for disease progression. In addition, our study provides evidence to suggest that greater benefits can be gained from treating such patients within the first 5 days from illness onset, which included reduced hospital admission and less need for any supplemental oxygen.

To our knowledge, this is the first study performed in a non-selected group of patients specifically focusing on factors associated with the receipt of early bamlanivimab and etesevimab combination therapy and the effects of early treatment on clinical outcomes. Our study includes a relatively large sample size of consecutive patients across three different hospitals and can therefore be considered representative for the current clinical practice in mild and moderate COVID-19.

Bamlanivimab and etesevimab are two monoclonal antibodies that are specifically directed against different but overlapping receptor binding sites of the spike protein of SARS-CoV-2, thus blocking its attachment to the human ACE2 receptor [10,11]. Previous studies evaluating bamlanivimab and etesevimab administered together have demonstrated that such combination significantly decreased SARS-CoV-2 log viral load as well as the need for hospital admission [12,13], leading to their approval from the EUA by the Food and Drug Administration for the treatment of mild and moderate COVID-19 in the outpatient setting [16]. Nevertheless, post-marketing information is very scarce [19–23], and at present, data regarding the correct timing for BEC infusion are lacking.

To the best of our knowledge, this study is the first to report the clinical use of bamlanivimab together with etesevimab in daily clinical practice, a few months after the drugs have been introduced in Italy. In our experience, the rate of hospital admission after BEC infusion was low (11.0%) and similar to the results obtained in other previous clinical experiences focusing on bamlanivimab alone, in which the percentage of hospital admission was reported to be up to 10% of the cases [20–23]. The reduction in the need for subsequent health resource utilization remained low despite the higher proportion of older persons with comorbidities included in the present study [20–23].

We are also the first to demonstrate that time to monoclonal antibodies administration, defined as the time from symptoms onset to monoclonal antibodies infusion, may have an important impact on clinical outcomes. Based on our experience, a larger reduction

in the requirement of any supplemental oxygen and hospital admission could be seen in patients who received early (<5 days) monoclonal antibodies. Accordingly, we suggest that monoclonal antibodies treatment should be started as soon as the diagnosis of SARS-CoV-2 has been established, theoretically within the first five days. Importantly, we should acknowledge that treatment bias is unlikely to explain our findings, as patients with multiple and severe underlying diseases were more likely to receive early BEC infusion.

Translating the main results of our study into clinical practice may be challenging; however, we believe that strategies aimed at early COVID-19 diagnosis and rapid access to monoclonal antibody therapies should be pursued [24]. Among them, infusion centers, pop-up sites or in-home visits should be considered and may be safer from a public health perspective (because of the reduced risk of potentially spreading SARS-CoV-2 in the community) while offering more convenience to the patient. In addition, if future studies will support our findings, we believe that early treatment with monoclonal antibodies should gather similar policy attention to that applied for early antiviral treatment for pandemic influenza [25].

The main limitation of our study is the retrospective analysis of the effect of bamlanivimab and etesevimab combination treatment timing on clinical outcomes, with the potential for residual confounding. Moreover, our study did not include information about the COVID-19 variants [26] as well as data on SARS-CoV-2 vaccination, which could have a significant impact on the clinical evolution of the patients.

5. Conclusions

In conclusion, this real-world study adds to the record of the benefits of bamlanivimab and etesevimab combination for patients with mild or moderate COVID-19 by demonstrating that earlier interventions increased treatment efficacy. Since monoclonal antibodies are relatively safe drugs [12,13], we encourage their early use among all patients with mild–moderate COVID-19 at risk for complications.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10204682/s1, Table S1: Description of patients who died after BEC infusion.

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or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Review

Immunomodulation and Reduction of Thromboembolic Risk in Hospitalized COVID-19 Patients: Systematic Review and Meta-Analysis of Randomized Trials

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Abstract: Background: We aimed to investigate the potential beneficial effect of immunomodulation therapy on the thromboembolic risk in hospitalized COVID-19 patients. Methods: We searched PubMed and Scopus for randomized trials reporting the outcomes of venous thromboembolism (VTE), ischemic stroke or systemic embolism, myocardial infarction, any thromboembolic event, and all-cause mortality in COVID-19 patients treated with immunomodulatory agents. Odds ratios (OR) and 95% confidence intervals (CI) were calculated using the Mantel–Haenszel random effects method. Results: Among 8499 patients hospitalized with COVID-19, 4638 were treated with an immunomodulatory agent, 3861—with usual care only. Among the patients prescribed immunomodulatory agents, there were 1.77 VTEs per 100 patient-months compared to 2.30 among those treated with usual care (OR: 0.84, 95% CI: 0.61–1.16; I^2: 0%). Among the patients who received an interleukin 6 (IL-6) antagonist, VTEs were reported in 12 among the 1075 patients compared to 20 among the 848 receiving the usual care (OR: 0.52, 95% CI: 0.22–1.20; I^2: 6%). Immunomodulators as an add-on to usual care did not reduce the risk of stroke or systemic embolism (OR: 1.10, 95% CI: 0.50–2.40; I^2: 0%) or of myocardial infarction (OR: 1.06, 95% CI: 0.47–2.39; I^2: 0%) and there was a nonsignificant reduction in any thromboembolic event (OR: 0.86, 95% CI: 0.65–1.14; I^2: 0%). Conclusions: We did not identify a statistically significant effect of immunomodulation on prevention of thromboembolic events in COVID-19. However, given the large effect estimate for VTE prevention, especially in the patients treated with IL-6 antagonists, we cannot exclude a potential effect of immunomodulation.

Keywords: COVID-19; thromboembolism; tocilizumab; anakinra; hydroxycholoroquine; immunomodulation

1. Introduction

Soon after the emergence of the SARS-CoV-2 pandemic and its associated COVID-19 disease, a high prevalence of thrombotic events, mostly consisting of venous thromboembolism (VTE), were observed in patients hospitalized with COVID-19. Meta-analyses of observational studies identified VTE prevalence ranging from 23.9% up to 40.3% in the patients who had undergone ultrasound screening [1,2]. Such thrombotic events occurred mainly in patients with severe disease, although they were also observed in mildly symptomatic or asymptomatic patients. It was postulated that thrombotic complications associated with COVID-19 were attributable, at least in part, to immune mechanisms that led to a hypercoagulable state [3–5].

Several proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL) 1β and chemotactic cytokines (e.g., IL-8 and macrophage chemoattractant protein-1 (MCP-1)) are upregulated during COVID-19, leading to a sustained increase in IL-6 levels [6–10]. The latter seems to play a major role in the maintenance of the virus-driven inflammatory process. Alongside this inflammatory process, a prothrombotic effect was postulated through multiple mechanisms that include endothelial inflammation, destabilization of atherosclerotic plaques, release of the von Willebrand factor, and upregulation of coagulation and complement pathways [11]. These pathogenic mechanisms could lead to the formation of microthrombi in various vascular beds and, eventually, the development of clinically overt venous and arterial thrombosis.

Several immunomodulatory agents targeting IL-6 and IL-1 blockade were proposed as potential therapeutic options for severe COVID-19 to inhibit the proinflammatory effect and its consequences on pulmonary and other organ function [12–21]. Our group recently showed in the SAVE study that early soluble urokinase plasminogen activator receptor (suPAR)-guided anakinra use decreased the severe respiratory failure but did not affect the thromboembolic risk [16]. Recent meta-analyses of randomized and observational studies exploring the effect of anakinra and tocilizumab in patients with COVID-19 showed a favorable effect on clinical outcomes, including mortality risk [22–26]. However, the impact of immunomodulation treatments on the occurrence of thromboembolic events in COVID-19 patients remains uncertain, representing a clinically important gap in knowledge given the biologically plausible association between COVID-19-mediated inflammation and thrombosis.

Against this background, we performed a systematic review and meta-analysis of randomized controlled trials (RCTs) to investigate the effect of immunomodulatory agents on thromboembolic events in patients hospitalized with COVID-19.

2. Materials and Methods

2.1. Search Strategy and Inclusion Criteria

We searched PubMed and Scopus until 16 October 2021 for RCTs of immunomodulatory agents in COVID-19 reporting thromboembolic events. We used search items "hydroxychloroquine, corticosteroids, dexamethasone, hydrocortisone, prednisolone, interleukin-6 inhibitor, tocilizumab, sarilumab, siltuximab, interleukin-1 inhibitor, canakinumab, anakinra, complement inhibitor, vilobelimab, JAK-2 inhibitors, baricitinib, IVIG, TNFa inhibitor, interferon gamma, GM-CSF" and "coronavirus or COVID-19 or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)" and "trial or randomized". In addition, we searched the references of the related letters, reviews, and editorials to identify other potentially eligible studies. To be eligible for the analysis, the studies should have been RCTs, published as full-text articles in English, and provided data on venous and arterial thromboembolic events in patients hospitalized with COVID-19. RCTs of several other

immunomodulatory agents not providing data on the incidence of thromboembolic events and pre-prints were not included in the analysis. This work was performed according to the PRISMA statement [27] and was submitted in PROSPERO (ID: CRD42021230346).

2.2. Outcomes, Data Extraction, and Assessment of the Risk of Bias

The following outcomes were assessed: venous thromboembolism (i.e., pulmonary embolism or deep vein thrombosis), myocardial infarction, ischemic stroke or systemic embolism, and the composite outcome of any thromboembolic event (TE) as reported in individual studies. Among the included studies, we assessed by means of a sensitivity analysis the outcome of mortality to explore potential correlations between thromboembolic events and mortality. In order to overcome the potential effect of hydroxychloroquine or corticosteroids which have been used as usual care in some studies, we prespecified one sensitivity analysis excluding hydroxychloroquine and corticosteroids treatment. Additionally, we assessed the outcome of pulmonary embolism among patients with VTE. Eligible studies were assessed independently by two authors (M.F. and P.T.), and the data were extracted using prespecified criteria and collection methods. An assessment of the risk of bias was performed by the same investigators with the use of the Cochrane Collaboration's tool focusing on sequence generation, allocation concealment, blinding, addressing incomplete outcome data, selective reporting and presence of other bias. Any discrepancy or uncertainty was resolved by consensus or discussion among all authors.

2.3. Statistical Analysis

The data were analyzed on an intention-to-treat basis. Odds ratios (OR) and 95% confidence intervals (CI) were calculated for each outcome using the Mantel–Haenszel random effects method. Heterogeneity between trials was assessed by measuring inconsistency using the I^2 index which measures the proportion of the variability in effect estimates that can be attributed to heterogeneity rather than chance. I^2 was calculated as follows: $I^2 = 100\% \times (Q - df)/Q$, where Q is the Cochran heterogeneity statistic and df is the degree of freedom. A value of 0% indicates no observed heterogeneity, and larger values show increasing heterogeneity [28]. The median follow-up duration was calculated using the follow-up duration reported in each trial.

We prespecified a subgroup analysis based on the immunomodulatory agent used in each study. Differences in pooled effect sizes between the subgroups were compared with a test of interaction (Cochran's Q test).

All the analyses were performed with Review Manager 5 (RevMan) version 5.3 (Copenhagen, Denmark: The Nordic Cochrane Centre, The Cochrane Collaboration, 2011).

3. Results

The initial literature search yielded 1875 potentially eligible articles, of which 22 met the inclusion criteria [12–14,29–47]. The flow diagram of study selection is presented in Supplementary Figure S1. The main characteristics of the included trials are summarized in Table 1. Overall, we did not identify the major risk of bias, except in two studies where we identified high risk of bias in allocation concealment and blinding of participants [12,14] (Supplementary Figures S2 and S3).

Among 8499 patients hospitalized with COVID-19, 4635 were treated with an immunomodulatory agent as an add-on to usual care, and 3,861 were treated with usual care only. The median (IQR) follow-up period of the included studies was 28 (21–28) days.

Table 1. Baseline characteristics of included studies.

Study	Drug	Included Patients	ICU	Male Gender	Age		Follow-Up (Days)	Standard of Care
					On Treatment	Control		
Stone et al., 2020 [35]	Tocilizumab	243	194	141	61.6 (46.4–69.7)	56.5 (44.7–67.8)	28	remdesivir
Vlaar A PJ et al., 2020 [38]	Vilobelimab	30	18	22	58	63	28	chloroquine, ganciclovir, azithromycin, nadroparin, LMWH, ASA, apixaban, rivaroxaban, clopidogrel, tinzaparin, dabigratan, edoxaban
Hermine O et al., 2020 [12]	Tocilizumab	130	0	88	64.0 (57.1–74.3)	63.3 (57.1–72.3)	28	antibiotics, antiviral agents, corticosteroids, vasopressor support, anticoagulants
Salvarani C et al., 2020 [14]	Tocilizumab	126	0	77	61.5 (51.5–73.5)	60 (54.0–69.0)	30	NA
Cavalcanti et al., 2020 [31]	HCQ	448	62	265	51.3 (36.8–65.8)	49.9 (34.8–65)	15	antibiotics, antiviral agents, corticosteroids
Dequin et al., 2020 [30]	Hydrocortisone	149	149	104	63.1 (51.5–70.8)	66.3 (53.5–72.7)	28	NA
Angus et al., 2020 [29]	Hydrocortisone	379	379	273	59.9 (47.7–72.1)	59.9 (45.3–74.5)	21	NA
Self et al., 2020 [32]	HCQ	479	96	267	58 (45–69)	57 (43–68)	28	NA
Tomazini et al., 2020 [40]	Dexamethasone	299	299	187	60.1 (15.8)	62.7 (13.1)	29	NA
Veiga et al., 2021 [36]	Tocilizumab	129	NA	88	57.4 (15.7)	57.5 (13.5)	29	HCQ, azithromycin, corticosteroids, antibiotics
Tharaux et al., 2021 [37]	Anakinra	114	NA	80	67 (55.5–74.3)	64.9 (59.5–78.3)	90	antibiotics, antiviral agents, corticosteroids, vasopressor support, anticoagulants
Salama et al., 2021 [13]	Tocilizumab	377	58	223	56 (14.3)	55.6 (14.9)	60	dexamethasone, remdesivir
Rosas I et al., 2021 [34]	Tocilizumab	438	0	306	60.9 (14.6)	60.6 (13.7)	60	remdesivir, glucocorticoids, convalescent plasma, supportive care

Table 1. *Cont.*

Study	Drug	Included Patients	ICU	Male Gender	Age		Follow-Up (Days)	Standard of Care
					On Treatment	Control		
Gordon et al., 2021 [33]	Tocilizumab + Sarilumab	865	865	629	61.7 (12.7)	61.1 (12.7)	90	corticosteroids, remdesivir, COVID-19 IG, anticoagulants, macrolides, antiplatelet, statins
Soin et al., 2021 [39]	Tocilizumab	180	118	152	56 (47–63)	54 (43–63)	28	corticosteroids, remdesivir
Kalil et al., 2021 [41]	Baricitinib	1033	NA	652	55.4 (15.7)	55 (15.4)	29	corticosteroids, remdesivir
Ali et al., 2021 [42]	IVIG	50	NA	35	55.9 (1.34)	59.1 (12.1)	28	remdesivir, enoxaparin, antibiotic, dexamethasone/ methylprednisolone
Caricchio et al., 2021 [44]	Canakinumab	454	0	267	59 (49–69)	57 (50–68)	29	heparin, dexamethasone, azithromycin, remdesivir, HCQ, convalescent plasma
Dubee et al., 2021 [43]	HCQ	250	0	121	76 (60–85)	78 (57–87)	28	azithromycin, other antibiotics, lopinavir-ritonavir, corticosteroids
Kyriazopoulou et al., 2021 [45]	Anakinra	594	42	344	62 (11.4)	61.5 (11.3)	28	dexamethasone, LMWH, remdesivir, antibiotics
Marconi et al., 2021 [46]	Baricitinib	1525	0	963	57.8 (14.3)	57.6 (13.8)	28	dexamethasone, remdesivir
Guimaraes et al., 2021 [47]	Tofacitinib	289	54	188	55 (14)	57 (14)	28	glucocorticosterotds, antibiotics, remdesivir

HCQ, Hydroxycloroquinw; NA, not applicable; IVIG, intravenous immunoglobulin; LMWH, low molecular-weight heparin; ASA, acetylsalicylic acid.

3.1. Venous Thromboembolic Events

Among the 7873 patients from 18 trials [12,29–33,35–41,43–47] who were included in the analysis of the outcome of VTE comprising deep vein thrombosis and pulmonary embolism, during a median follow-up period of 28 (IQR: 21–28) days, the outcome occurred in 170 patients during the overall follow-up period of 8435 patient-months (2.01 per 100 patient-months). There were 81 VTE events among the COVID-19 patients prescribed an immunomodulatory agent (1.77 per 100 patient-months) and 88 among the placebo-assigned patients (2.30 per 100 patient-months) (OR: 0.84, 95% CI: 0.61–1.16; I^2: 0%) (Figure 1). This effect remained consistent after excluding hydroxychloroquine and corticosteroid treatment (OR: 0.88, 95% CI: 0.61–1.27, I^2: 3%). In the subgroup analysis of trials of IL-6 antagonists, there were 12 VTE events among the 1075 patients prescribed an IL-6 antagonist compared to 20 among the 848 patients in the placebo group (OR: 0.52, 95% CI: 0.22–1.20, RRR: 52%, ARR: 1.2%, NNT: 80) without heterogeneity (I^2: 6%). In the sensitivity analysis of the outcome of pulmonary embolism, neither the overall im-

munomodulatory agents nor IL-6 antagonists significantly reduced the risk of pulmonary embolism (Supplementary Figure S4).

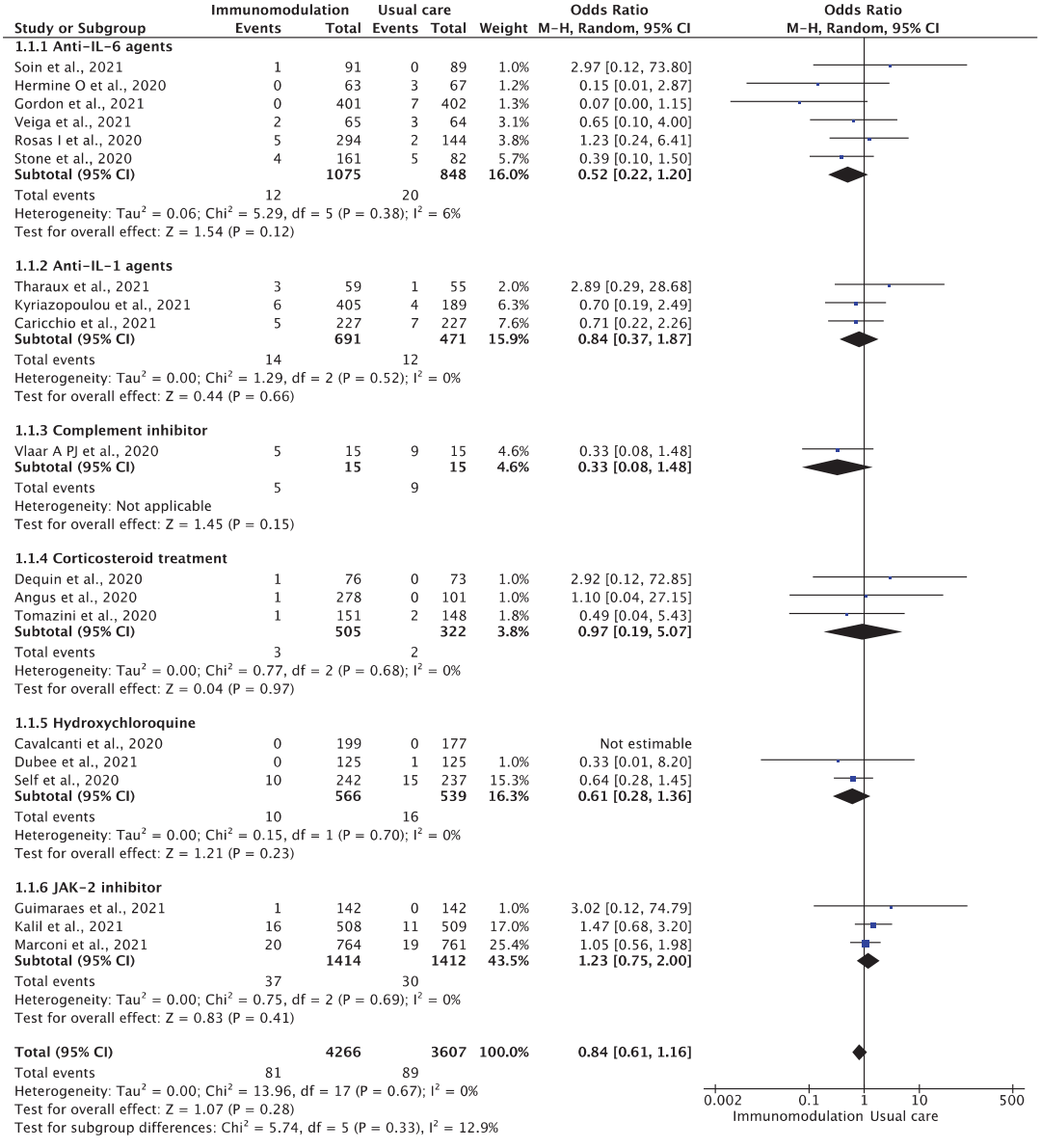

Figure 1. Odds ratio (OR) and 95% confidence intervals for the occurrence of venous thromboembolic events among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to usual care vs. usual care only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I^2: heterogeneity.

3.2. Ischemic Stroke or Systemic Embolism

Among the 4352 patients from nine trials [13,32,34–38,41,46] who were included in the analysis of ischemic stroke or systemic embolism, during a median follow-up period of 28 (IQR: 28–29) days, the outcome occurred in 26 patients during the follow-up period of 4663 patient-months (0.56 per 100 patient-months). There were 16 ischemic strokes or systemic embolic events among the patients prescribed immunomodulatory agents (0.63 per 100 patient-months) and 10 among the patients treated with SOC (0.46 per 100 patient-months) (OR: 1.10, 95% CI: 0.50–2.40; I^2: 0%) (Figure 2). This effect remained consistent after excluding hydroxychloroquine treatment (OR: 1.03, 95% CI: 0.46–2.31, I^2: 0%).

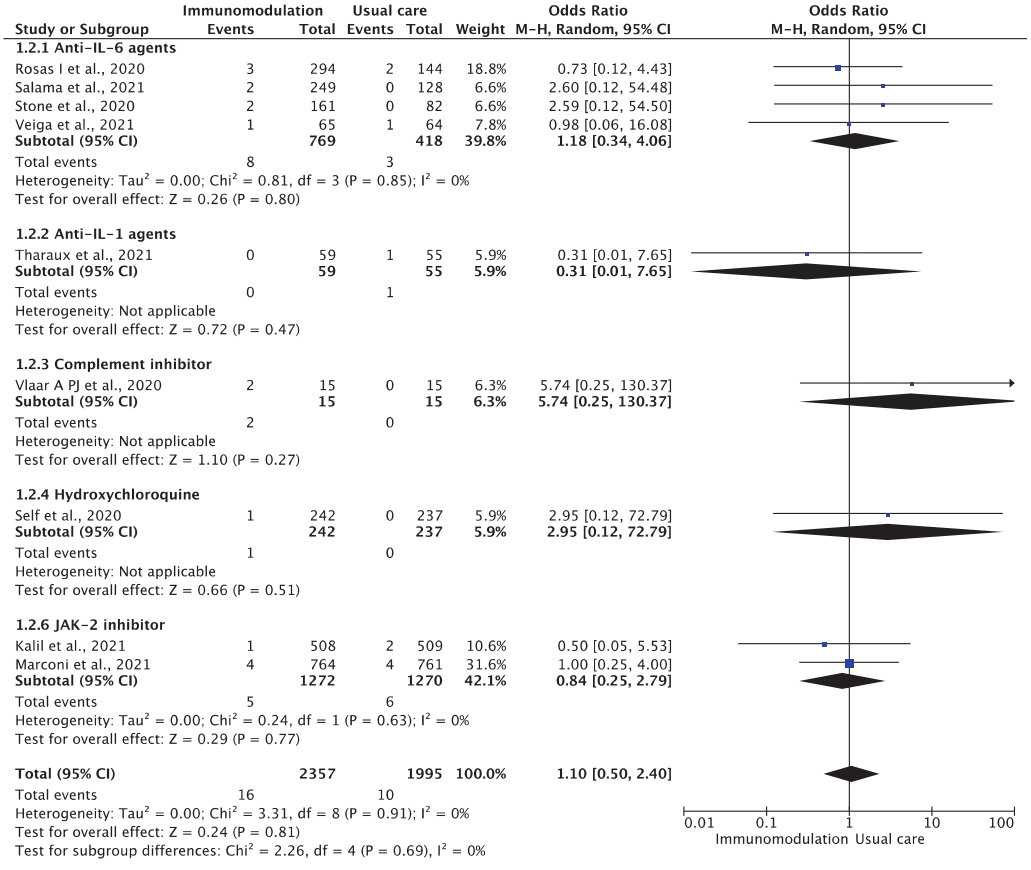

Figure 2. Odds ratio (OR) and 95% confidence intervals for the occurrence of ischemic stroke or systemic embolism among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to usual care vs. usual care only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I2: heterogeneity.

3.3. Myocardial Infarction

Among the 5438 patients from nine trials [13,31–35,39,40,46] who were included in the analysis of myocardial infarction, during a median follow-up period of 28 (IQR: 21–28) days, this outcome occurred in 23 patients during the overall follow-up period of 5826 patient-months (0.39 per 100 patient-months). There were 14 myocardial infarction events among the patients prescribed immunomodulatory agents (0.45 per 100 patient-months) and nine among the patients treated with usual care (0.33 per 100 patient-months) (OR: 1.06, 95% CI: 0.47–2.39; I^2: 0%) (Figure 3). This effect remained consistent after excluding hydroxychloroquine treatment (OR: 0.99, 95% CI: 0.43–2.29, I^2: 0%).

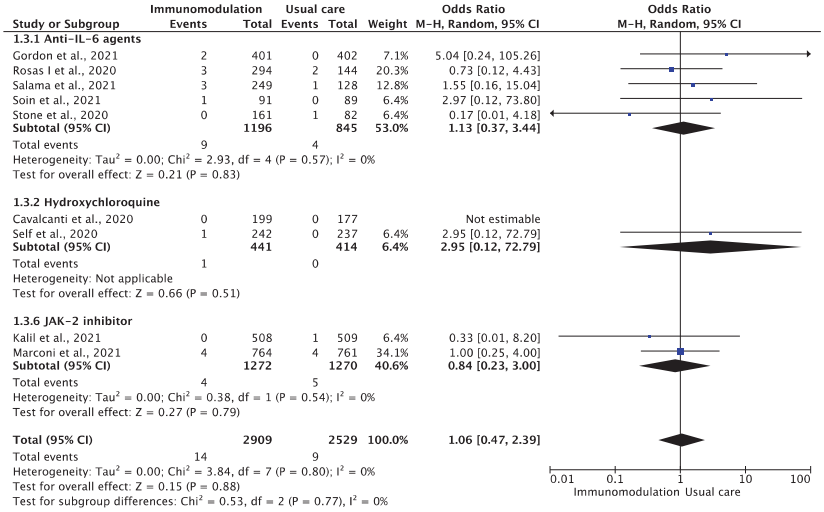

Figure 3. Odds ratio (OR) and 95% confidence intervals for the occurrence of myocardial infarction among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to SOC vs. SOC only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I^2: heterogeneity.

3.4. Any Thromboembolic Event and All-Cause Mortality

Among the 8499 patients from 22 trials [12–14,29–47] who were included in the analysis of the composite outcome of any thromboembolic event, the outcome occurred in 224 patients during the follow-up period of 9106 patient-months (2.46 per 100 patient-months). There were 112 thromboembolic events among the patients prescribed immunomodulatory agents (2.25 per 100 patient-months) and 112 among the patients treated with the standard of care (2.71 per 100 patient-months) (OR: 0.86, 95% CI: 0.65–1.14; I^2: 0%) (Figure 4). This effect remained consistent after excluding hydroxychloroquine and corticosteroid treatment (OR: 0.90, 95% CI: 0.66–1.22, I^2: 0%). In the analysis of all-cause mortality, immunomodulation significantly reduced the risk of all-cause mortality in the included studies (OR: 0.76, 95% CI: 0.66–0.86, I^2: 9%) (Supplementary Figure S5). In the sensitivity analysis excluding the effect of hydroxychloroquine and corticosteroid treatment, the results remained consistent (OR: 0.74, 95% CI: 0.64–0.87, I^2: 18%).

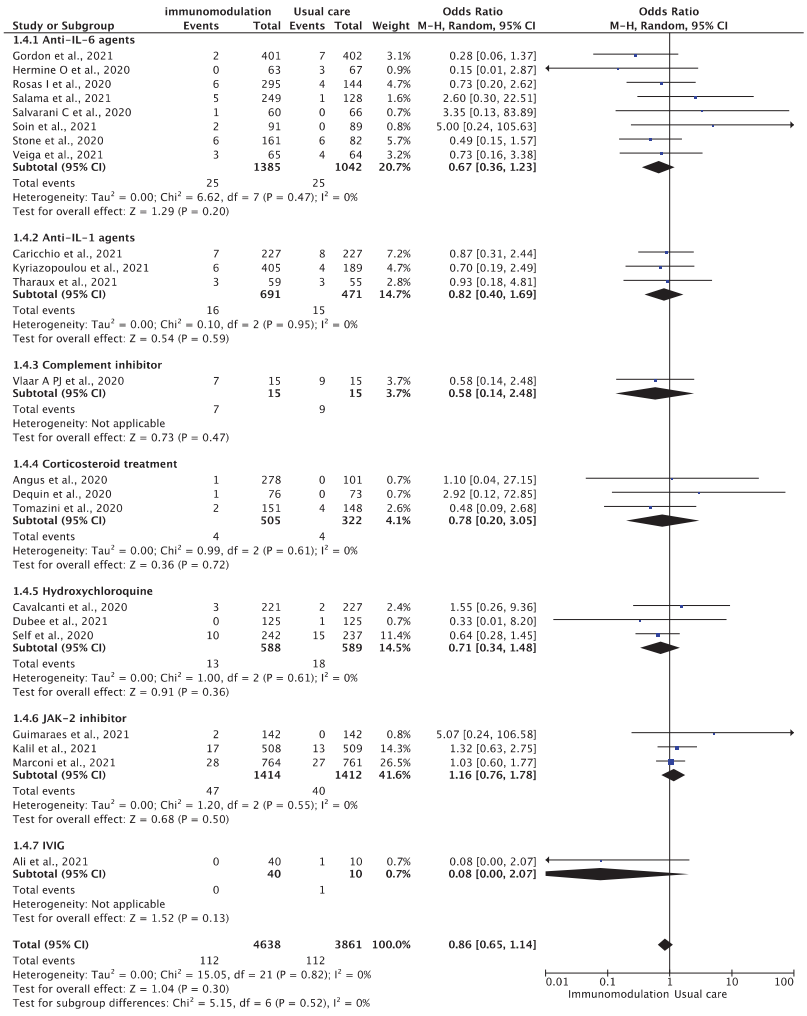

Figure 4. Odds ratio (OR) and 95% confidence intervals for the occurrence of any thromboembolic event among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to SOC vs. SOC only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I^2: heterogeneity.

4. Discussion

This meta-analysis did not identify a statistically significant effect of immunomodulation on the prevention of thromboembolic events in patients hospitalized with COVID-19. However, given the large effect estimate for the prevention of VTE, especially in patients treated with IL-6 antagonists, we cannot exclude an effect of immunomodulation on VTE occurrence. The effect of immunomodulation therapy on any thromboembolism, driven largely by VTE events, was a nonsignificant reduction in this outcome.

The potency of SARS-CoV-2 to trigger a proinflammatory cascade leading to a pulse of proinflammatory cytokines is the first step towards a vicious cycle of hyper-inflammation and cytokine release syndrome [6–10,48]. During this phase, TNF-α and IL-1β facilitate a sustained increase of IL-6. Furthermore, recent histopathological data suggest sustained systemic activation of the complement pathway in the microvascular network [49]. More-

over, locally activated platelets were shown to induce the release of neutrophil extracellular traps covered with the tissue factor, which in turn activates the extrinsic coagulation cascade leading to thrombin formation [50]. This cross-talk between innate immunity, platelets, and endothelial cells in the maladaptive host immune system can lead to the development of microvascular immune-mediated thrombosis and hypercoagulability [51]. Towards this direction, the effects of several immunomodulatory agents in COVID-19 are being investigated in ongoing randomized trials (Supplementary Table S1). In this context, it can be hypothesized that the administration of immunomodulatory agents such as IL-6 or IL-1 antagonists, complement inhibitors, corticosteroids, IVIG, and hydroxychloroquine acting on these pathogenic pathways could potentially mitigate the development of microvascular thrombosis with a corresponding reduction of VTE. Although our results did not confirm this hypothesis, we identified a trend towards fewer VTEs among patients treated with IL-6 inhibitors. Although this effect was not statistically significant, a potential effect of IL-6 antagonists on VTE risk cannot be excluded given the large effect estimate. We should not oversee that these results represent a potential additive effect of these agents on the antithrombotic effect of low-molecular-weight heparin, which was used in these studies. This was driven by RCTs of IL-6 inhibitors, but it should be noted that the number of events and patients in the IL-1-antagonist trials was low, and therefore a positive association cannot be excluded.

On the other hand, we did not identify any effect of these agents on arterial thromboembolic events comprising ischemic stroke, systemic embolism, and myocardial infarction. Compared to VTE, stroke is a heterogeneous syndrome comprising various pathophysiological mechanisms (e.g., atherosclerosis, atrial fibrillation, patent foramen ovale, dissection, small vessel disease), which frequently overlap [52–56]. This complexity may explain why immunomodulatory agents did not show any decrease in the risk of stroke or systemic embolism in the COVID-19 patients in our analysis.

Despite the potential myocardial injury which frequently occurs in patients with COVID-19 either due to myocardial infarction or because of inflammatory injury to the myocardial cells [57,58], we did not identify any effect of immunomodulatory agents on the risk of myocardial infarction. Although recently the RESCUE trial reported a marked reduction in inflammation and thrombosis biomarkers with a novel IL-6 inhibitor [59] and previous studies showed a beneficial effect of immunomodulatory agents in cardiovascular outcomes [60,61], this effect was not identified in our analysis of patients hospitalized with COVID-19. Currently, the effects of several immunomodulatory agents in COVID-19 are being investigated in ongoing randomized trials (Supplementary Table S1).

Strengths of this meta-analysis include the conduct and report of the analysis according to the PRISMA recommendations for reviews evaluating randomized trials [27]. In addition, our analysis did not consider observational studies, but focused only on RCTs characterized by their prospective design, which minimizes recall errors and selection bias, and the rigorous blind assessment of pre-defined and adjudicated outcome events, especially in this patient population, reduced the possible confounding effect of antithrombotic treatment.

There are potential limitations of this meta-analysis. Firstly, apart from the variations in the definitions of comorbidities used across trials, differences in patient selection criteria and differences in the length of follow-up across trials, the included immunomodulatory agents had highly distinctive immunological properties, which may have affected the outcomes of this study. Even though thromboembolic events and especially VTE were not systematically investigated in the included studies, most COVID-19-related thromboembolic events occur during the most severe, in-hospital period of the disease, and thus it is unlikely that these events were undocumented. Moreover, as these events were objectively diagnosed across treatment arms, no discrepancy would be likely to affect the observed treatment effects. Second, we acknowledge that the studies included in this meta-analysis were not designed to investigate the effect of immunomodulatory agents on thromboembolic events. As a result, the studies did not provide detailed data of thromboembolic events related to patient comorbidities and characteristics; thus, we were not

able to perform further analyses, while the patients' cardiovascular comorbidities may have affected the incidence of thromboembolic events in each arm. Additionally, the studies did not include extensive reports on antithrombotic treatment modalities used in these patients, and the outcomes were not independently adjudicated. However, since these events typically require objective diagnostic testing for confirmation and immunomodulation treatments are not known to have antithrombotic properties, it is unlikely that diagnostic suspicion bias may have affected detection of such events across the treatment arms. Lastly, as a significant number of clinical trials did not report results on thromboembolic events, some immunomodulatory agents were underrepresented in the analysis, potentially affecting the results of the study. Due to the increased research interest and publication rate related to COVID-19, additional RCTs may become available in the future and provide further insights in the effect of immunomodulation on the thromboembolic risk of COVID-19 patients.

5. Conclusions

In conclusion, we did not identify a statistically significant effect of immunomodulation on the prevention of thromboembolic events in patients hospitalized with COVID-19. However, given the large effect estimate for the prevention of VTE, especially in patients treated with IL-6 antagonists, we cannot exclude an effect of immunomodulation on VTE occurrence. The effect of immunomodulation on thromboembolic risk warrants further research.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10225366/s1, Figure S1: Flow diagram of the studies identified, screened, and included in the meta-analysis, Figure S2: (A) Risk of bias graph: review of the authors' judgements about each risk of bias item presented as percentages across all the included studies; (B) risk of bias summary: review of the authors' judgements about each risk of bias item for each included study, Figure S3: Diagnostic plots for each outcome based on the use of immunomodulatory agents or placebo. Panel (A): venous thromboembolism, Panel (B): ischemic stroke or systemic embolism, Panel (C): myocardial infarction, Panel (D): Any thromboembolic event, Figure S4: Odds ratio (OR) and 95% confidence intervals for the occurrence of pulmonary embolism among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to SOC vs. SOC only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I^2: heterogeneity, Figure S5: Odds ratio (OR) and 95% confidence intervals for the occurrence of all-cause mortality in the included studies among the hospitalized COVID-19 patients prescribed immunomodulatory agents as an add-on to SOC vs. SOC only. Boxes represent the OR and lines represent the 95% CIs for individual studies. The diamonds and their width represent the pooled ORs and the 95% CIs, respectively. CI, confidence interval for the Mantel–Hansen estimator, I^2: heterogeneity; Table S1: Studies of immunomodulatory agents for COVID-19.

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Review

High versus Standard Intensity of Thromboprophylaxis in Hospitalized Patients with COVID-19: A Systematic Review and Meta-Analysis

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Abstract: Thromboprophylaxis in hospitalized patients with COVID-19 has been associated with a survival benefit and is strongly recommended. However, the optimal dose of thromboprophylaxis remains unclear. A systematic review and meta-analysis (PubMed/EMBASE) of studies comparing high (intermediate or therapeutic dose) versus standard (prophylactic dose) intensity of thromboprophylaxis with regard to outcome of hospitalized patients with COVID-19 was performed. Randomized and non-randomized studies that provided adjusted effect size estimates were included. Meta-analysis of 7 studies comparing intermediate versus prophylactic dose of thromboprophylaxis (2 randomized and 5 observational, n = 2009, weighted age 61 years, males 61%, ICU 53%) revealed a pooled adjusted relative risk (RR) for death at 0.56 (95% confidence intervals (CI) 0.34, 0.92) in favor of the intermediate dose. For the same comparison arms, the pooled RR for venous thromboembolism was 0.84 (95% CI 0.54, 1.31), and for major bleeding events was 1.63 (95% CI 0.79, 3.37). Meta-analysis of 17 studies comparing therapeutic versus prophylactic dose of thromboprophylaxis (2 randomized and 15 observational, n = 7776, weighted age 64 years, males 54%, ICU 21%) revealed a pooled adjusted RR for death at 0.73 (95% CI 0.47, 1.14) for the therapeutic dose. An opposite trend was observed in the unadjusted analysis of 15 observational studies (RR 1.24 (95% CI 0.88, 1.74)). For the same comparison arms, the pooled RR for venous thromboembolism was 1.13 (95% CI 0.52, 2.48), and for major bleeding events 3.32 (95% CI 2.51, 4.40). In conclusion, intermediate compared with standard prophylactic dose of thromboprophylaxis appears to be rather safe and is associated with additional survival benefit, although most data are derived from observational retrospective analyses. Randomized studies are needed to define the optimal thromboprophylaxis in hospitalized patients with COVID-19.

Keywords: anticoagulation; COVID-19; COVID-19 therapeutics; dose; meta-analysis; mortality; thromboprophylaxis; treatment

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1. Introduction

Venous thromboembolic events (VTE) constitute one of the major complications of critical COVID-19 and are associated with adverse outcome [1–3]. Furthermore, thrombosis and microvascular disease in small pulmonary blood vessels and capillaries has been found in several autopsy studies of patients whose cause of death was COVID-19 [4]. Moreover, the administration of thromboprophylaxis in hospitalized patients with COVID-19 has been associated with survival benefit [5,6]. Based on such available evidence, current guidelines recommend thromboprophylaxis in all hospitalized patients with COVID-19, mainly in the form of prophylactic dose of low molecular weight heparin (LMWH) [7].

However, some of these guidelines qualify a higher (intermediate) than prophylactic dose of anticoagulation in patients with severe COVID-19 and increased thromboembolic risk [7], despite the fact that the latter recommendation represents mainly expert opinion rather than evidence [6]. Indeed, the available evidence is weak since this is derived mainly from observational studies, where the selection of higher versus prophylactic doses of anticoagulation has been decided for patients with critical disease. However, in these patients, the benefit of this strategy might be blunted by the adverse prognosis of severe COVID-19. It might be argued that the benefit of the anticoagulation strategy is gained only with early administration and before the establishment of irreversible lung damage [8]. Recent randomized controlled trials provide higher quality data but their findings are controversial, mainly due to the heterogeneity in the characteristics of the study population (general ward or intensive care unit (ICU) patients, degree of COVID-19 severity), as well as the time of the initiation of thromboprophylaxis, which might affect the outcome [9–13].

The aim of this systematic review and meta-analysis was to assess the risk of in-hospital mortality in hospitalized patients with COVID-19 receiving high (intermediate or therapeutic) versus prophylactic doses of thromboprophylaxis, by using data from randomized or observational studies providing adjusted analyses.

2. Materials and Methods

2.1. Registration and Reporting

This systematic review and meta-analysis was performed according to preferred reporting items for systematic reviews and meta-analyses (PRISMA) guidelines [14]. The PRISMA 2020 checklist for the present meta-analysis is presented in Supplementary File, Table S1. The PRISMA 2020 abstracts checklist is presented in Supplementary File, Table S2. The protocol was registered in the PROSPERO international prospective register of systematic reviews (CRD42021286921).

2.2. Search Strategy

A systematic search of PubMed and EMBASE databases was performed until October 1st, 2021 using the following search algorithm: ("coronavirus 2019" OR "2019-nCoV" OR "SARS-CoV-2" OR "COVID-19" OR COVID OR COVID19) AND (anticoag* OR dosing OR dose OR intensity OR thromboprophyla* OR intermediate OR prophylactic) AND (thrombotic OR thrombosis OR "deep vein" OR "pulmonary embolism" OR thromboemboli* OR death OR mortality OR fatal OR survival OR outcome OR intubation OR bleed* OR hemorrhag* OR haemorrhag*). Articles were also identified from reference lists of previously conducted relevant systematic reviews and meta-analyses and relevant papers and websites through snowball procedure.

2.3. Study Selection

The study selection was performed independently by five investigators (K.G.K., I.P.T., V.R., I.G.K., and C.A.T.). Discrepancies were resolved by consensus with a senior author (A.K.). Eligible studies were full-text articles in English language including $\geq$20 patients (not case series) that had either a randomized design or were observational but reported both unadjusted (or provided the number of events in each group) and adjusted hazard or odds ratios or relative risks (RR) for mortality (primary endpoint) for high (either intermediate or therapeutic dose) versus standard (prophylactic dose) intensity of thromboprophylaxis in hospitalized COVID-19 patients. No restriction was applied concerning the type of anticoagulant used. Doses were defined and categorized according to each study definitions as prophylactic, intermediate, and therapeutic.

2.4. Data Extraction

Five investigators (K.G.K., I.P.T., V.R., I.G.K., and C.A.T.) extracted and tabulated, independently, data concerning study design, main characteristics of included populations,

and that regarding the primary (adjusted hazard/odds ratio or RR for mortality) and secondary (VTE and bleeding events) outcomes of interest.

2.5. Risk of Bias Assessment

The risk of bias was assessed in terms of selection of patients, exposure measurement, confounding factors identification, outcome measurement, methodology, and analysis, independently, by five investigators (K.G.K., I.P.T., V.R., I.G.K., and C.A.T.). Checklists for cohort studies and for randomized controlled trials from Joanna Briggs Institute Critical Appraisal Tools were used [15]. Observational studies fulfilling $\geq$8 and randomized controlled trials fulfilling $\geq$9 of the quality domains were deemed as low risk of bias.

2.6. Certainty (Confidence) of the Outcome

The certainty of the body of evidence for the outcome of death was independently assessed by two investigators (K.G.K. and A.K.) using the GRADE approach (grading of recommendations assessment, development and evaluation) described in Chapter 14 of *The Cochrane Handbook for Systematic Reviews of Interventions* [16]. The certainty of evidence was deemed as high, moderate, low, or very low, depending on factors that either decrease the confidence of the outcome—such as the risk of bias, the publication bias, the inconsistency, the indirectness, and the imprecision of results—or factors that increase the certainty—such as the large effect size, the dose response, and the effect of plausible residual confounding [17].

2.7. Statistical Analysis

Meta-analysis was performed using the Stata/SE 11 (Texas) software. Logarithms of adjusted RR and corresponding standard errors were used for the analysis (fixed-effects meta-analysis when I^2 statistic value <50%). Odds ratios were converted to RR according to appropriate formula [18]. Hazard ratios were treated as RR. Results were graphically displayed as forest plots. Sensitivity analysis was performed to investigate the impact of different thromboprophylaxis doses in studies conducted exclusively in ICU or not (general wards or mixed settings). Meta-regression analysis was performed for assessing associations of the RR for mortality with mean age, mean d-dimer value, and percentage of males, diabetics, and ICU patients. Mean values of subgroups were combined where feasible [19]. Median (interquartile range) values were converted to mean values (standard deviation) using appropriate formulas [20]. Heterogeneity was tested using I^2 statistics. Publication bias was assessed by inspecting funnel plots, as well as Egger's test (linear regression method) and Begg's test (rank correlation method) [21,22]. Two-sided p values of <0.05 were considered statistically significant. Missing information was retrieved after communication with the corresponding authors.

3. Results

3.1. Literature Search and Inclusion of Studies

Among the 8318 articles initially retrieved through our literature search, 21 fulfilled the inclusion criteria and were included in our analysis [11,12,23–41]. The PRISMA 2020 flow diagram for systematic reviews and meta-analyses study selection is presented in Supplementary File, Figure S1. A total of 7 studies reported data for intermediate versus prophylactic dose [11,27–30,34,35], while 17 studies reported data for therapeutic versus prophylactic dose of thromboprophylaxis [12,23–26,29,31–41]. Three studies contributed data for both intermediate versus prophylactic and therapeutic versus prophylactic dose analyses [29,34,35]. The main characteristics of the included studies are shown in Table 1. A list of the adjustment variables included in the multivariate analyses of the observational studies is presented in Supplementary File, Table S3.

Table 1. Main characteristics of included studies that compared intermediate or therapeutic versus prophylactic dose of thromboprophylaxis in terms of outcomes in hospitalized COVID-19 patients.

Study	Design	N	ICU (%)	Males (%)	I/P or T/P (%)	Type of Anticoagulation
Intermediate* versus *prophylactic dose						
Peperu et al. [30]	R	173	62	56	50/50	LMWH
Sadeghipour et al. [11]	R	562	100	58	49/51	LMWH/UFH
Jimenez-Soto et al. [29]	O	244	0	66	55/45	LMWH
Jonmarker et al. [35]	O	115	100	82	42/58	LMWH
Hsu et al. [34]	O	393	NR	55	4/96	LMWH/UFH/DOAC/VKA
Paolisso et al. [28]	O	450	0	63	20/80	LMWH
Stessel et al. [27]	O	72	100	68	36/64	LMWH
Therapeutic* versus *prophylactic dose						
Lopes et al. [33]	R	615	6	60	51/49	LMWH/DOAC
Lemos et al. [12]	R	20	100	80	50/50	LMWH/UFH
Matli et al. [31]	O	82	0	62	38/62	LMWH/UFH/DOAC/Fondaparinux
Copur et al. [32]	O	115	0	50	40/60	LMWH
Jimenez-Soto et al. [29]	O	186	0	67	41/59	LMWH
Roomi et al. [26]	O	176	NR	NR	19/81	NR
Di Castelnuovo et al. [41]	O	1577	NR	NR	30/70	UFH
Motta et al. [25]	O	374	17	59	20/80	LMWH/UFH
Canoglu et al. [40]	O	154	NR	62	36/64	LMWH
Jonmarker et al. [35]	O	104	100	87	36/64	LMWH
Bolzetta et al. [39]	O	81	0	60	30/70	LMWH/UFH/Fondaparinux
Lynn et al. [38]	O	402	27	54	38/62	LMWH/UFH/DOAC
Ionescu et al. [24]	O	3119	20	49	32/68	LMWH/UFH/DOAC/VKA
Hsu et al. [34]	O	425	NR	55	11/89	LMWH/UFH/DOAC/VKA
Ferguson et al. [37]	O	141	100	55	33/67	LMWH/UFH
Secco et al. [23]	O	112	NR	70	43/57	LMWH/DOAC/VKA/Fondaparinux
Bousquet et al. [36]	O	93	0	NR	34/66	NR

DOAC, direct oral anticoagulants; I, intermediate dose; LMWH, low molecular weight heparin; NR, not reported; O, observational; P, prophylactic dose; R, randomized; T, therapeutic dose; UFH, unfractionated heparin; VKA, vitamin K antagonists.

3.2. Data Synthesis

3.2.1. Intermediate versus Prophylactic Dose of Anticoagulation

There were 2 randomized [11,30] and 5 observational studies [27–29,34,35] (n = 2009, weighted age 61 years, males 61%, ICU 53%) that reported the RR for death in patients with COVID-19 administered intermediate versus prophylactic dose of thromboprophylaxis. Meta-analysis of these 7 studies (use of adjusted estimates for the non-randomized) revealed a pooled adjusted RR for death of 0.56 (95% confidence intervals [CI] 0.34, 0.92; I^2 66%) (Figure 1). Meta-analysis of the 5 observational studies [27–29,34,35] showed pooled unadjusted RR at 0.45 (95% CI 0.29, 0.69; I^2 28%), whereas the adjusted pooled RR remained the same at 0.45 (95% CI 0.28, 0.72; I^2 36%).

Regarding the secondary outcomes, meta-analysis of 6 studies [11,27,29,30,34,35] revealed a pooled unadjusted RR for VTE at 0.84 (95% CI 0.54, 1.31; I^2 0%) and meta-analysis of 7 studies [11,27–30,34,35] revealed a pooled RR for major bleeding events at 1.63 (95% CI 0.79, 3.37; I^2 0%) for intermediate versus prophylactic dose of thromboprophylaxis.

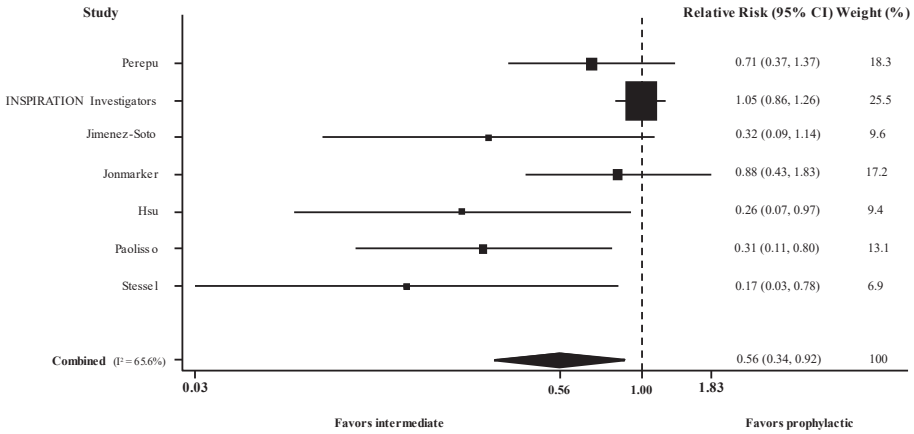

Figure 1. Forest plot of adjusted risk ratios for death in hospitalized patients with COVID-19 administered intermediate versus prophylactic dose of thromboprophylaxis. CI, confidence intervals; I^2, test for heterogeneity.

3.2.2. Therapeutic versus Prophylactic Dose of Thromboprophylaxis

There were 2 randomized [12,33] and 15 observational studies [23–26,29,31,32,34–41] (*n* = 7776, weighted age 64 years, males 54%, ICU 21%) that reported the RR for death in patients with COVID-19 administered therapeutic versus prophylactic dose of thromboprophylaxis. Meta-analysis of these 17 studies (use of adjusted estimates for non-randomized) revealed a pooled adjusted RR for death at 0.73 (95% CI 0.47, 1.14; I^2 87%) (Figure 2). Meta-analysis of the 15 observational studies showed pooled unadjusted RR for death at 1.24 (95% CI 0.88, 1.74; I^2 87%), whereas the adjusted pooled RR was 0.71 (95% CI 0.44, 1.15; I^2 88%).

Regarding the secondary outcomes, meta-analysis of 6 studies [12,29,31,33–35] revealed a pooled unadjusted RR for VTE at 1.13 (95% CI 0.52, 2.48; I^2 58%) and meta-analysis of 9 studies [24,25,29,31,33–35,37,38] revealed a pooled unadjusted RR for major bleeding events at 3.32 (95% CI 2.51, 4.40; I^2 0%) for therapeutic versus prophylactic dose of thromboprophylaxis.

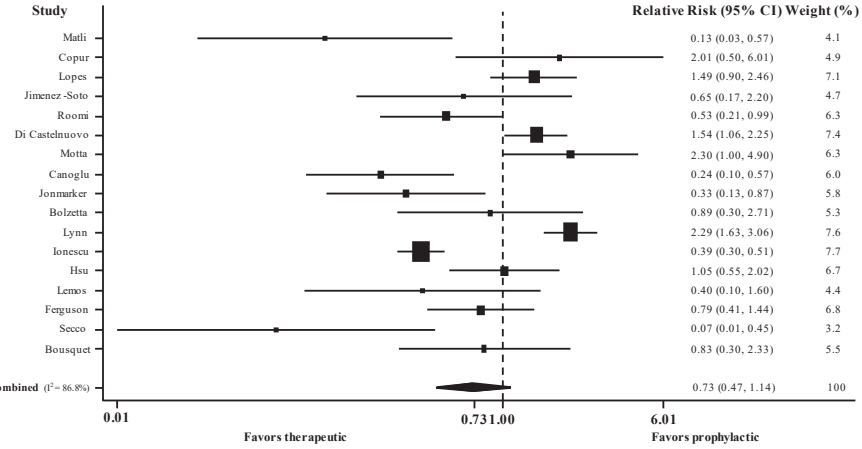

Figure 2. Forest plot of adjusted risk ratios for death in hospitalized patients with COVID-19 administered therapeutic versus prophylactic dose of thromboprophylaxis. CI, confidence intervals; I^2, test for heterogeneity.

3.3. Sensitivity and Meta-Regression Analyses

In sensitivity analyses, meta-analysis of 3 studies conducted exclusively in ICU [11,27,35] revealed a pooled adjusted RR for death in patients with COVID-19 administered intermediate versus prophylactic dose of thromboprophylaxis at 0.80 (95% CI 0.43, 1.50; I^2 59%), whereas meta-analysis of 4 studies conducted in general wards or mixed settings (general ward/ICU) [28–30,34] revealed a pooled adjusted RR at 0.47 (95% CI 0.29, 0.75; I^2 12%). Meta-analysis of 3 studies conducted exclusively in ICU [12,35,37] revealed a pooled adjusted RR for death in patients with COVID-19 administered therapeutic versus prophylactic dose of thromboprophylaxis at 0.58 (95% CI 0.35, 0.94; I^2 24%), whereas meta-analysis of 14 studies conducted in general wards or mixed settings (general ward/ICU) [23–26,29,31–34,36,38–41] revealed a pooled adjusted RR at 0.79 (95% CI 0.48, 1.30; I^2 89%).

Multivariate meta-regression analysis did not reveal any significant associations between RR for death for intermediate versus prophylactic dose and mean age (regression coefficient (RC) −0.04, 95% CI −0.32, 0.23), percentage of male (RC 0.02, 95% CI −0.15, 0.19) and diabetic (RC 0.02, 95% CI −0.16, 0.19) patients. In addition, there was no association between the RR and the mean d-dimer value (RC 0.001, 95% CI −0.002, 0.004), but there was a trend for lower RR with lower percentage of ICU patients (RC 0.01, 95% CI −0.0004, 0.02; $p = 0.06$) (these variables were examined in univariate meta-regression analyses due to insufficient observations). Multivariate meta-regression analysis did not reveal any significant associations between RR for death for therapeutic versus prophylactic dose and mean age (RC 0.03, 95% CI −0.31, 0.37), percentage of male (RC −0.009, 95% CI −0.27, 0.25), diabetic (RC 0.14, 95% CI −0.68, 0.95), and ICU (RC 0.004, 95% CI −0.06, 0.07) patients, as well as with the mean d-dimer value (RC −0.001, 95% CI −0.007, 0.005).

3.4. Risk of Bias, Publication Bias, and Certainty of the Evidence Assessment

The assessment of the risk of bias of the included studies comparing intermediate or therapeutic versus prophylactic dose of thromboprophylaxis is presented in Supplementary File, Tables S4–S6. All studies were deemed as low risk of bias, mainly due to their randomized design or the strict inclusion criteria of the observational studies providing adjusted analyses for several confounders.

Egger's test and Begg's funnel plots revealed a small study effect ($p = 0.02$ and 0.05, respectively) for intermediate versus prophylactic dose but not for therapeutic versus prophylactic ($p = 0.61$ and 0.07, respectively) (Supplementary File, Figure S2).

The certainty of the evidence on the outcome of death was low in terms of a beneficial effect of intermediate or therapeutic versus prophylactic dose of thromboprophylaxis in hospitalized COVID-19 patients (Supplementary File, Table S7).

4. Discussion

This meta-analysis summarized the available evidence on the efficacy and safety of enhanced (intermediate or therapeutic) versus standard (prophylactic) dose of thromboprophylaxis in hospitalized patients with COVID-19. The main findings include the following: (i) intermediate dose of thromboprophylaxis seems to be associated with additional benefit in terms of survival compared with prophylactic dose; (ii) therapeutic versus prophylactic dose of thromboprophylaxis seems to be associated with an increased risk for major hemorrhage, whereas the benefit in terms of survival is questionable; (iii) the evidence is mainly derived from observational studies; (iv) LMWH is the main anticoagulant that has been used for thromboprophylaxis.

The majority of the available guidance documents recommend standard prophylactic low dose of thromboprophylaxis in all hospitalized patients; however, higher doses can be selectively recommended on an individualized basis for patients at high or very high thrombotic risk, provided they also have a low risk of bleeding [7]. The available evidence, mainly derived from observational studies, is heterogeneous regarding the beneficial role of higher doses since the latter are administered in patients with critical disease and unfavor-

able prognostic factors [7,42]. Recent randomized studies have been published providing a high level of evidence, but their findings seem to be heterogeneous as well [9–13,30,33]. The current meta-analysis included only studies that were either randomized or observational that provided adjusted effect size estimates for high versus standard dose of thromboprophylaxis, which might mitigate the above-mentioned methodological challenges.

The present analysis included mainly observational studies. Most studies used LMWH for thromboprophylaxis. Intermediate compared with prophylactic dose appeared to be associated with an about 45% decrease in mortality. A trend towards increased incidence of major bleeding events with intermediate dose was observed; however, this did not reach statistical significance. On the other hand, therapeutic dose was not observed to show a significant effect on reducing mortality compared to prophylactic dose. However, opposite trends were observed in the unadjusted and adjusted analyses. More specifically, a trend towards harm was observed in the unadjusted analysis (RR 1.24), whereas a trend towards benefit was observed in the adjusted analysis, including data from the same studies (RR 0.71). This finding highlights the indication bias of the included observational studies: higher doses were selectively administered in patients with higher risk for severe disease due to their baseline risk factors and/or high levels of indices of COVID-19 severity. Thus, their adverse prognosis might mitigate the benefit of this strategy or even mislead to a link between high dose and mortality. Adjustment for appropriate confounders seems to be necessary with this respect; however, randomized trials are the most appropriate studies for providing the highest level of evidence.

Evidence from randomized trials has become available lately; however, their findings should be interpreted with caution. In the Intermediate versus Standard-Dose Prophylactic Anticoagulation in Critically-ill Patients with COVID-19: An Open Label Randomized Controlled Trial (INSPIRATION), 562 ICU patients were randomized to receive either intermediate or therapeutic dose of thromboprophylaxis [11]. Intermediate dose did not offer significant benefits either in the primary composite outcome (acute VTE, arterial thrombosis, treatment with extracorporeal membrane oxygenation, or all-cause mortality), or in each one of its components. It should be noticed however, that all patients had critical disease and randomization was performed after a median of 13 days from symptoms onset, with no available data regarding their previous anticoagulation regimen [8,11]. A plausible explanation could be that microvascular disease in small pulmonary blood vessels and capillaries may have already been established in critically ill patients, rendering intensified anticoagulation non-efficacious at this timepoint [43]. Similarly, a landmark study including data of 1098 critically ill patients from 3 different platforms (Randomized, Embedded, Multifactorial Adaptive Platform Trial for Community-Acquired Pneumonia (REMAP-CAP); A Multicenter, Adaptive, Randomized Controlled Platform Trial of the Safety and Efficacy of Antithrombotic Strategies in Hospitalized Adults with COVID-19 (ACTIV-4a); The Antithrombotic Therapy to Ameliorate Complications of COVID-19 (ATTACC) trial) failed to show clinical benefits with therapeutic versus standard dose and was prematurely terminated due to the prespecified futility criteria [9]. Interestingly, in the study derived by the same platforms but including non-critically ill patients, a significant clinical benefit was observed for patients receiving therapeutic doses [10]. In the latter trials, patients were randomly assigned to receive therapeutic dose of anticoagulation with unfractionated heparin or LMWH or to receive usual-care pharmacologic thromboprophylaxis which included either standard low dose or enhanced intermediate dose of thromboprophylaxis [9,10]. In another recent randomized clinical trial, therapeutic dose of LMWH reduced major thromboembolism and death compared with institutional standard prophylactic or intermediate dose of LMWH or unfractionated heparin among hospitalized patients with COVID-19 with very elevated D-dimer levels, but interestingly this treatment effect was not evident in ICU patients [13]. It should be noted that the above-mentioned studies comparing therapeutic versus standard dose of thromboprophylaxis were not included in the present meta-analysis because both prophylactic and intermediate doses were used in the standard arm. However, all these findings support a benefit in favor of a more intensive

thromboprophylaxis when this is administered early in selected patients with adverse prognostic factors and before the advent of critical disease.

In the present meta-regression analysis, there was a trend for an inverse association between the observed benefit with intermediate versus prophylactic dose of thromboprophylaxis and the percentage of ICU patients. This was additionally confirmed in sensitivity analyses, including studies conducted exclusively in ICU, compared with general wards or mixed settings (general ward/ICU). This is in line with previous observations and highlights the important issue of the prompt initiation of thromboprophylaxis. However, this observation was not valid for therapeutic versus prophylactic dose of thromboprophylaxis and could be attributed to the ecological bias of the meta-regression analysis. Unfortunately, data regarding the time of initiation of thromboprophylaxis in relation to symptoms onset were not available in the majority of included studies.

An interesting finding in the present analysis was that no difference in the risk for VTE was observed for higher versus prophylactic dose of thromboprophylaxis. However, all these analyses regarded unadjusted estimates and details on the screening or the diagnostic algorithm strategies for VTE were absent. Indeed, the VTE rate differs considerably among the studies, with higher rates among studies implementing universal screening [2]. Thus, minor VTE might be uncaptured in most of the studies. Furthermore, LMWH, apart from its anticoagulant action, has anti-inflammatory effects, which might justify its beneficial role in terms of mortality, above and beyond simply reducing VTE [44,45].

The issue of safety is of paramount importance. This analysis confirmed a higher risk of major bleeding events with therapeutic versus prophylactic dose of thromboprophylaxis, whereas this was not valid for the intermediate dose. However, these analyses were unadjusted and patients with critical disease are frail with complex hematological dysregulations and at risk for complications. In the REMAP-CAP, ACTIV-4a, and ATTACC trial with critically ill patients, hemorrhagic events were more common in patients receiving therapeutic dose compared with the standard arm [9].

Two relevant meta-analyses have been identified through our literature search [46,47]. Both of them analyzed studies comparing the efficacy and safety of therapeutic versus prophylactic dose of thromboprophylaxis and confirmed a trend towards clinical benefits of therapeutic dose. However, mixed adjusted and unadjusted estimates were used, rendering these analyses inconclusive [46,47]. In the present meta-analysis, only high-quality studies with adjusted effect size estimates were included. Moreover, to the best of our knowledge, this is the first time that a comparison between intermediate and prophylactic dose has been performed.

The findings of this analysis should be examined in light of the fact that the available evidence was derived from studies with high heterogeneity regarding the patients' characteristics, as well as the treatment strategies applied. Combining estimates from different types of studies can be problematic, but it should be mentioned that this meta-analysis applied strict methodological criteria and included studies with high quality. Furthermore, the performance of RR in studies with high mortality rates can be challenging, but meta-regression and sensitivity analyses confirmed the consistency of our findings across heterogeneous studies. Moreover, a small study effect was observed in the comparison of the intermediate versus prophylactic dose. Yet, it should be mentioned that when fewer than 10 studies are included in the meta-analysis, the power of the test for funnel plot asymmetry is too low to distinguish chance from asymmetry. Lastly, the definition of the intensity of thromboprophylaxis might differ among studies with the implemented protocols adjusted for weight and creatinine clearance. For example, the dose of LMWH might be escalated in obese patients but can still be regarded as standard prophylactic dose.

5. Conclusions

Evidence on the optimal thromboprophylaxis for hospitalized patients with COVID-19 is derived mainly from observational studies with significant methodological limitations. This meta-analysis of randomized and non-randomized studies with adjusted analyses

showed a potentially beneficial impact of enhanced intensity of thromboprophylaxis compared with the standard one. Thus, higher than prophylactic doses of thromboprophylaxis, mainly in the context of an intermediate dose, can be considered for selected patients with COVID-19 at high thrombotic risk. In addition, prompt initiation of thromboprophylaxis appears to be as important as the optimal dose. Randomized trials with strict methodological criteria are needed to provide the highest level of evidence.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jcm10235549/s1, Figure S1: The PRISMA 2020 flow diagram for systematic reviews and meta-analyses study selection, Figure S2: Begg's funnel plot for the assessment of publication bias of included studies comparing (**a**) intermediate versus prophylactic and (**b**) therapeutic versus prophylactic thromboprophylaxis dose, Table S1: The PRISMA 2020 for Abstracts Checklist for the present meta-analysis, Table S2: The PRISMA 2020 for Abstracts Checklist for the present meta-analysis, Table S3: List of adjustment variables regarding the included observational studies, Table S4: The assessment of the risk of bias of the included observational studies (comparing intermediate versus prophylactic dose) for the present meta-analysis using a checklist from Joanna Briggs Institute Critical Appraisal Checklists for Cohort Studies, Table S5: The assessment of the risk of bias of the included observational studies (comparing therapeutic versus prophylactic dose) for the present meta-analysis using a checklist from Joanna Briggs Institute Critical Appraisal Checklists for Cohort Studies, Table S6: The assessment of the risk of bias of the included randomized clinical trials in the present meta-analysis using a checklist from Joanna Briggs Institute Critical Appraisal Checklists for Randomized Clinical Trials, Table S7: Certainty of the evidence on the outcome of death for the present meta-analysis using the GRADE approach.

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Brief Report

Humoral and T-Cell Response before and after a Fourth BNT162b2 Vaccine Dose in Adults ≥60 Years

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Abstract: Both humoral and cellular anamnestic responses are significant for protective immunity against SARS-CoV-2. In the current study, the responses in elderly people before and after a fourth vaccine dose of BNT162b2 were compared to those of individuals immunized with three vaccine doses. Although a boost effect was observed, the high response following the third administration questions the necessity of an early fourth boost.

Keywords: SARS-CoV-2; BNT162b2; COVID-19

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1. Introduction

The SARS-CoV-2 B.1.1.529 variant (Omicron) wave in Israel led to the early authorization of a fourth dose of the BNT162b2 vaccine (BioNTech/Pfizer) to individuals with age ≥60 years who had received a third dose at least 4 months earlier. The potential benefit of a third vaccine dose of BNT162b2 or mRNA-1273 was demonstrated by lower rates of breakthrough infection and effectiveness against emerging variants of concern (VOCs) [1]. Although currently, there are no clear correlates of protection, the involvement of both humoral and T-cell immunity in protection from COVID-19 was shown [2]. Age-associated immune system dysfunction, manifested by compromised immunity parameters such as declined lymphocyte function may eventually predispose one to severe COVID-19. Consequently, the vaccination of such individuals might be beneficial [3].

We characterized the humoral and cellular immune responses prior and following a fourth BNT162b2 vaccine dose and compared them to the responses amongst individuals four months following a third vaccine dose.

2. Materials and Methods

Participants ≥60 years (n = 16) without prior SARS-CoV-2 infection or active malignancy were recruited in the Rabin Medical Center (RMC) vaccination center. The study was approved by the ethics committee of RMC, and all participants provided written informed consent.

Anti-spike IgG titers and T-cell response against the ancestral and Omicron spike proteins were determined as previously described [4,5]. Anti-S IgG titers were determined in the serum with the SARS-CoV-2 IgG II Quant assay (Abbott Laboratories, Lake Forest, IL, USA) with strict adherence to the manufacturer's protocol. Seropositivity was defined as ≥50 arbitrary units (AU)/mL.

For T-cell response, blood was collected into sodium-heparin tubes (vacutainer, BD, Franklin Lakes, NJ, USA) and processed within 2 h of collection. Peripheral blood mononuclear cells (PBMCs) were isolated with density gradient sedimentation using Ficoll-Paque (Sigma-Aldrich, Rehovot, Israel) according to the manufacturer's protocol. PBMCs were stimulated with commercially available peptide pools (15-mer sequences with an overlap of 11 amino acids) covering the full length of the Wuhan-1 SARS-CoV-2 (wild-type) or Omicron B.1.1.529 variant spike (Peptides & Elephants GmbH, Hennigsdorf, Germany). Interferon gamma (IFNγ)-secreting cells were quantified using a fluorescent ELISPOT assay (ImmunoSpot, Cleveland, OH, USA) with strict adherence to the manufacturer's protocol. Data were acquired with the ImmunoSpot S6 Ultimate reader and analyzed with ImmunoSpot software version 7.0.30.2 (ImmunoSpot). A positive T-cell response was defined as ≥10 IFNγ-secreting cells per 10^6 PBMCs. The presented T response is the average of four measurements minus background response without antigen stimulation. Samples with background responses ≥25 spots were excluded (not applicable, NA).

3. Results and Discussion

All 16 participants in the study were evaluated 20 (T1) and 22 (T2) weeks after the third dose. Among the 16 participants, 5 participants received a fourth dose at week 20 (after the blood draw); 9 received three doses only, and 2 who received only three doses had a polymerase chain reaction (PCR)-confirmed SARS-CoV-2 infection between T1 and T2.

In the five participants with four doses, who were all seropositive before the fourth dose (T1), the anti-spike IgG levels increased (4.0–11.3-fold) after the fourth dose (T2). At T1, four and two of the five participants had a T-cell response to the ancestral and Omicron spike protein, respectively. At T2, all five had a T-cell response against both spike proteins that was generally higher than before the fourth dose (Table 1).

All nine participants with three vaccine doses were seropositive at both timepoints, although a decrease in anti-spike IgG levels was noted from T1 to T2 (1.1–1.3-fold). In T2, of the nine participants with three vaccine doses, eight had a T-cell response against the ancestral spike protein and eight had a response against the Omicron protein.

The two participants with a documented SARS-CoV-2infection demonstrated an increase in anti-spike IgG titers following the infection. For one of these participants, data on T-cell response before and after the infection were available, and an increased T-cell response against both the ancestral and Omicron spike proteins was noted.

Among all 16 participants, the average response to the ancestral spike protein was similar to that against the Omicron spike protein (average [SD] of 261.4 [401.5] vs. 80.7 [100.4]).

Sample 1 of the PCR-confirmed SARS-CoV-2 denoted an opportunity to follow the boost response following infection, most probably by the Omicron variant. Interestingly, the boost response to the Omicron spike was significantly higher than to the ancestral spike (14.5 vs. 2.8-fold increase, respectively), possibly a result of novel T-cell epitopes in the Omicron variant.

Data on the efficacy of the fourth dose are limited, and our study is the first to examine the immune response following a fourth BNT162b2 vaccine dose. The available data suggest that the fourth dose lowers the risk of infection and severe disease by 2- and 4-fold, respectively, compared to three doses [6]. In another study, a limited protective effect of the fourth vaccine against Omicron was described, in parallel to immunological boost [7]. Our study, although limited by the small sample size, provides immunogenicity data demonstrating that the majority of participants had a detectable T-cell response 20–22 weeks after the third dose regardless of the fourth dose and that the T-cell response against the Omicron spike protein was comparable to that against the ancestral spike protein. T-cell

response varies between individuals due to HLA polymorphism. Additionally, it was shown that along the spike protein, for each individual, there is a median of 11 and 10 recognized epitopes of CD4 and CD8 T-cell populations, respectively [8]. Therefore, it could be speculated that T-cell response may be maintained against VOCs [5,8].

Table 1. Anti-spike IgG titers and T-cell response in participants who received 3 or 4 BNT162b2 vaccine doses.

#	Age	Sex	Anti-Spike IgG, AU/mL		T-Cell Response, IFNγSecreting Cells Per 10^6 PBMC			
					Ancestral		Omicron	
			T1 (20 wks after Dose 3)	T2 (22 wks after Dose 3)	T1 (20 wks after Dose 3)	T2 (22 wks after Dose 3)	T1 (20 wks after Dose 3)	T2 (22 wks after Dose 3)
Participants with 4 vaccine doses (4th dose was on T1, immediately after blood draw)								
1	66	F	11,295	80,000	205	1823	117	905
2	68	F	4906	26,951	75	151	56	136
3	72	F	3696	19,711	53	372	0	219
4	65	F	2971	33,420	3	102	0	34
5	69	M	897	3547	22	29	8	11
Participants with 3 vaccine doses								
1	73	M	12,033	10,816	148	7	100	17
2	74	F	9113	7882	NA	78	NA	55
3	64	F	6980	6473	180	199	149	144
4	64	F	6230	5574	7	31	0	20
5	68	F	5519	4980	NA	10	NA	7
6	77	M	4476	3597	196	86	106	48
7	76	F	4009	3238	1450	1577	68	144
8	71	M	3432	2641	358	88	414	80
9	75	F	2328	2016	NA	1030	NA	108
Participants with 3 vaccine doses and confirmed COVID-19 infection (by PCR) between T1 and T2								
1	77	M	1348	7883	243	691	27	393
2	72	M	587	1050	NA	100	NA	56

Taken together, our data show a significant humoral and cellular immune response among elderly individuals 20 weeks after a third BNT162b2 vaccine dose. Thus, given the low decay kinetics of memory B and T cells [9], our findings, as those of other studies do [7], question the benefit of an early boost.

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Institutional Review Board Statement: The study was approved by the ethics committee of Rabin Medical Center, Petah Tikva, 49100, Israel, and all participants provided written informed consent.

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

Conflicts of Interest: S.M.S. received research grants (to the Institution) from CAN-FITE, AstraZeneca, Bioline RX, BMS, Halozyme, Clovis Oncology, CTG Pharma, Exelexis, Geicam, Halozyme, Incyte, Lilly, Moderna, Teva pharmaceuticals, and Roche, and owns stocks and options in CTG Pharma, DocBoxMD, Tyrnovo, VYPE, Cytora, and CAN-FITE. The remaining authors declare no conflict of interest.

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Article

Lung Aeration in COVID-19 Pneumonia by Ultrasonography and Computed Tomography

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Abstract: We conducted a prospective single-center observational study to determine lung ultrasound reliability in assessing global lung aeration in 38 hospitalized patients with non-critical COVID-19. On admission, fixed chest CT scans using visual (CTv) and software-based (CTs) analyses along with lung ultrasound imaging protocols and scoring systems were applied. The primary endpoint was the correlation between global chest CTs score and global lung ultrasound score. The secondary endpoint was the association between radiographic features and clinical disease classification or laboratory indices of inflammation. Bland–Altman analysis between chest CT scores obtained visually (CTv) or using software (CTs) indicated that only 1 of the 38 paired measures was outside the 95% limits of agreement (−4 to +4 score). Global lung ultrasound score was highly and positively correlated with global software-based CTs score (r = 0.74, CI = 0.55–0.86; $p < 0.0001$). Significantly higher median CTs score ($p = 0.01$) and lung ultrasound score ($p = 0.02$) were found in severe compared to moderate COVID-19. Furthermore, we identified significantly lower ($p < 0.05$) lung ultrasound and CTs scores in those patients with a more severe clinical condition manifested by SpO_2 < 92% and C-reactive protein > 58 mg/L. We concluded that lung ultrasound is a reliable bedside clinical tool to assess global lung aeration in hospitalized non-critical care patients with COVID-19 pneumonia.

Keywords: lung ultrasound; COVID-19 pneumonia; imaging; radiology; computed tomography

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1. Introduction

The histopathology of early severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) interstitial pneumonia is characterized by an exudative inflammation consisting of an accumulation of monocytes in the alveolar cavities and by monocytes and lymphocytes centered on small blood vessels infiltrating widened alveolar septa [1,2]. The common radiographic manifestation of pneumonia in the setting of COVID-19 is the appearance of parenchymal opacities in the periphery of the lungs. Chest X-ray is often insensitive in early phase parenchymal lung disease [3]. Low-dose chest computed tomography (CT) is considered in the diagnostic algorithm of hospitalized symptomatic patients with COVID-19 infection and pneumonia [4,5], even though it is not always required for the initial diagnosis or treatment of non-critical COVID-19 infection. CT images acquired early in the course of pneumonia typically reveal a unilateral focal ground-glass opacity, quickly evolving into

bilateral multilobar ground-glass opacities with peripheral or posterior distribution. As the disease progresses, ground-glass opacities may progress to consolidative pulmonary opacities and mixed patterns [6–8].

CT scanning is not available in all emergency departments. Therefore, alternative imaging modalities to identify and manage these infections are highly desirable. Indeed, lung ultrasound (LUS) can provide information on interstitial-alveolar syndrome, lung consolidation and pleural effusion, and has an established value in the evaluation of pulmonary edema, ARDS, and pneumonia [9,10]. According to the International Consensus Conference on lung ultrasound, B-lines are discrete laser-like vertical hyperechoic artefacts that appear from the pleural line and move synchronously with lung sliding [9].

Along these lines, lung interstitial syndrome is defined by an increased number of B-lines indicating the accumulation of extravascular fluid or inflammatory cells in the pulmonary interstitial space or alveoli and having a ground-glass opacity appearance with or without thickening of the interlobular septa on a chest CT scan. LUS is more accurate than standard chest X-ray or physical examination, and nearly as accurate as chest CT scan to detect community-acquired pneumonia [10–13], with the main disadvantage being its lower sensitivity in detecting deeper consolidations. The bedside utility of LUS has, therefore, consistently been suggested during the COVID-19 pandemic [14]. A prospective description of LUS findings in COVID-19 is not yet available. In addition, a LUS score can give a quantitative global assessment of lung aeration: an increase in LUS score indicates a decrease in lung parenchymal aeration.

Accordingly, this study aimed to prospectively compare LUS with CT to detect loss of lung aeration in patients with COVID-19 pneumonia with lung opacity on chest CT scan as the gold standard, the latter computed with specific software (CTs) and validated with the visual method (CTv). It was reasoned that the LUS score would be associated with CTs scan measurements for evaluating lung aeration to assist the management of patients with COVID-19.

2. Materials and Methods

2.1. Study Design

This was a prospective single-center observational study on non-critical hospitalized patients admitted to a COVID-19 ward between 2 April 2020 and 24 April 2020. The study is part of a larger observational study that was registered at ClinicalTrials.gov, number NCT04327570. Data in Table 1 (baseline demographics, clinical and laboratory findings) as well as the LUS scores of the 38 patients of this study appeared in a recent publication of our group, which aimed to explore the LUS scores for the rapid assessment of the severity of SARS-CoV-2 pulmonary infection in patients hospitalized with COVID-19 pneumonia [15]. Furthermore, chest computed tomography data have not appeared anywhere in that, or in any other, report. Eligible patients for lung ultrasound evaluation met the following inclusion criteria: SARS-CoV-2 infection confirmed by a positive RT-PCR for SARS-CoV-2 RNA of a nasopharyngeal swab; low dose chest CT scan performed on admission to the emergency room; low dose chest CT scan performed within 24 h from the LUS; and presence of CT image abnormalities consistent with viral pneumonia. Exclusion criteria were: pulmonary edema; >24 h interval since chest CT scan; CT findings known to the sonographer; and patients admitted to the non-critical COVID-19 ward for reason of palliative care or sedation. This study was approved by the Institutional Review Board of the University Hospitals KU Leuven (study ID s60207). All participants provided written informed consent.

2.2. Demographic, Clinical, and Laboratory Data

Patient demographic, clinical, and laboratory data were collected on the day of emergency room admission and collected from the electronic medical file of the patients. Clinical classification was applied to summarize patients' condition at the time of hospital admission, according to the American Thoracic Society (ATS) $\geq$ 3 minor criteria for defining

severe community-acquired pneumonia, and the China National Health Commission (NHC) clinical case classification considering severe COVID-19 cases who met at least one of the following conditions: (1) respiratory rate $\geq$ 30/min, or (2) oxygen saturation (resting state) $\leq$ 93%, or (3) PaO2/FiO2 $\leq$ 300 mmHg [16,17].

Table 1. Baseline demographics, clinical and laboratory data.

Variables	% or Median (IQR 25–75%)
Age, years	64 (57–72)
Gender, % male	63%
BMI, kg/m^2	27 (25–31)
Presenting symptom, % fever or respiratory	82%
Days from onset of illness to lung ultrasound	6.5 (4–10)
ATS pneumonia severity, % severe	18%
China NHC clinical classification, % severe	76%
Pulse oximetry (SpO2), %	92 (91–93)
Supplemental Oxygen NC, L/min	2 (1–3)
White blood cell count, $10^3/\mu$L	5890 (4010–7645)
Neutrophil count, $10^3/\mu$L	4000 (2550–6100)
Lymphocyte count, $10^3/\mu$L	900 (600–1450)
Neutrophil-to-lymphocyte ratio	4 (3–7)
Platelet count, $10^3/\mu$L	191,000 (156,500–270,750)
C-reactive protein, mg/L	58 (25–106)
Lactate dehydrogenase, U/L	300 (245–442)
Haemoglobin A1c, %	6 (5.7–6.9)
D-dimer, ng/mL	673 (372–1106)
Creatinine Clearance, mL/min/1.73 m^2	80 (63–97)

BMI, body mass index; ATS, American Thoracic Society; NHC, National Health Commission; SpO2, oxygen saturation measured by pulse oximeter; NC, nasal cannula; IQR, interquartile range.

2.3. Chest Computed Tomography and Score Assessment

All CT scans were performed using a Siemens SOMATOM Definition Flash, dedicated to the COVID-19 emergency department of our institution. This was part of the emergency department (ED) planning of our tertiary university hospital, in order to immediately isolate patients with flu-like symptoms while all the needed tests were performed. All patients underwent a low-dose non-contrast CT of the chest in the inspiration phase with the following scan protocol: slice thickness and increment: 1 mm/0.7 mm (lung and mediastinal window), pitch: 1.2, collimation: 128 $\times$ 0.6 mm, rotation time: 0.5 s. The dose protocol (kV and mAs) was: <50 kg: 80 kV and 30 mAs; between 50 and 80 kg: 120 kV and 20 mAs; >80 kg: 140 kV and 28 mAs.

Chest radiologists independently and blinded to the lung ultrasound findings performed qualitative and quantitative evaluations of lung parenchyma opacities on the CT scan. Opacities of interest were low-density ground-glass opacity (GGO) and high-density consolidations. GGO was defined as hazy increased lung attenuation with preservation of bronchial and vascular margins, whereas consolidation was defined as an increase in parenchymal opacification with obscuration of margins of vessels and airway walls. The Syngo. VIA CT Pneumonia Analysis software program prototype was used to measure the percentage of lung parenchyma opacity [18]. Based on 3D segmentations of lungs, lobes, and pneumonia lesions, an artificial intelligence algorithm of the CT Pneumonia Analysis program automatically identified and quantified increased attenuation areas of the lung

parenchyma (GGO and consolidations) on axial CT data with slice thicknesses up to 5 mm, and quantified, lobe-wise, the extent of these lung parenchyma opacities.

A CT score was assigned by converting the estimated (visual or CTv) and the measured (software or CTs) percentage of lung parenchyma opacity for each lobe into a 5-point scale: a score of 0 for 0% lung opacity, 1 for 1% to <5% lung opacity, 2 for 5% to 25% lung opacity, 3 for 26 to 50% lung opacity, 4 for 51 to 75% lung opacity, and 5 for 76 to 100% lung opacity [19]. The total CT score is the sum of the individual lobar scores and can range from 0 (no area with an increase in lung opacity) to 25 (all five lobes show more than a 75% increase in lung opacity) (see Figures A1–A5).

2.4. Lung Ultrasonography and Score Assessment

Lung ultrasound was performed using a GE Healthcare LOGIQ E9 ultrasound system, dedicated to exclusive use at the non-critical COVID-19 wards of our institution with all unnecessary parts removed, and a curved 3.5-MHz array probe. All LUS examinations were performed within 12 h of the initial CT scan. To correctly identify the artifactual images of the lungs, the harmonic imaging was removed, and the reject post-processing was lowered. The focus was set at the level of the pleural line and depth was set at 15 cm from the pleural line.

All lung ultrasound examinations were performed bedside in full personal protection equipment (PPE) and scored by one physician who remained blinded to the chest CT images. A 12-region lung ultrasound scanning method was used [20,21]. Each systematically examined hemithorax consisted of six regions: anatomical landmarks set by anterior and posterior axillary lines defined anterior, lateral, and posterior regions, which were each divided into superior and inferior. Patients were examined in supine and lateral position; the latter to examine the posterior regions. All intercostal spaces in all 12 regions were explored via both longitudinal and cross-sectional views, to perform a comprehensive examination [22].

In each of the 12 regions and during an entire respiratory cycle the most pathologic out of 4 ultrasound lung aeration patterns was considered representative for the entire region and classified as a score of 0 for normal aeration (lung sliding with A-lines); a score of 1 for moderate loss of aeration ($\geq$3 well-spaced B-lines, or B1); a score of 2 for severe loss of aeration (coalescent B-lines including white lung, or B2); and a score of 3 for consolidation (hyperechoic lung tissue). A LUS score ranging from 0 to 36 (LUS) was calculated as the sum of each of the 12 regions. In addition, pleural fluid was registered if present. Representative ultrasound images from each of the 12 regions were extracted from the machine and stored in the electronic medical file of each patient.

2.5. Statistical Analysis

A Shapiro–Wilk test was applied to test the normality of the data. This analysis identified that CT (visually and software-based) and LUS scores were normally distributed. We did not perform a sensitivity analysis on the different sample sizes required for different levels of correlation between LUS and CT scores to determine the optimal sample size of this study to detect statistical significance. Nevertheless, the sample size calculation was based on the objective to detect at least a correlation coefficient of 0.5 for LUS and CT scores based on a previous study from our group, which indicated significant associations between LUS score and clinical outcomes (correlation r ranged between 0.48 and 0.58) in patients hospitalized for COVID-19 pneumonia [15]. A minimum required sample size for this study was 37 for a power of 90% and alpha level of significance of 0.05 [23]. Given the possibility of 5% dropouts, a sample size of 40 patients were recruited to address the aim of the study. Quantitative variables are summarized as mean (and SD) or median (and interquartile range, IQR 25–75%) for Gaussian or skewed distribution, respectively. Comparisons were performed with the Mann–Whitney test for skewed distributions. All tests were two-sided and statistical significance was determined as p-value < 0.05. Correlations between LUS and CT scores were evaluated by the Pearson's correlation coefficient in case of two quantitative

normally distributed variables. The Bland–Altman analysis was utilized to measure the agreement in aeration assessment between the two CT scoring methods, and 95% limits of agreement were calculated as the mean difference (1.96 × SD). A satisfactory agreement between CT scores measured visually (CTv) and CT scores calculated by the software (CTs) was considered when the difference between CTv and CTs measurements did not significantly vary from zero. For this purpose, one-sample *t*-test among the difference between CTv and CTs measurements and zero value was performed. All statistical analyses were performed with a statistical software package, GraphPad Prism version 5.0 for Mac, GraphPad Software, San Diego, CA, USA.

3. Results

3.1. Patient Characteristics

Forty consecutive patients were eligible and consented. Two subjects were excluded as they were not meeting the study entry criteria and 38 subjects were analyzed. Demographic, clinical, and laboratory data for the 38 study participants are presented in Table 1. In >90% of hospitalized subjects fever and/or respiratory symptoms (cough, dyspnea) were the presenting symptoms, while in the remaining subjects atypical symptoms, such as loss of appetite or confusion, were attributed to COVID-19 pneumonia. The WHO clinical disease state score, the China NHC clinical case classification, and the ATS community-acquired pneumonia severity index demonstrated a mild disease state with score of four in 79% of subjects, a severe clinical case in 76% of subjects, and a severe pneumonia in 18% of subjects, respectively. All subjects were admitted to the COVID-19 ward for disease monitoring.

3.2. Chest Computed Tomography (CT) and Lung Ultrasound (LUS) Scores

Chest CT and lung ultrasound findings are depicted in Table 2. The median time interval between low-dose CT scan and LUS was 20 h (IQR 16–22). In all subjects the lung parenchymal opacities on the chest CT scan were located at least in the outer part of the hemithorax. Major descriptive radiographic findings included ground-glass opacity on the chest CT scan in 36 (95%) of subjects, and B-lines on LUS in 37 (97%) of subjects (Figures 1 and 2). While GGO on the chest CT scan was present in 95% of subjects, this was the predominant abnormal CT finding in 84%. Similarly, LUS observed a B1 or B2 pattern in at least one of the twelve regions in 92% and 82% of subjects, respectively. A predominant presence out of 12 regions for the B1 or B2 pattern was observed in 61% and 42% of subjects, respectively, as in some subjects both patterns were equally predominantly present (e.g., 5 out of 12 regions for both B1 and B2 pattern). Consolidation on the chest CT scan was observed in 29% and the predominant abnormal finding in 16% of subjects. Similarly, LUS observed consolidation in 29% and was the predominant abnormal finding in 11% of subjects. A more global assessment of lung aeration was provided by quantification with a scoring system. Mean (±SD) loss of aeration score was 6.6 (±2.8) out of 25 points for CT_V, 6.6 (±3.2) out of 25 points for CT_S, and 11 (±5.3) out of 36 points for LUS.

Table 2. Chest computed tomography and lung ultrasound descriptive findings.

Appearance of CT Findings	Any, n (%)	Predominant, n (%)
Ground-glass opacity (±crazy paving)	36 (95%)	32 (84%)
Consolidation (±ground-glass opacity)	11 (29%)	6 (16%)
Distribution of CT findings		
Peripheral (±central)	38 (100%)	
Bilateral	36 (95%)	

Table 2. *Cont.*

Appearance of CT Findings	Any, n (%)	Predominant, n (%)
Number of lobes affected, mean	4 ± 1	
1 or 2	4 (10%)	
3	7 (18%)	
4	10 (26%)	
5	17 (45%)	
Appearance of LUS findings	Any, n(%)	Predominant, n (%)
Interstitial Edema (B1 pattern)	35 (92%)	23 (61%)
Alveolar Edema (B2 pattern)	31 (82%)	16 (42%)
Consolidation (C)	11 (29%)	4 (11%)
Pleural fluid	2 (5%)	na
Distribution of LUS findings		
Bilateral	37 (97%)	
N of regions (out of 12) affected, mean	7 ± 3	

n, number per variable; na, not applicable; N, total number.

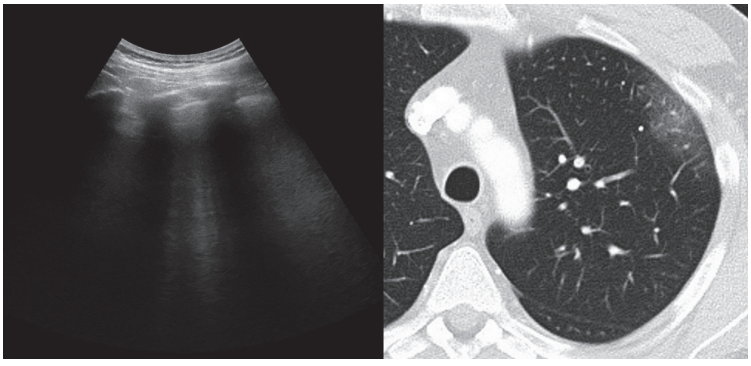

Figure 1. Left panel: LUS demonstrating thickening of the pleural line and intercostal predominantly well-spaced B-lines or B1 pattern. **Right panel**: CT scan demonstrating bilateral pure GGO.

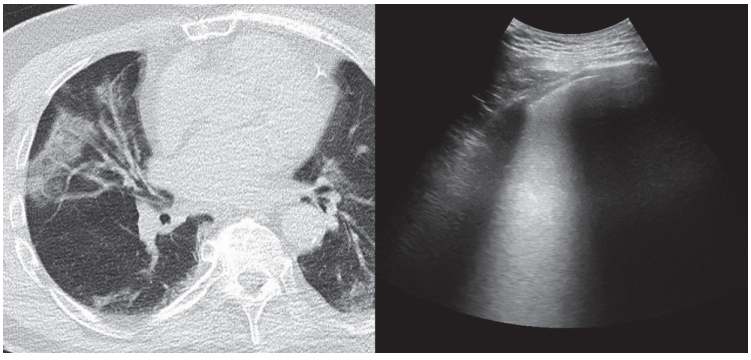

Figure 2. Left panel: CT scan demonstrating bilateral GGO with tendency of consolidation. **Right panel**: LUS demonstrating thickening of the pleural line and intercostal predominantly coalescent B-lines or B2 pattern.

3.3. Agreement between CT-Software (CTs) and CT-Estimated (CTV) Scores

The CTv score was strongly correlated with the CTs score (r = 0.76, $p < 0.0001$; Figure 3). The mean difference between CT-software (CTs) and CT-estimated (CTv) score was zero points, indicating no average systematic measurement error or bias; the difference between the two methods did not vary statistically significantly from zero ($p = 1.0$, Figure 4). The size of the measurement error or 95% limits of agreement from −4 to +4 points was rather wide, implicating disagreement between the two scoring methods. The software-based method was used as the gold standard to compare with LUS.

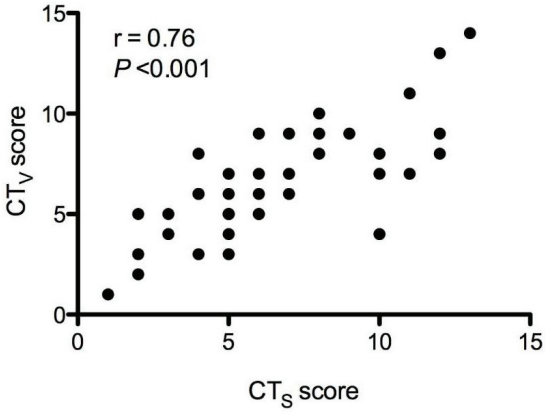

Figure 3. Pearson's correlation for CTs and CTv scores.

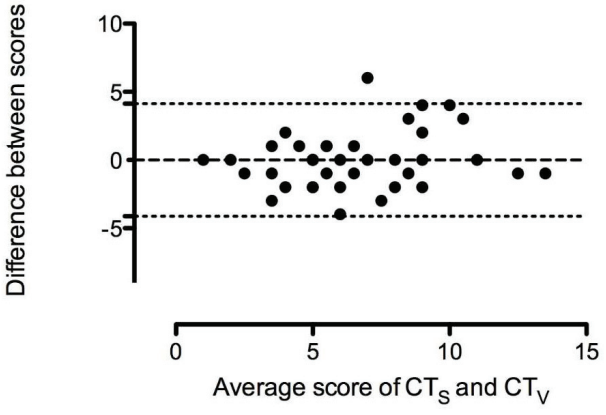

Figure 4. Bland–Altman plot of difference (CTs–CTv) against mean for CTs lung opacity score versus CTv lung opacity score. The dashed line represents the mean difference (bias 0.000), and the dotted lines represent the 95% limits of agreement (−4 to +4) between the paired measurements.

3.4. Correlation between Lung Ultrasound Score (LUS) and CT-Software (CT_S) Score and Other Clinical Variables

The CT_S score was highly and positively correlated with the global LUS score (r = 0.74, CI = 0.55–0.86, $p < 0.0001$; Figure 5).

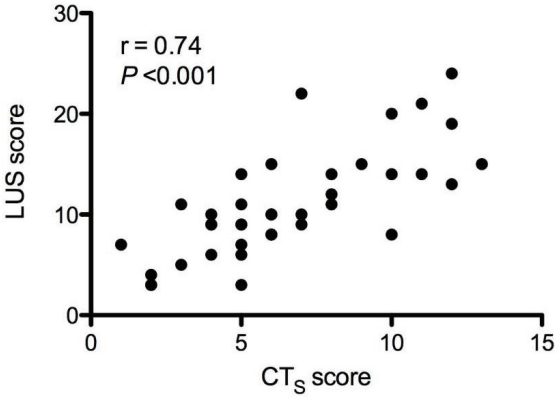

Figure 5. Pearson's correlation for CTs and LUS score.

The association between the radiographic assessment of loss of lung aeration and baseline demographics, clinical classifications, or laboratory findings of inflammation is depicted in Table 3. No association was found between a radiographic quantitative evaluation and demographic findings of age, sex, or BMI. The median CTs and LUS score in China NHC severe-type clinical COVID-19 cases was 7 (IQR 5–10) and 11 (IQR 8–15), respectively, which was significantly higher ($p < 0.05$) than that of moderate severity cases (four with IQR 3–6, and nine with IQR 3–10, respectively). Furthermore, we identified significantly lower LUS and CTs scores in those patients with worsened clinical condition manifested by SpO2 < 92% and CRP > 58 mg/L (Table 3). No significant differences were found in LUS and CTs scores in patients with lower, as compared to patients with higher, neutrophil-to-lymphocyte ratio or D-dimers, probably related to a clinical context of hospitalized patients excluding critical-type intensive care unit COVID-19 cases.

Table 3. Association between baseline radiographic features of lung aeration and demographic data, clinical classification, or laboratory indices of inflammation.

	Computed Tomography Software (CTs)		**Lung Ultrasound(LUS)**	
	Global ScoreMedian (IQR)	***p*-Value**	**Global ScoreMedian (IQR)**	***p*-Value**
Age				
<median (64 years, n = 17)	7 (5–10)	0.38	11 (8–15)	0.54
≥median (64 years, n = 21)	5 (2–8)		9 (8–14)	
Gender				
male (n = 24)	7 (4–10)	0.39	11 (8–15)	0.21
female (n = 14)	6 (4–7)		9 (6–14)	
BMI				
<median (27 kg/m^2, n = 19)	6 (5–10)	0.65	10 (8–14)	0.99
≥median (27 kg/m^2, n = 19)	5 (4–9)		10 (7–14)	
O2 saturation				
>median (92%, n = 17)	5 (4–6)	0.012	9 (7–11)	0.018
≤median (92%, n = 21)	8 (5–11)		14 (8–17)	
ChinaNHC classification				
moderate cases (n = 9)	4 (3–6)	0.007	9 (3–10)	0.023
severe cases (n = 29)	7 (5–10)		11 (8–15)	

Table 3. *Cont.*

	Computed Tomography Software (CTs)		Lung Ultrasound(LUS)	
	Global ScoreMedian (IQR)	***p*-Value**	**Global ScoreMedian (IQR)**	***p*-Value**
ATS severity				
non-severe (n = 31)	5 (4–8)	0.045	10 (7–14)	0.14
severe (n = 7)	10 (6–12)		15 (8–20)	
Neutrophil count				
<median (4000 $10^3/\mu L$, n = 19)	5 (4–8)	0.17	9 (6–12)	0.20
≥median (4000 $10^3/\mu L$, n = 19)	7 (5–10)		12 (8–15)	
NLR				
<median (4, n = 19)	6 (4–9)	0.20	9 (6–14)	0.11
≥median (4, n = 19)	7 (5–11)		12 (10–14)	
C-reactive protein				
<median (58 mg/L, n = 19)	5 (3–7)	0.017	9 (5–10)	0.002
≥median (58 mg/L, n = 19)	8 (5–10)		14 (9–15)	
D-dimer				
<median (673 ng/mL, n = 19)	5 (4–7)	0.04	9 (6–11)	0.06
≥median (673 ng/mL, n = 19)	7 (5–11)		13 (8–19)	

BMI, body mass index; NHC, National Health Commission; ATS, American Thoracic Society; NLR, neutrophil-to-lymphocyte ratio.

4. Discussion

Our study demonstrated satisfactory agreement between CTs and CTv in the assessment of lung aeration in patients with COVID-19; hence, the software-based method (CTs) was used as the gold standard to compare with LUS. We found a strong correlation for loss of lung aeration between a quantitative chest CTs score and a global LUS score obtained by a sonographer blinded to the chest CT scan.

Two types of disagreement between the two scoring systems used should be acknowledged. First, different scoring mechanisms and scales were developed and applied. The LUS scoring system assigns a score to a certain region (not anatomical lobe) considering the worst finding for rating, independently of its dimension [22]. Second, the depth of inspection for LUS is limited to the outer part of the hemithorax, while a CT scan evaluates the entire hemithorax [13]. This may lead to an overestimation of loss of aeration with higher LUS scores, certainly for the non-critical patients with a severe disease stage that is often limited to the outer hemithorax. Despite these flaws, our clinical findings support a global LUS score as a reliable bedside clinical assessment tool in hospitalized non-critical patients with COVID-19. Baseline higher than median (>92%) SpO2 and lower than median blood CRP value was significantly associated with lower radiographic (both CTs and LUS) scores of loss of lung aeration. The median CTs score and LUS score in severe-type was significantly higher than moderate COVID-19, a finding that was not observed when a non-COVID-19 ATS community-acquired pneumonia severity classification was applied.

The adoption of lung ultrasound at a point-of-care setting in a COVID-19 internal medicine ward has been proposed as the stethoscope of the 21st century to visualize the global lung aeration in SARS-CoV-2 pneumonia and assess changes or resolution of lung opacities over time [14,24]. Our findings contribute to the potential application of LUS as a bedside clinical tool for longitudinal monitoring of SARS-CoV-2 pneumonia, which should be evaluated in further prospective studies.

Different point-of-care lung ultrasound scanning protocols have been described for qualitative evaluation of the lung. The BLUE-protocol decision tree is performed on acute dyspneic patients who will be admitted to the ICU. A systematic six-regions lung ultrasound examination is performed for immediate diagnosis of the main causes of acute respiratory failure [25]. A systematic 12-regions lung ultrasound examination has been

prospectively evaluated in an intensive care unit setting for early diagnosis and monitoring of ventilator-associated pneumonia, and for the determination of lung aeration changes during spontaneous breathing weaning from mechanical ventilation [26–28]. A lung ultrasound scoring system has been described for this 12-region method to quantify the assessment of the lung [21,22,28]. The calculation of a LUS score allows semi-quantification of the global assessment of lung aeration regardless of etiology: an increase in LUS score indicates a decrease in lung aeration. Inter-observer agreements between physicians for 12-region LUS analysis and scoring was kappa 0.77 to 0.84 in blinded prospective research [29,30]. We decided to use the 12-region lung ultrasound examination with global LUS score. Patients hospitalized in a non-critical COVID-19 ward are likely in a stable clinical condition and able to turn into the lateral position used for the posterior lung surface examination. This extended range of examination is essential as COVID-19 is often characterized by bilateral multilobar opacities with a peripheral and/or posterior distribution. This LUS method examines the entire lung surface and gives a detailed image of aeriation loss, making it suitable for a qualitative and quantitative correlation with chest CT scan in a COVID-19 population.

The strengths of this study are the prospective design with fixed imaging protocols including detailed evaluations with scoring system, and a LUS operator blinded to the chest CT images. Our study also has limitations. We acknowledge that our data are preliminary and larger studies are necessary to confirm the role of lung ultrasound in the management of COVID-19. Nevertheless, our data support the previous literature and further indicates the use of bedside ultrasound for the early diagnosis in patients who presented to the emergency department with COVID-19 pneumonia [24,31,32]. Moreover, this is a single-center study, and one expert sonographer performed all image acquisitions, the latter in order to minimize the exposure of health-care professionals and use of PPE. This can also justify the necessary time delay between the performance of the CT and LUS in our study. Larger studies are needed to support the significant associations between LUS and clinical outcomes we found in our study and further promote the use of LUS as prognostication in terms of severity of COVID-19 pneumonia and its trajectory. In our study, we do not conduct repeat imaging at different time points during the hospital stay to examine whether improvements in GGO were also correlated with improvements in LUS. Additionally, we cannot generalize our findings to more severe patients admitted directly to critical care. Finally, the sensitivity of LUS is the highest for the diagnosis of normally aerated tissue or pleural effusion, while false negative findings can be seen for alveolar-interstitial and consolidated tissue not reaching the pleural borders or when an affected lung area is surrounded by alveolar gas.

5. Conclusions

In conclusion, we found that the lung ultrasound score correlated strongly with the chest CT scan for the evaluation of COVID-19 with the added advantage of ease of use at point-of-care and the absence of radiation exposure. LUS is a reliable bedside clinical tool to evaluate global lung aeration and might be suitable as an alternative imaging modality for COVID-19 lung disease monitoring.

6. Patents

No patents resulting from the work are reported in this manuscript.

Author Contributions: Conceptualization, A.K. and C.D.; methodology, A.K., C.D., Z.L., D.T. and E.W.; software, A.K., C.S., L.G., P.V.M. and W.D.W.; validation, A.K., C.S., Z.L., L.G., E.W., D.T., N.L., P.V.M., J.W., W.D.W. and C.D.; formal analysis, A.K. and C.D.; investigation, A.K. and C.D.; resources, A.K., D.T. and C.D.; data curation, A.K and C.D.; writing—original draft preparation, A.K., C.S., Z.L., L.G., E.W., D.T., N.L., P.V.M., J.W., W.D.W. and C.D.; writing—review and editing, A.K. and Z.L.; visualization, A.K. and Z.L.; supervision, A.K. and C.D.; project administration, A.K.; funding acquisition, N.L., J.W., E.W. and L.G. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of the University Hospitals KU Leuven (study ID s60207).

Informed Consent Statement: Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

Acknowledgments: We thank the patients for participating in this investigation.

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

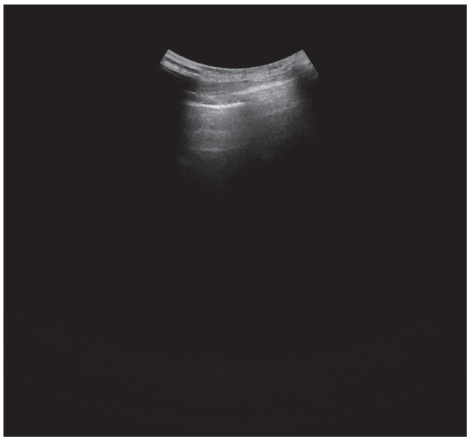

Figure A1. A-lines (Normal)—score 0.

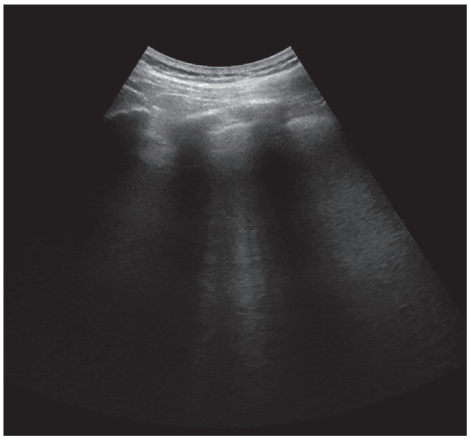

Figure A2. Well-spaced B-lines (B1)—score 1.

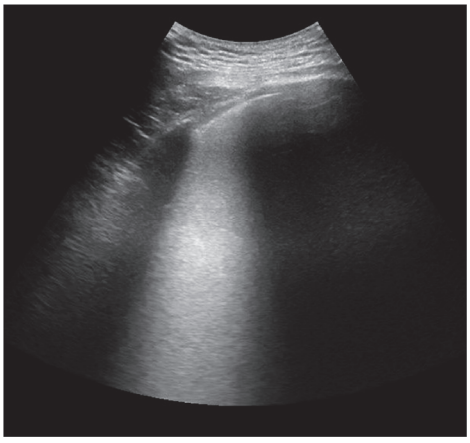

Figure A3. Coalescent B-lines (B2)—score 2.

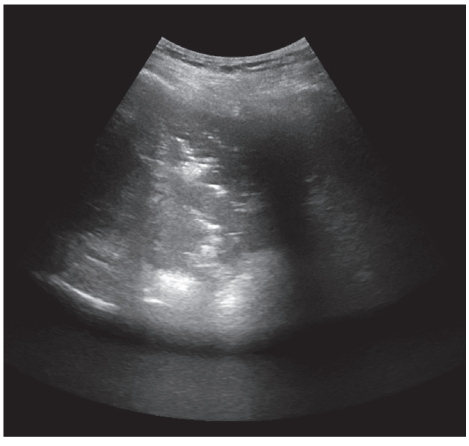

Figure A4. Consolidation—score 3.

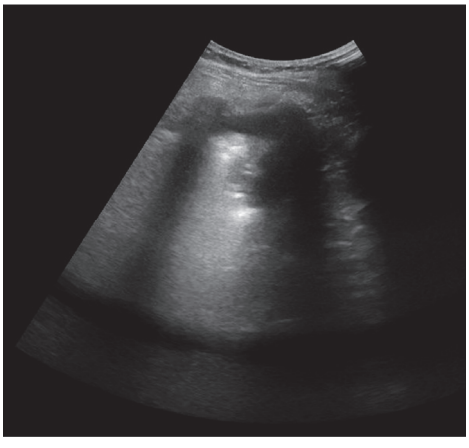

Figure A5. Pleural fluid.

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Remdesivir in the Treatment of COVID-19: A Propensity Score-Matched Analysis from a Public Hospital in New York City Assessing Renal and Hepatic Safety

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Abstract: While the relative efficacy of remdesivir as a therapeutic agent in selected patients with COVID-19 has been established, safety concerns have been raised regarding potential nephrotoxicity and hepatotoxicity. Our main objective was to investigate the kidney- and liver-related safety outcomes in patients with COVID-19 treated with remdesivir in a public hospital in New York. A propensity score-matched retrospective study was conducted in hospitalized patients with COVID-19 from 1 June 2020 to 10 March 2021. A total of 927 patients were included in this study (remdesivir: 427, non-remdesivir: 500; women: 51.8%; median age 61 years; median BMI: 28.5 kg/m^2). Matching without replacement yielded a cohort of 248 patients (124 in each group). In the matched cohort, the remdesivir group had a significantly lower rate of acute kidney injury (AKI) (12.1% vs. 21.8%, $p = 0.042$), a lower rate of acute liver injury (ALI) on the verge of statistical significance (7.3% vs. 14.5%, $p = 0.067$), and non-significantly lower death rate (13.7% vs. 16.1%, $p = 0.593$) compared to the non-remdesivir group. Multivariable analyses revealed that patients treated with remdesivir were found to be associated with a significantly lower likelihood for AKI (OR: 0.40; 95% CI: 0.24–0.67, $p < 0.001$), no association was found for ALI (OR: 0.68; 95% CI: 0.35–1.30, $p = 0.241$), while a trend towards an association of patients treated with remdesivir with a lower likelihood for in-hospital death was observed (OR: 0.57; 95% CI: 0.32–1.01, $p = 0.053$). In conclusion, no safety concerns with regards to renal and liver outcomes were raised in patients with COVID-19 treated with remdesivir. Instead, there were signals of possible nephroprotection and improved in-hospital mortality.

Keywords: remdesivir; COVID-19; safety; adverse events; acute kidney injury; nephrotoxicity

1. Introduction

Viral replication is the main characteristic of the early infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and additionally plays a central role in the subsequent pulmonary phase of Coronavirus Disease 2019 (COVID-19) [1]. Therefore, remdesivir, which had originally been developed for the treatment of Ebola virus disease

and found to inhibit the replication of various coronaviruses in preclinical studies, was one of the first therapeutics to receive attention at the beginning of the pandemic [2].

Remdesivir is an inhibitor of the RNA-dependent RNA polymerase, which is essential for viral replication [3]. It is phosphorylated by cellular kinases to form the pharmacologically active nucleoside triphosphate that can be integrated into viral RNA-dependent polymerase, which then induces premature termination of viral RNA transcription [3]. In the ACTT-1 trial, hospitalized patients with COVID-19 that received remdesivir had a significantly shorter recovery time, higher likelihood of clinical improvement at day 15, and non-significant lower death rate by day 29 compared to patients that received placebo [4]. The preliminary findings of ACTT-1 findings made remdesivir the first drug to receive emergency use authorization by the FDA initially for the treatment of patients with severe COVID-19 [5]. In contrast, the WHO Solidarity trial did not show positive results [6]. However, a subsequent meta-analysis for the American College of Physicians revealed that remdesivir offered mortality benefits in patients that were on supplemental oxygen but not on mechanical ventilation [7]. Finally, the recently published PINETREE trial showed that an early three-day course of remdesivir decreased substantially the risk of hospitalization in outpatients with risk factors for COVID-19 progression and led to an expansion of indications for use of remdesivir [8,9].

While the relative efficacy of remdesivir as a therapeutic agent in selected patients with COVID-19 has been established, safety concerns have been raised mainly regarding potential nephrotoxicity and hepatotoxicity [10–12]. The possible mechanism behind the presumed nephrotoxicity of remdesivir is the prolonged plasma half-life of its metabolites and the accumulation of sulfobutylether-β-cyclodextrin (SBECD) carrier which is the solubilizing excipient used to prepare the intravenous formulation as remdesivir has limited water solubility [13]. While remdesivir itself may not be nephrotoxic, there are concerns that SBECD accumulation in tubular cells may be responsible for renal injury [4,13]. In the ACTT-1 trial, the Acute Kidney Injury (AKI) rates were similar in the remdesivir and placebo groups, 3.9% and 4.1%, respectively [4]. In the PINETREE trial, the mean change from baseline in creatinine clearance was lower in the remdesivir group compared to the placebo group (0.26 ± 21.2 mL per minute vs 1.9 ± 18.6 mL per minute) [8]. However, both landmark randomized studies excluded patients with creatinine clearance <30 mL/min, Refs. [4,8] while available real-world studies are small or obtained data from adverse events reporting system databases [14–16].

Potential remdesivir-induced liver injury has been another safety concern [17,18]. The metabolism of remdesivir occurs via CYP3A4 in the liver, which can be one of the targeted organs by SARS-CoV-2 since Angiotensin-converting enzyme 2 (ACE2) is present in hepatocytes and cholangiocytes [19]. No liver-related safety signals were detected in ACTT-1 and PINETREE trials but patients with significant elevation of liver enzymes at baseline were excluded [4,8].

Therefore, well-designed real-world studies are needed to further assess the renal and liver outcomes of patients on remdesivir with special emphasis on patients with AKI, chronic kidney, or liver disease. The primary objective of this analysis was to investigate the kidney- and liver-related safety outcomes of patients with COVID-19 treated with remdesivir in a public hospital in the Bronx, New York. Our secondary objective was to investigate the efficacy of remdesivir with regard to hard in-hospital outcomes.

2. Materials and Methods

2.1. Study Design, Study Setting, Patient Population

This was a propensity score-matched observational cohort study performed at New York City Health and Hospitals/Jacobi, an inner-city hospital in the Bronx, New York. Patients $\geq$ 18 years of age who were admitted to an inpatient service, including the intensive care unit (ICU), with laboratory-confirmed COVID-19 from 1 June 2020 to 10 March 2021 were included. We excluded patients who met any one of the following criteria: (i) patients < 18 years old; (ii) patients without laboratory-confirmed COVID-19; (iii) patients

who were still hospitalized at the time of data collection; (iv) women who were pregnant at the time of the index hospitalization. Per our institutional protocol for the diagnosis and management of COVID-19, all patients had to be tested for COVID-19 immediately upon arrival to the emergency room and no remdesivir could be initiated without approval, for which laboratory confirmation of COVID-19 was needed. The study was approved by the Biomedical Research Alliance of New York (BRANY) Institutional Review Board with a waiver of informed consent (IRB #20-12-103-373). Data were fully de-identified and anonymized before the data was accessed and the IRB waived the requirement for informed consent.

2.2. Data Sources

Study data were obtained from electronic health records via appropriate diagnostic codes (Epic Systems, Verona, WI, USA). The initial dataset was reviewed by two independent investigators for accuracy (HL and SN). Two pairs of additional independent investigators reviewed individual charts to obtain additional information (LP-CB, MP-NV). The extracted data included age, gender, body mass index (BMI), history of hypertension, hyperlipidemia, diabetes, coronary artery disease (CAD), congestive heart failure (CHF), stroke, chronic kidney disease (CKD) including stage, end-stage renal disease (ESRD), chronic liver disease (none, alcohol hepatitis, hepatitis B or C), liver cirrhosis (none, compensated, decompensated), sequential laboratory data including blood urea nitrogen (BUN), creatinine (Cr), albumin, total bilirubin, alkaline phosphatase (ALP), aspartate transaminase (AST) and alanine transaminase (ALT), COVID-19 severity on presentation, remdesivir administration during the index hospitalization (our institutional guidelines suggested a treatment duration of up to five days with the option to extend to up to ten days for patients with critical COVID-19), and outcomes including invasive mechanical ventilation, admission to intensive care unit (ICU), acute kidney injury (AKI), initiation of dialysis, or acute liver injury (ALI) during the index hospitalization, death, and hospital discharge. COVID-19 severity on presentation was adjudicated by two independent attending physicians (LP and AA) based on the NIH COVID-19 treatment guidelines (moderate: evidence of lower respiratory disease and oxygen saturation $\geq$94% on room air; severe: oxygen saturation <94% on room air; critical: respiratory failure requiring intubation and/or multiple organ dysfunction requiring ICU admission) [20]. AKI during the index hospitalization was adjudicated by two independent nephrologists (NV and AA) based on Kidney Disease: Improving Global Outcomes (KDIGO) guidelines on AKI (AKI of any stage was defined by an increase in serum creatinine by 0.3 mg/dL or more within 48 hours or an increase in serum creatinine to 1.5 times baseline or more within the last 7 days; stage 2 AKI was defined by an increase in serum creatinine 2–2.9 times baseline, and stage 3 AKI by an increase in serum creatinine more than three times baseline or increase to $\geq$ 4 mg/dL or need for initiation of renal replacement therapy; accurate data on urine output were not expected to be available) [21]. The glomerular filtration rate was estimated based on the CKD-EPI equation. ALI during the index hospitalization was adjudicated by two independent attending physicians (LP and NP) based on serum ALT and/or AST levels equal to or greater than 2.5 times the upper limit normal level which corresponds to grade 2 moderate liver injury [22]. Baseline laboratory tests were obtained while patients were located in the emergency room and before initiation of any medication as defined by our institutional protocol regarding COVID-19 treatment. The data were processed and analyzed without any personal identifiers to maintain patient confidentiality as per the Health Insurance Portability and Accountability Act (HIPAA).

2.3. Exposure of Interest and Outcomes

The exposure of interest was remdesivir. Patients were classified into two groups based on remdesivir administration: patients that received remdesivir and patients that did not receive remdesivir. The primary endpoints were AKI and ALI. The secondary

endpoints were initiation of dialysis, invasive mechanical ventilation, admission to ICU, and in-hospital death.

2.4. Statistical Analysis

Propensity score matching was conducted to create comparable groups [23]. The propensity scores were estimated using a logistic regression model, in which fourteen covariates were used: age, gender, BMI, hypertension, hyperlipidemia, diabetes, CAD, CHF, stroke, CKD, chronic liver disease, liver cirrhosis, chronic alcohol use disorder, and COVID-19 severity on admission. The estimated propensity score was the predicted probability of receiving remdesivir derived from the fitted model.

We performed a nearest-neighbor matching without (one-to-one) replacement. Once a remdesivir-treated patient had been matched with a non-remdesivir patient, the latter was no longer available as a potential match for subsequent remdesivir patients. An optimal caliper width of 0.2 of the pooled standard deviation of the logit of the propensity score was used [24].

Continuous data were presented as median with interquartile range (IQR) and categorical data as absolute and relative frequencies. The *t*-test was used to compare continuous variables and chi-square for dichotomous variables. To further assess the balance of covariates between the remdesivir and non-remdesivir groups before and after propensity-score matching, standardized mean differences (SMD) were also calculated. In contrast to significance testing, SMD does not depend upon the size of the sample [25]. A standardized mean difference lower than the absolute value of 10% was considered to support the assumption of balance between groups.

For both cohorts (before matching and after matching without replacement) the outcomes of mortality, AKI, and ALI were compared between groups using logistic regression models resulting in an odds ratio (OR) with a 95% confidence interval. We applied univariate analyses and one multivariate model for each cohort and outcome that included remdesivir and baseline characteristics that were found significant ($p < 0.05$) in the univariate.

Statistical analysis was performed with STATA (version 14·1; STATA Corporation, College Station, TX, USA) and for matching, the psmatch2 module was used [26]. A nominal p-value < 0.05 was considered statistically significant.

3. Results

3.1. Baseline Patient Characteristics

In total, 927 patients were included in this study (remdesivir: 427, non-remdesivir: 500), 480 women (51.8%) and 447 men (48.2%). The median age was 61 (IQR 47–73) years and the median BMI was 28.5 (IQR 24.4–33.5) kg/m^2. Hypertension, diabetes, and hyperlipidemia were the most common comorbidities being prevalent in 56.9%, 38.9%, and 26.5% of our patients, respectively. A total of 12.5% had CKD IIIA-V or ESRD on dialysis (CKD IIIA: 5.7%, CKD IIIB: 2.7% had CKD IV: 1.2%, CKD V: 1.9%, ESRD on dialysis: 1%). A total of 3% had chronic liver disease and 1.6% had liver cirrhosis. Regarding COVID-19 severity on admission, 52.5% were considered to have moderate disease, 35% had severe disease, and 11.8% had critical COVID-19. The rate of severe or critical COVID-19 in the remdesivir group was significantly higher compared to the non-remdesivir group ($p < 0.001$). Matching without replacement yielded a cohort of 248 patients. There were no missing data. Detailed baseline patient characteristics of the original cohort and the cohort after matching without replacement are presented in Table 1 and the density of propensity scores is presented in Figure 1.

Table 1. Baseline Patient Characteristics.

Characteristics	Before Matching					After Matching without Replacement				
		Remdesivir					Remdesivir			
	Total	No	Yes			Total	No	Yes		
	n = 927	*n* = 500	*n* = 427	*p*-Value	SMD	*n* = 248	*n* = 124	*n* = 124	*p*-Value	SMD
Gender—*n* (%)				0.285	0.070				0.525	0.080
Male	447 (48.2)	233 (46.6)	214 (50.1)			127 (51.2)	61 (49.2)	66 (53.2)		
Female	480 (51.8)	267 (53.4)	213 (49.9)			121 (48.8)	63 (50.8)	58 (46.8)		
Age—median (IQR)	61.0 (47.0–73.0)	59.0 (39.5–73.0)	63.0 (53.0–73.0)	<0.001	0.304	62.00 (50.5–73.5)	64.50 (51.0–74.0)	62.00 (49.0–72.5)	0.569	0.072
Age Category—*n* (%)				<0.001	0.249				0.801	0.075
18–44	195 (21.0)	148 (29.6)	47 (11.0)			40 (16.1)	19 (15.3)	21 (16.9)		
45–54	129 (13.9)	55 (11.0)	74 (17.3)			42 (16.9)	21 (16.9)	21 (16.9)		
55–64	198 (21.4)	93 (18.6)	105 (24.6)			50 (20.2)	22 (17.7)	28 (22.6)		
65–74	203 (21.9)	91 (18.2)	112 (26.2)			61 (24.6)	34 (27.4)	27 (21.8)		
≥75	202 (21.8)	113 (22.6)	89 (20.8)			55 (22.2)	28 (22.6)	27 (21.8)		
BMI—median (IQR)	28.51 (24.4–33.5)	27.46 (23.8–31.9)	30.02 (25.5–34.5)	<0.001	0.365	28.19 (24.4–33.7)	28.09 (25.5–33.5)	28.75 (23.5–33.8)	0.871	0.021
BMI Category—*n* (%)				<0.001	0.349				0.022	0.090
<25	259 (28.3)	165 (33.7)	94 (22.1)			64 (25.8)	25 (20.2)	39 (31.5)		
25–29.9	273 (29.8)	158 (32.2)	115 (27.0)			83 (33.5)	51 (41.1)	32 (25.8)		
≥30	384 (41.9)	167 (34.1)	217 (50.9)			101 (40.7)	48 (38.7)	53 (42.7)		
HTN—*n* (%)				<0.001	0.267				0.609	0.064
No	400 (43.2)	246 (49.2)	154 (36.1)			110 (44.4)	53 (42.7)	57 (46.0)		
Yes	527 (56.9)	254 (50.8)	273 (63.9)			138 (55.7)	71 (57.3)	67 (54.0)		
HLD—*n* (%)				0.582	0.036				0.780	0.035
No	681 (73.5)	371 (74.2)	310 (72.6)			176 (80.0)	89 (71.8)	87 (70.2)		
Yes	246 (26.5)	129 (25.8)	117 (27.4)			72 (29.0)	35 (28.2)	37 (29.8)		
DM—*n* (%)				0.004	0.188				0.372	0.113
No	567 (61.2)	327 (65.4)	240 (56.2)			135 (54.4)	64 (51.6)	71 (57.3)		
Yes	360 (38.8)	173 (34.6)	187 (43.8)			113 (45.6)	60 (48.4)	53 (42.7)		
CAD—*n* (%)				0.926	0.006				0.718	0.045
No	834 (90.1)	449 (90.0)	385 (90.2)			212 (85.5)	107 (86.3)	105 (84.7)		
Yes	92 (9.9)	50 (10.0)	42 (9.8)			36 (14.5)	17 (13.7)	19 (15.3)		
CHF—*n* (%)				0.518	0.042				1.000	0.000
No	823 (88.8)	447 (89.4)	376 (88.1)			214 (86.3)	107 (86.3)	107 (86.3)		
Yes	104 (11.2)	53 (10.6)	51 (11.9)			34 (13.7)	17 (13.7)	17 (13.7)		
Stroke—*n* (%)				0.052	0.128				0.527	0.080
No	846 (91.3)	448 (89.6)	398 (93.2)			223 (89.9)	113 (91.1)	110 (88.7)		
Yes	81 (8.7)	52 (10.4)	29 (6.8)			25 (10.1)	11 (8.9)	14 (11.3)		
CKD—*n* (%)				0.006	0.133				0.040	0.088
No	809 (87.5)	433 (87.0)	376 (88.1)			199 (80.2)	103 (83.1)	96 (77.4)		
IIIA	53 (5.7)	20 (4.0)	33 (7.7)			22 (8.9)	5 (4.0)	17 (13.7)		
IIIB	25 (2.7)	16 (3.2)	9 (2.1)			10 (4.0)	4 (3.2)	6 (4.8)		
IV	11 (1.2)	9 (1.8)	2 (0.5)			4 (1.6)	2 (1.6)	2 (1.6)		
V	18 (2.0)	15 (3.0)	3 (0.7)			11 (4.4)	9 (7.3)	2 (1.6)		
ESRD with HD—*n* (%)	9 (1.0)	5 (1.0)	4 (0.9)			2 (0.8)	1 (0.8)	1 (0.8)		
Chronic liver disease—*n* (%)				0.113	0.110				1.000	0.000
No	897 (97.0)	477 (95.8)	420 (98.4)			240 (96.8)	120 (96.8)	120 (96.8)		
Alcoholic hepatitis	25 (2.7)	19 (3.8)	6 (1.4)			8 (3.2)	4 (3.2)	4 (3.2)		
HepB	1 (0.1)	1 (0.2)	0 (0.0)							
HepC	2 (0.2)	1 (0.2)	1 (0.2)							

Table 1. *Cont.*

Characteristics	Before Matching					After Matching without Replacement				
		Remdesivir					Remdesivir			
	Total	No	Yes			Total	No	Yes		
	n = 927	*n* = 500	*n* = 427	*p*-value	SMD	*n* = 248	*n* = 124	*n* = 124	*p*-value	SMD
Cirrhosis—*n* (%)				0.123	0.129				0.845	0.038
No	911 (98.4)	487 (97.6)	424 (99.3)			243 (98.0)	121 (97.6)	122 (98.4)		
Compensated	10 (1.1)	8 (1.6)	2 (0.5)			3 (1.2)	2 (1.6)	1 (0.8)		
Decompensated	5 (0.5)	4 (0.8)	1 (0.2)			2 (0.8)	1 (0.8)	1 (0.8)		
Chronic alcohol use disorder—*n* (%)				0.002	0.236				0.485	0.021
No	882 (95.4)	464 (93.2)	418 (97.9)			234 (94.4)	118 (95.2)	116 (93.6)		
In remission	22 (2.4)	16 (3.2)	6 (1.4)			7 (2.8)	2 (1.6)	5 (4.0)		
Active	21 (2.3)	18 (3.6)	3 (0.7)			7 (2.8)	4 (3.2)	3 (2.4)		
COVID-19 severity on admission—*n* (%)				<0.001	1.655				0.456	0.057
Moderate	486 (52.5)	428 (85.8)	58 (13.6)			117 (47.2)	59 (47.6)	58 (46.8)		
Severe	331 (35.8)	50 (10.0)	281 (65.8)			97 (39.1)	45 (36.3)	52 (41.9)		
Critical	109 (11.8)	21 (4.2)	88 (20.6)			34 (13.7)	20 (16.1)	14 (11.3)		

BMI in kg/m^2. Abbreviations and symbols: BMI = body mass index; kg = kilograms; m= meter; *n* = number; IQR = interquartile range; HTN = hypertension; HLD = hyperlipidemia; DM = Diabetes Mellitus; CAD = coronary artery disease; CHF = Congestive Heart Failure; CKD: chronic kidney disease; Hep B = Hepatitis B; Hep C = Hepatitis C.

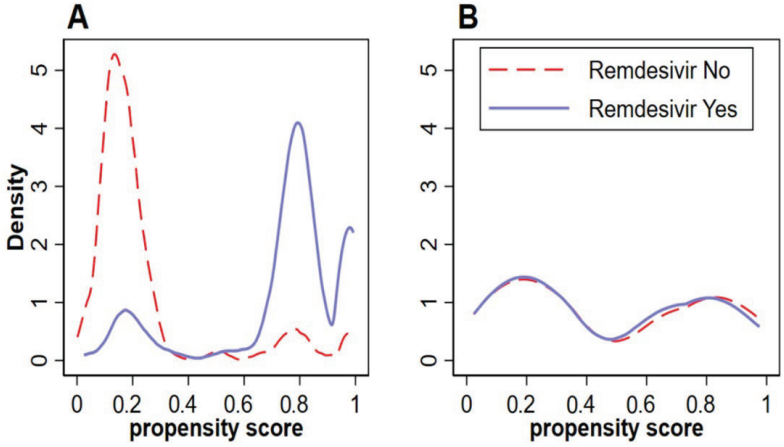

Figure 1. Density of Propensity Scores (**A**): Before Matching, (**B**): After Matching without Replacement.

3.2. Laboratory Markers on Presentation

In the overall cohort, median BUN was 15 (IQR 11–24) mg/dL, median Cr was 1.0 (IQR 0.8–1.3) mg/dL, median AST was 34 (IQR 24–60) U/L, and median ALT was 26 (IQR 16–45.5) U/L. Concentrations of baseline laboratory markers of the original cohort and the cohort after matching without replacement are presented in Table 2.

Table 2. Laboratory tests on presentation.

Laboratory Tests	Before Matching					After Matching without Replacement				
	Remdesivir					Remdesivir				
	Total—*n* (%)	No—*n* (%)	Yes—*n* (%)			Total—*n* (%)	No—*n* (%)	Yes—*n* (%)		
	n = 927	*n* = 500	*n* = 427	*p*-Value	SMD	*n* = 248	*n* = 124	*n* = 124	*p*-Value	SMD
BUN (mg/dL)—median (IQR)	15.00 (11.0–24.0)	14.00 (10.0–22.0)	15.00 (11.0–25.0)	0.081	0.115	16.00 (11.0–29.0)	16.00 (11.0–33.0)	15.00 (11.0–27.0)	0.200	0.164
Cr (mg/dL)—median (IQR)	1.00 (0.8–1.3)	0.90 (0.7–1.3)	1.00 (0.8–1.3)	0.782	0.018	1.10 (0.8–1.6)	1.10 (0.8–1.6)	1.00 (0.8–1.5)	0.157	0.181
Albumin (g/dL)—median (IQR)	3.80 (3.5–4.2)	3.90 (3.5–4.3)	3.80 (3.5–4.0)	0.005	0.189	3.80 (3.3–4.1)	3.70 (3.2–4.1)	3.80 (3.5–4.1)	0.559	0.075
Total Bilirubin (mg/dL)—median (IQR)	0.40 (0.3–0.6)	0.40 (0.3–0.7)	0.40 (0.3–0.5)	0.002	0.214	0.40 (0.3–0.6)	0.40 (0.3–0.7)	0.30 (0.2–0.5)	0.023	0.297
ALP (U/L)—median (IQR)	79.00 (62.0–109.0)	85.00 (64.0–119.0)	75.00 (58.0–97.0)	0.002	0.214	79.00 (60.0–111.0)	82.00 (61.0–133.0)	74.00 (58.0–93.0)	0.007	0.359
AST (U/L)—median (IQR)	34.00 (24.0–60.0)	29.00 (21.0–49.0)	42.00 (28.0–64.0)	0.251	0.079	38.00 (27.0–63.0)	34.00 (25.0–62.0)	42.00 (30.0–63.0)	0.029	0.281
ALT (U/L)—median (IQR)	26.00 (16.0–45.5)	23.50 (14.0–42.0)	28.00 (19.0–48.0)	0.256	0.078	29.00 (18.0–53.0)	29.50 (17.0–67.0)	27.00 (20.0–46.0)	0.019	0.303

Results of liver and kidney function tests in patients who received remdesivir and patients who did not receive remdesivir, before and after matching are provided. Abbreviations and symbols: *n* = number; mg = milligram; dL = deciliter; U/L = Unit/Litre BUN = Blood urea nitrogen; Cr = Creatinine; ALP = Alkaline phosphatase; AST = Aspartate aminotransferase; ALT = Alanine aminotransferase; IQR = Interquartile range.

3.3. Outcomes

In-hospital, AKI was observed in 13.2% of patients in the original cohort (remdesivir 15.5%, non-remdesivir 11.2%, $p = 0.055$). After matching, AKI had a significantly lower rate in the remdesivir group compared to the non-remdesivir group (12.1% vs. 21.8%, $p = 0.042$). Only 9 patients (0.9%) required initiation of dialysis without significant differences observed in the original cohort or the cohort after matching. The mean serum creatinine was decreased from 1.37 mg/dL before treatment with remdesivir to 1.21 mg/dL after completion of treatment with remdesivir in the cohort before matching ($p < 0.001$) and from 1.34 mg/dL to 1.19 mg/dL in the cohort after matching ($p = 0.007$). ALI was observed in 8.2% of patients in the original cohort (remdesivir 9.6%, non-remdesivir 7%, $p = 0.150$). After matching, a signal towards lower ALI incidence in the remdesivir group compared to the non-remdesivir group was noted (7.3% vs. 14.5%, $p = 0.067$). In the overall cohort, a total of 11.9% died during hospitalization, 12.7% required intubation, and 19.3% required admission to the ICU. The rates of in-hospital death, intubation, and ICU admission were significantly higher in the remdesivir group (18%, 19.4%, 28.8%, respectively) compared to the non-remdesivir group (6.6%, 7%, 11.2%, respectively) ($p < 0.001$). After matching, however, the rates of in-hospital death, intubation, and ICU admission were higher in the non-remdesivir group (16.1%, 19.4%, 25%, respectively) compared to the remdesivir group (13.7%, 11.3%, 21.8%, respectively) but these differences were not statistically significant. In-hospital outcomes and AKI per stage are presented in Table 3 and Supplementary Table S1 respectively.

Subgroup analyses of the patients with CKD and chronic liver disease for the outcomes of AKI and ALI, respectively, were conducted. In the matched cohort, the AKI rates were similar in patients that received remdesivir to those that did not receive it (27.3% vs. 26.7%, $p = 0.967$). One patient developed ALI (1/4) among patients with chronic liver disease that did not receive remdesivir in the cohort after matching and no patients among those that were treated with remdesivir (0/4). The subgroup analysis is presented in Table 4.

Table 3. In-hospital outcomes.

Outcomes	Before Matching					After Matching without Replacement				
	Remdesivir					Remdesivir				
	Total—*n* (%)	No—*n* (%)	Yes—*n* (%)			Total—*n* (%)	No—*n* (%)	Yes– *n* (%)		
	n = 927	*n* = 500	*n* = 427	*p*-Value	SMD	*n* = 248	*n* = 124	*n* = 124	*p*-Value	SMD
Intubation—*n* (%)				<0.001	0.373				0.078	0.22
No	809 (87.3)	465 (93.0)	344 (80.6)			210 (84.7)	100 (80.7)	110 (88.7)		
Yes	118 (12.7)	35 (7.0)	83 (19.4)			38 (15.3)	24 (19.4)	14 (11.3)		
Admission to ICU—*n* (%)				<0.001	0.451				0.548	0.08
No	748 (80.7)	444 (88.8)	304 (71.2)			190 (76.6)	93 (75.0)	97 (78.2)		
Yes	179 (19.3)	56 (11.2)	123 (28.8)			58 (23.4)	31 (25.0)	27 (21.8)		
Death—*n* (%)				<0.001	0.352				0.593	0.07
No	817 (88.1)	467 (93.4)	350 (82.0)			211 (85.1)	104 (83.9)	107 (86.3)		
Yes	110 (11.9)	33 (6.6)	77 (18.0)			37 (14.9)	20 (16.1)	17 (13.7)		
AKI during hospitalization				0.056	0.125				0.042	0.26
No	805 (86.8)	444 (88.8)	361 (84.5)			206 (83.1)	97 (78.2)	109 (87.9)		
Yes	122 (13.2)	56 (11.2)	66 (15.5)			42 (16.9)	27 (21.8)	15 (12.1)		
New dialysis during hospitalization				0.055	0.123				0.156	0.180
No	918 (99.0)	498 (99.6)	420 (98.4)			246 (99.2)	122 (98.4)	124 (100.0)		
Yes	9 (1.0)	2 (0.4)	7 (1.6)			2 (0.8)	2 (1.6)	0 (0.0)		
ALI during hospitalization				0.150	0.094				0.067	0.23
No	851 (91.8)	465 (93.0)	386 (90.4)			221 (89.1)	106 (85.5)	115 (92.7)		
Yes	76 (8.2)	35 (7.0)	41 (9.6)			27 (10.9)	18 (14.5)	9 (7.3)		

(1) The outcomes are presented as *n* (%), (2) Presence or absence of each outcome is indicated by 'yes' and 'no' below it, (3) Before Matching, out of a total of 927 patients, 427 received remdesivir and 500 did not. After matching, a total of 248 patients were divided into two equal groups based on the administration of remdesivir. Abbreviations and symbols: *n* = number; ICU = Intensive Care Unit; AKI = Acute Kidney Injury; ALI = Acute Liver Injury.

Table 4. Subgroup Analysis for patients with chronic kidney disease and chronic liver disease.

	Before Matching					After Matching without Replacement				
	Remdesivir					Remdesivir				
	Total—*n* (%)	No—*n* (%)	Yes—*n* (%)			Total—*n* (%)	No—*n* (%)	Yes—*n* (%)		
Patients with CKD	*n* = 107	*n* = 60	*n* = 47	*p*-value	SMD	*n* = 37	*n* = 15	*n* = 22	*p*-value	SMD
AKI during hospitalization				0.141	0.291				0.967	0.013
No	84 (78.5)	44 (73.3)	40 (85.1)			27 (73.0)	11 (73.3)	16 (72.4)		
Yes	23 (21.5)	16 (26.7)	7 (14.9)			10 (27.0)	4 (26.7)	6 (27.3)		
Patients with Chronic Liver Disease	*n* = 28	*n* = 21	*n* = 7			*n* = 8	*n* = 4	*n* = 4		
ALI during hospitalization				0.111	0.872				0.285	0.707
No	22 (78.6)	15 (71.4)	7 (100.0)			7 (87.5)	3 (75.0)	4 (100.0)		
Yes	6 (21.4)	6 (28.6)	0 (0.0)			1 (12.5)	1 (25.0)	0 (0.0)		

Subgroup Analysis of patients with chronic kidney disease and chronic liver disease with and without acute kidney injury and acute liver injury respectively. Abbreviations and symbols: CKD: Chronic Kidney Disease, AKI: Acute Kidney Injury; ALI: Acute Liver Injury.

3.4. Logistic Regression Analyses

3.4.1. Acute Kidney Injury

In the multivariable analysis for the outcome of AKI, patients treated with remdesivir were found to be associated with a significantly lower likelihood of AKI (OR: 0.40; 95% CI:

0.24–0.67, $p < 0.001$) in the overall cohort, while the association was on the verge of statistical significance in the smaller post-matching cohort (OR: 0.48; 95% CI: 0.23–1.01, $p = 0.054$). Higher age group, hypertension, and higher COVID-19 severity on presentation were all associated with a higher likelihood of AKI. The univariate and multivariate analyses for the outcome of AKI in the overall and post-matching cohorts are presented in Table 5.

Table 5. Logistic Regression Analysis for Acute Kidney Injury.

Outcomes	Before Matching		After Matching without Replacement	
	Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
		n = 926		*n* = 248
	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value
Female	0.73 (0.50–1.08) $p = 0.113$		0.84 (0.43–1.64) $p = 0.614$	
Age Category	1.57 (1.36–1.80) $p < 0.001$	1.36 (1.15–1.62) $p < 0.001$	1.42 (1.09–1.85) $p = 0.009$	1.26 (0.93–1.69) $p = 0.131$
BMI	1.02 (0.99–1.04) $p = 0.134$		1.02 (0.98–1.06) $p = 0.290$	
Hypertension	2.88 (1.85–4.50) $p < 0.001$	1.82 (1.08–3.07) $p = 0.026$	2.27 (1.10–4.68) $p = 0.027$	1.67 (0.79–3.50) $p = 0.177$
Hyperlipidemia	1.48 (0.99–2.23) $p = 0.059$		1.45 (0.72–2.93) $p = 0.298$	
Diabetes	1.97 (1.34–2.89) $p = 0.001$	1.09 (0.69–1.74) $p = 0.712$	1.75 (0.90–3.43) $p = 0.102$	
CAD	1.09 (0.59–2.04) $p = 0.775$		1.50 (0.63–3.57) $p = 0.364$	
CHF	2.52 (1.54–4.13) $p < 0.001$	1.44 (0.84–2.46) $p = 0.181$	2.82 (1.25–6.38) $p = 0.013$	1.59 (0.67–3.78) $p = 0.297$
Stroke	1.29 (0.69–2.42) $p = 0.422$		1.26 (0.44–3.57) $p = 0.668$	
CKD or ESRD	1.17 (1.00–1.37) $p = 0.051$		1.57 (0.72–3.40) $p = 0.254$	
Chronic liver disease	1.03 (0.60–1.78) $p = 0.906$		3.09 (0.71–13.51) $p = 0.134$	
Cirrhosis	1.40 (0.63–3.08) $p = 0.410$		2.32 (0.57–9.46) $p = 0.241$	
COVID-19 severity on admission	2.86 (2.14–3.81) $p < 0.001$	3.69 (2.61–5.21) $p < 0.001$	3.28 (1.94–5.53) $p < 0.001$	2.99 (1.76–5.09) $p < 0.001$
Remdesivir	1.45 (0.99–2.12) $p = 0.057$	0.40 (0.24–0.67) $p = 0.000$	0.49 (0.25–0.98) $p = 0.045$	0.48 (0.23–1.01) $p = 0.054$

(1) BMI in kg/m^2, (2) age in years. Abbreviations and symbols: BMI: Body Mass Index, CAD: Coronary Artery Disease, CHF: Congestive Heart Failure, CKD: Chronic Kidney Disease; ESRD: End-Stage Renal Disease, OR: Odd's ratio; CI: Confidence index.

3.4.2. Acute Liver Injury

In the multivariable analysis for the outcome of ALI, no association between treatment with remdesivir and ALI was noted in either cohort (overall cohort OR: 0.68; 95% CI: 0.35–1.30, $p = 0.241$; post-matching cohort OR: 0.47; 95% CI: 0.20–1.11, $p = 0.087$). Higher COVID-19 severity on presentation was the only variable associated with a higher likelihood for ALI in this analysis, whereas female sex and hypertension were the only variables associated with a lower likelihood for ALI. The univariate and multivariate analyses for the outcome of ALI in the overall and post-matching cohorts are presented in Table 6.

3.4.3. Mortality

In the multivariable analysis for the outcome of in-hospital mortality, a trend towards an association of patients treated with remdesivir with a lower likelihood for in-hospital death was observed in the overall cohort (OR: 0.57; 95% CI: 0.32–1.01, $p = 0.053$) that was lost in the smaller post-matching cohort (OR: 0.97; 95% CI: 0.42–2.22, $p = 0.941$). Higher age group, CKD/ESRD, and higher COVID-19 severity on presentation were associated with a higher likelihood of death. The univariate and multivariate analyses for the outcome of death in the overall and post-matching cohorts are presented in Table 7.

Table 6. Logistic Regression Analysis for Acute Liver Injury.

Outcomes	Before Matching		After Matching without Replacement	
	Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
		n = 924		*n* = 248
	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value
Female sex	0.45 (0.28–0.74) $p = 0.002$	0.58 (0.35–0.98) $p = 0.041$	0.69 (0.31–1.56) $p = 0.378$	
Age Category	0.90 (0.78–1.04) $p = 0.151$		0.84 (0.64–1.09) $p = 0.191$	
BMI	1.01 (0.98–1.04) $p = 0.446$		1.00 (0.96–1.04) $p = 0.963$	
Hypertension	0.52 (0.33–0.84) $p = 0.008$	0.57 (0.32–1.02) $p = 0.060$	0.60 (0.27–1.35) $p = 0.219$	
Hyperlipidemia	0.44 (0.23–0.86) $p = 0.015$	0.66 (0.32–1.35) $p = 0.255$	0.84 (0.34–2.09) $p = 0.707$	
Diabetes	0.58 (0.34–0.97) $p = 0.039$	0.67 (0.36–1.23) $p = 0.199$	0.56 (0.24–1.31) $p = 0.182$	
CAD	0.48 (0.17–1.35) $p = 0.164$		0.71 (0.20–2.51) $p = 0.597$	
CHF	0.66 (0.28–1.56) $p = 0.341$		0.47 (0.11–2.10) $p = 0.324$	
Stroke	1.07 (0.47–2.40) $p = 0.879$		1.66 (0.52–5.26) $p = 0.392$	
CKD or ESRD	0.69 (0.31–1.54) $p = 0.363$		0.91 (0.33–2.56) $p = 0.864$	
Chronic liver disease	1.49 (0.89–2.51) $p = 0.131$		1.18 (0.14–9.98) $p = 0.882$	
Cirrhosis	0.69 (0.17–2.88) $p = 0.613$		cannot be estimated	
COVID-19 severity on admission	2.19 (1.57–3.05) $p < 0.001$	2.75 (1.81–4.16) $p < 0.001$	2.20 (1.23–3.92) $p = 0.008$	2.16 (1.22–3.82) $p = 0.008$
Remdesivir	1.41 (0.88–2.26) $p = 0.152$	0.68 (0.35–1.30) $p = 0.241$	0.46 (0.20–1.07) $p = 0.072$	0.47 (0.20–1.11) $p = 0.087$

(1) BMI in kg/m^2, (2) age in years. Abbreviations and symbols: BMI: Body Mass Index, CAD: Coronary Artery Disease, CHF: Congestive Heart Failure, CKD: Chronic Kidney Disease; ESRD: End-Stage Renal Disease, OR: Odd's ratio; CI: Confidence index.

Table 7. Logistic Regression Analysis for In-hospital Mortality.

Outcomes	Before Matching		After Matching without Replacement	
	Univariate Analysis	Multivariate Analysis	Univariate Analysis	Multivariate Analysis
		n = 924		*n* = 248
	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value	OR, 95% CI, *p*-Value
Female sex	0.72 (0.48–1.07) $p = 0.107$		0.87 (0.43–1.76) $p = 0.708$	
Age Category	1.49 (1.28–1.72) $p < 0.001$	1.36 (1.11–1.67) $p = 0.003$	1.22 (0.94–1.59) $p = 0.130$	
BMI	1.01 (0.99–1.04) $p = 0.295$		1.02 (0.97–1.06) $p = 0.484$	
Hypertension	1.32 (0.87–1.99) $p = 0.186$		1.20 (0.59–2.45) $p = 0.614$	
Hyperlipidemia	1.04 (0.67–1.63) $p = 0.852$		1.40 (0.67–2.93) $p = 0.378$	
Diabetes	1.20 (0.80–1.80) $p = 0.373$		1.31 (0.65–2.65) $p = 0.445$	
CAD	0.90 (0.45–1.78) $p = 0.753$		0.91 (0.33–2.51) $p = 0.851$	
CHF	2.09 (1.23–3.54) $p = 0.006$	1.17 (0.62–2.22) $p = 0.627$	1.96 (0.81–4.76) $p = 0.136$	
Stroke	1.80 (0.99–3.27) $p = 0.056$		4.83 (1.97–11.87) $p = 0.001$	3.34 (0.94–11.80) $p = 0.061$
CKD or ESRD	1.29 (1.10–1.51) $p = 0.001$	1.29 (1.08–1.54) $p = 0.006$	1.25 (0.98–1.60) $p = 0.071$	
Chronic liver disease	0.96 (0.52–1.76) $p = 0.889$		3.64 (0.83–15.96) $p = 0.087$	
Cirrhosis	0.88 (0.32–2.42) $p = 0.799$		1.69 (0.48–5.95) $p = 0.410$	
COVID-19 severity on admission	8.23 (5.46–12.43) $p < 0.001$	9.25 (5.99–14.29) $p < 0.001$	10.89 (4.50–26.36) $p < 0.001$	10.24 (4.14–25.31) $p < 0.001$
Remdesivir	3.11 (2.02–4.79) $p < 0.001$	0.57 (0.32–1.01) $p = 0.053$	0.83 (0.41–1.67) $p = 0.594$	0.97 (0.42–2.22) $p = 0.941$

(1) BMI in kg/m^2, (2) age in years. Abbreviations and symbols: BMI: Body Mass Index, CAD: Coronary Artery Disease, CHF: Congestive Heart Failure, CKD: Chronic Kidney Disease; ESRD: End-Stage Renal Disease, OR: Odd's ratio; CI: Confidence index.

4. Discussion

Our propensity score-matched study investigated the renal and liver safety outcomes and in-hospital mortality of patients treated with remdesivir in a cohort of 927 patients admitted with COVID-19 in a public hospital in the Bronx, New York. We found that the remdesivir group had a significantly lower rate of AKI and remdesivir was associated with a lower likelihood for AKI. In addition, an indication towards lower ALI rates in the remdesivir group was observed, while remdesivir itself was not associated with a higher or lower likelihood of ALI. Patients with CKD and chronic liver disease that were treated with remdesivir did not have higher rates of AKI or ALI, respectively, compared to those that did not receive remdesivir. Regarding in-hospital mortality, the remdesivir group had a non-significantly lower death rate compared to the non-remdesivir group and a trend towards an association of patients treated with remdesivir with a lower likelihood for in-hospital death was observed.

Our findings demonstrated that remdesivir not only is safe from the renal standpoint but might even be nephroprotective. No safety concerns were raised in patients with CKD that were treated with remdesivir either. COVID-19 initially thought to be primarily a respiratory disease, is actually a multisystem disease with several organs, often being involved including the kidneys [27–29]. While factors such as hemodynamic instability, shock, or hypovolemia leading to tubular injury are common mechanisms that might play a role in COVID-19-associated AKI, direct injury of the renal parenchyma by SARS-CoV-2 is likely [30,31]. Reports from autopsies of patients with COVID-19 with kidney injury revealed the presence of viral particles within both the tubular epithelium and the podocytes on electron microscopy [30]. A recent animal study showed that remdesivir may be nephroprotective in COVID-19 via effective inhibition of inflammatory immune responses, which specifically repress NLRP3 inflammasome activation in lipopolysaccharide (LPS)-activated macrophages in mice models [32]. Our findings line up with the SIMPLE-Moderate study that showed lower rates of AKI in patients receiving remdesivir compared to standard care (7% vs 10%) [4,33]. Therefore, it is likely that remdesivir improves renal outcomes both via direct inhibition of viral replication in the kidneys and through halting the inflammatory response and overall progression of COVID-19.

Our findings do not raise concerns regarding hepatotoxicity of remdesivir. Instead, the remdesivir group had lower rates of ALI compared to the non-remdesivir group but the statistical significance threshold was not reached. Our results are consistent with the ACTT-1 trial which had shown no difference in liver function test changes between remdesivir and non-remdesivir groups [4]. Similarly, a randomized controlled trial by Wang et al. reported a higher incidence of AST elevation in the placebo group compared to the remdesivir group (12% vs. 5%) [17]. Since liver injury in COVID-19 is likely caused by direct viral toxicity due to high ACE2 expression on hepatocytes and cholangiocytes [19,34], it is plausible the possibly lower ALI rate in patients who received remdesivir can be partially explained by inhibition of viral replication systemically and in the liver per se.

In the matched cohort of our study, patients that received remdesivir had a modestly lower in-hospital death rate without reaching statistical significance. The logistic regression analysis in the overall cohort revealed that remdesivir was on the verge of statistical significance to be associated with a lower likelihood for death after adjusting for important covariates including COVID-19 severity on presentation. Likely, our sample size did not provide adequate power to reveal a clear association. For instance, the RECOVERY trial which showed that Dexamethasone decreased mortality in patients with COVID-19 employed an almost seven times larger patient population [35]. The signal of possible mortality benefit depicted in our study is consistent with the results of a meta-analysis of randomized trials which demonstrated that remdesivir offered a modest decrease in mortality in patients that were on supplemental oxygen but not on mechanical ventilation [7].

Our study has several strengths. First, our patient population is of low socioeconomic status which is often underrepresented in literature. Second, we employed robust statistical analysis using the propensity-matched scoring system before estimating the

treatment effects. We should acknowledge that our study has several limitations. This was a retrospective cohort involving electronic medical records, hence, there are risks related to observational bias and unmeasured confounding that cannot be mitigated by a propensity-matched scoring system [36]. However, we employed a robust independent review process and strict methodology in our efforts to minimize bias. Second, our sample size, particularly after matching, was relatively low limiting its power to detect significant associations. Third, given the relatively low sample size, we were not able to take into consideration other important variables such as concomitant treatments.

5. Conclusions

In conclusion, our propensity score-matched study revealed that remdesivir was safe in our patient population including patients with and without CKD and chronic liver disease. Actually, some of our findings revealed that remdesivir might be nephroprotective. In addition, a signal was noted suggesting that remdesivir might have offered a survival benefit. Overall, our real-world study findings encourage the liberal use of remdesivir in the treatment of hospitalized patients with moderate-to-severe COVID-19.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11113132/s1, Table S1: Acute Kidney Injury per Stage.

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In-Hospital Antibiotic Use for COVID-19: Facts and Rationales Assessed through a Mixed-Methods Study

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Abstract: It is well known that during the coronavirus disease 2019 (COVID-19) pandemic, antibiotics were overprescribed. However, less is known regarding the arguments that have led to this overuse. Our aim was to understand the factors associated with in-hospital antibiotic prescription for COVID-19, and the rationale behind it. We chose a convergent design for this mixed-methods study. Quantitative data was prospectively obtained from 533 adult patients admitted in six hospitals (services of internal medicine, infectious diseases and pneumology). Fifty-six percent of the patients received antibiotics. The qualitative data was obtained from interviewing 14 physicians active in the same departments in which the enrolled patients were hospitalized. Thematic analysis was used for the qualitative approach. Our study revealed that doctors based their decisions to prescribe antibiotics on a complex interplay of factors regarding the simultaneous appearance of consolidation on the chest computer tomography together with a worsening of clinical conditions suggestive of bacterial infection and/or an increase in inflammatory markers. Besides these features which might suggest bacterial co-/suprainfection, doctors also prescribed antibiotics in situations of uncertainty, in patients with severe disease, or with multiple associated comorbidities.

Keywords: COVID-19; SARS-CoV-2; antibiotics; antibacterial agents; mixed methods; qualitative; quantitative

1. Introduction

Widespread antibiotic use leads over time to antimicrobial resistance, affecting all the countries, independent of their level of development. The Centers for Disease Control and Prevention reported that more than 2.8 million antibiotic-resistant infections occur in the U.S. every year, resulting in more than 35,000 deaths, but also in prolonged hospitalization, which represents a burden for the economy of any state [1]. Although the World Health

Organization is constantly drawing attention towards the need for new antibacterial drugs to be developed, if advances in preventing the selection and the spreading of new resistant strains are not made and measures are not be implemented, then any new drug will have the same fate as the older ones [2,3]. One of the most common conditions in which antibacterial agents have been misused is in the management of patients (inpatients as well as outpatients) with various viral respiratory tract infections (RTIs). A previous study which enrolled 196 hospitalized patients with confirmed viral RTIs reported that 67% began antibiotic therapy, and 64% continued it after the confirmation of the viral infection, while 63% of the latter had normal chest-imaging findings [4]. At present, there is growing interest in the ways antibacterial agents were prescribed during the coronavirus disease 2019 (COVID-19) pandemic. It was shown that a minority of patients had a coinfection at admission (3.5 to 18.5%) or developed a secondary bacterial infection (3.8 to 14.3%), but more than 70% received antibiotics while hospitalized [5,6].

Currently, it is quite clear that antibiotics were overprescribed for both in- and outpatients with COVID-19 despite the relatively low rate of confirmed bacterial infections [5,6]. However, less is known about the rationale that has led to this antibiotic overuse. When reporting this high rate of antibiotic prescription to the enormous number of patients confirmed with COVID-19 since the pandemic emerged, it becomes obvious that we need to understand why the clinicians felt so tempted to give antibiotics. This information could be useful for further guidelines and antimicrobial stewardship programs that explore the aforementioned principles to be elaborated and implemented as support for doctors in guiding their decisions when managing future viral infections.

We aimed to understand the rationale behind antibiotic prescriptions in COVID-19, both by analyzing which factors proved to be associated with antibiotic treatment and by exploring the complex reasoning which ultimately served as grounds in this decision.

2. Materials and Methods

Research Design

In our mixed-methods study we chose a convergent design to investigate antibiotic prescription during the COVID-19 pandemic. In a convergent design, quantitative and qualitative data are collected and analyzed separately, the final step consisting of mixing the results during the interpretation of the data in order to achieve a more comprehensive analysis [7]. Our aim was to assess if the results from the qualitative analysis are in agreement with the results from the quantitative approach. Moreover, we also wished to explore the potential disagreements that may arise given the fact that in clinical practice there is a complex cognitive process involving multiple pros and cons behind the decisions, which are almost impossible to be evaluated only through quantitative instruments. Therefore, our research question was the following: "What are the factors associated with in-hospital antibiotic prescriptions during the COVID-19 pandemic, and what are the doctors' reasonings when deciding to administer antibacterial drugs?".

3. Quantitative Approach

3.1. Study Design and Population

For this study, we used the same database as for the study of Pinte et al., which had the primary objective of assessing the impact of antibiotic treatment on the mortality of hospitalized patients with COVID-19 [8]. This was a prospective, multicenter, cohort study conducted in six institutions in Romania. We included adult patients confirmed with COVID-19, admitted between January 2021 and May 2021, who were divided into two groups according to the prescription of antibiotics (dependent variable, outcome). The study participants were enrolled from the departments of Internal Medicine, Pneumology, and Infectious Diseases.

The inclusion criteria for the study were patients 18 years of age or older confirmed with SARS-CoV-2 infection by a positive real-time polymerase chain reaction (RT-PCR) test or rapid antigen test. The exclusion criteria were patients initially admitted in the

intensive care units (ICU), patients with end-stage kidney disease undergoing hemodialysis or peritoneal dialysis, and patients with hematologic malignancies. The treatment decision remained at the discretion of the attending physician. For the quantitative analysis we adhered to Strengthening the Reporting of Observational Studies in Epidemiology reporting guidelines [9].

3.2. Variables and Data Measurement

Patients were classified according to disease severity in agreement with the National Institutes of Health guidelines [10] as follows: mild (normal O_2 saturation and normal chest X-ray), medium (radiological evidence of COVID-19 pneumonia), and severe disease (at least one of the following criteria: peripheral oxygen saturation (SpO_2) $\leq$ 93% in ambient air, respiratory rate (RR) > 30/min, arterial oxygenation partial pressure to fractional inspired oxygen ratio (PaO_2/FiO_2 ratio) < 300, or lung infiltrates > 50% of lung parenchyma). During hospitalization, complete blood count, inflammation markers, and d-dimer values were obtained daily. We used the admission values for all patients and those prior to antibiotic administration (for patients who received antibiotics) and from the day with the greatest C-reactive protein (CRP) value (for patients who did not receive antibiotics). Patients who received oral vancomycin for *Clostridioides difficile* colitis were included in the non-antibiotic group.

3.3. Data Analysis

Demographic, clinical, biological, and imaging data of the enrolled patients were analyzed descriptively. Continuous and categorical variables were presented as median (min, max) and absolute numbers (percentage), respectively. The variables associated with $p \leq 0.10$ in bivariate analysis with antibiotic prescription were introduced into a logistic regression model (forward stepwise selection) with prescription of antibiotics (yes/no) as the dependent variable. Statistical significance was set at $p < 0.05$. We analyzed the collected data using the Statistical Package for Social Sciences (SPSS version 20, IBM Corp., Armonk, NY, USA) and Microsoft Excel 2018 (Microsoft Corporation, Redmond, WA, USA).

4. Qualitative Approach

4.1. Methodology

For the qualitative analysis we used semi-structured, in-depth interviews with physicians from the same departments from where the patients were included. The Consolidated Criteria for Reporting Qualitative Research were used to report the methodology of the qualitative design [11]. Participants' recruitment was directed via telephone, while the information and the consent forms were sent via e-mail. The interview was based on five questions which are presented in "Table 1".

Table 1. Interview topic guide.

In Your Opinion, How Often Do You Prescribe Antibiotics to COVID-19 Patients?
1. Which arguments/settings represent in your opinion a clear indication for antibiotic prescription in COVID-19 patients?
2. What are the arguments, or in which situations do you prescribe antibiotics in COVID-19 patients without having a certainty regarding the presence of an associated bacterial infection?
3. How do you differentiate between colonization and infection?
4. Do you consider your antibiotic prescription practices changed during the pandemic? How about when comparing the emergence of the pandemic with the actual moment when we have some experience in treating COVID-19 patients?

Additional questions were asked to ensure rich data collection. It was initially piloted on one person to establish if the questions we had designed would provide the needed data, but no modifications were made regarding the topic guide. The interviews were

audio recorded and conducted face-to-face or over the phone according to the participants preference. All the interviews were transcribed verbatim by the interviewer, with the anonymization of the transcript. After the publication of the article, all the audio recordings will be destroyed.

4.2. Sample and Data Collection

Participants were not involved in the development of the research questions, study design, and recruitment process. Since the decision to prescribe or not antibiotics may vary with age and experience, we purposely selected respondents towards achieving maximum of variation in age. Volunteers received no remuneration.

4.3. Analysis

Given the research question, we conducted a primarily experiential form of thematic analysis using an inductive, data-driven approach, while focusing on both latent and semantic levels. We followed the stages described by Braun and Clarke, which consist of familiarization with the data, generating initial codes, actively searching for the themes, reviewing potential themes, defining and naming themes, and finally writing up the themes into a report [12]. We included in our report codes not only based on the saturation principle but also on the saliency analysis principle [13]. After familiarizing with the data, the interviewer (first author) generated the codes and presented them to the last author, who was also the supervisor of the study; together we matched the codes into themes in three meetings. The report was then written and sent to three randomly selected participants to perform member checking. We achieved data saturation after 14 interviews.

4.4. Ethical Considerations

This study was performed in line with the principles of the Declaration of Helsinki and accepted by the Ethics Committee of the involved medical centers (32/08.12.2020). Patients signed an written informed consent during their hospital admissions, while the doctors who were enrolled in the qualitative analysis signed the informed consent before the interviews.

5. Results

5.1. Quantitative Approach

A total of 553 patients were included in the study of which 311 (56.2%) received antibiotics. The median time from admission until antibiotics prescription was 0 (min 0, max 24) days. Patients' characteristics at admission and at the moment of antibiotic initiation (for the patients who received antibiotics)/the day with the highest CRP value (for the patients who did not receive antibiotics), together with routine tests results, and the treatment they received for COVID-19 are presented in Table 2.

In our study, the variables associated with antibiotic prescription were older age, higher Charlson Comorbidity Index, COVID-19 severity, the presence of pulmonary infiltrates and pulmonary consolidation on CT scan, higher procalcitonin, WBC and neutrophils levels, but not higher inflammation markers values (CRP, ferritin). However, after adjusting for the pulmonary consolidation, the pulmonary infiltrates were no longer associated with antibiotic prescription. In Table 3, after adjusting for the factors related to antibiotic administration in bivariate analysis, only the presence of pulmonary consolidation, a higher Charlson Comorbidity Index, and higher neutrophil count remained independent factors associated with antibiotic prescription. (Table 3). This regression model predicted antibiotic prescription with an AUROC (95% CI) of 0.791 (0.751, 0.830).

Table 2. The distribution of the variables according to antibiotic prescription.

Variable	Antibiotics N = 311	Non-Antibiotics N = 242	AUROC (95% CI)	*p*-Value
Gender, male, N (%)	159 (51.1)	124 (51.2)		1
Age, median (min, max)	70 (32, 94)	65 (18, 92)	0.599 (0.551, 0.647)	<0.001
Charlson Comorbidity Index, median (min, max)	4 (0, 12)	3 (0, 12)	0.668 (0.622, 0.713)	<0.001
Disease severity, N (%)	311 (56.2)	242 (43.8)		<0.001
Mild	19 (6.1)	25 (10.3)		
Moderate	148 (47.6)	149 (61.6)		
Severe	144 (46.3)	68 (28.1)		
Pulmonary infiltrates, N (%)	298 (95.8)	217 (89.7)		0.006
Corticosteroid treatment, N (%)	237 (76.2%)	194 (80.2)		0.301
Tocilizumab, N (%)	13 (6.8%)	13 (5.4%)		0.594
Anakinra, N (%)	48 (15.4%)	41 (16.9)		0.643
Fever *, N (%)	48 (15.4)	44 (18.2)		0.421
Productive cough, N (%)	28 (9)	14 (5.8)		0.196
Symptoms of UTI, N (%)	5 (1.6)	2 (0.8)		0.476
Pulmonary consolidation on CT, N (%)	173 (55.6)	30 (12.4)		<0.001
SpO_2 at ATB p, median (min, max)	93 (53, 99)	93 (56, 99)		0.309
CRP *, median (min, max)	66.2 (0.2, 390.6)	61.5 (0.26, 312.2)	0.513 (0.462, 0.564)	0.614
Procalcitonin *, median (min, max)	0.15 (0.02, 24.8)	0.08 (0.02, 5)	0.671 (0.610, 0.732)	<0.001
Ferritin, median (min, max)	615.2 (58, 5887)	496 (6, 3993)	0.548 (0.496, 0.600)	0.089
WBC *, median (min, max)	8810 (1060, 29,760)	7100 (1205, 25,100)	0.634 (0.585, 0.683)	<0.001
Neutrophils *, median (min, max)	7160 (650, 26,400)	5240 (660, 20,000)	0.638 (0.589, 0.686)	<0.001
Lymphocytes *, median (min, max)	1005 (150, 5930)	1065 (260, 3500)	0.493 (0.441, 0.544)	0.788

* At the moment of antibiotic initiation (for the patients who received antibiotics)/the day with the highest CRP value (for the patients who did not receive antibiotics). Abbreviations: ATB—antibiotic, UTI—urinary tract infection, CT—computed tomography, SpO_2—oxygen saturation level, CRP—C-reactive protein, WBC—white blood count.

Table 3. Factors associated with antibiotic prescription (logistic regression).

Variables	B	OR	95% CI for OR		*p*
			Upper	Lower	
Charlson Comorbidity Index	0.177	1.193	1.071	1.330	0.001
Pulmonary consolidation	1.907	6.732	3.323	13.641	<0.001
Neutrophil count	0	1.000	1	1	0.001

5.2. Qualitative Approach

For the qualitative part of the study, we interviewed 14 physicians. The ages ranged from 29 to 57 years old; further characteristics of the doctors whom we interviewed are presented in Table 4.

Table 4. Participants' characteristics.

Participants		Numbers
Age	<30	2
	30–50	7
	>50	5
Gender	F	6
	M	8
Function	Senior Physician	12
	Resident Physician	2
Specialty	Internal Medicine	8
	Pneumology	1
	Infectious Diseases	5

After we analyzed and coded the transcripts, we identified two themes which are defined in Table 5: "Times have changed" and "Justifying antibiotic prescription" with the second theme having two subsequent subthemes.

Table 5. Overview of themes.

Themes Titles	Themes Definitions	Subthemes
Times have changed	This theme explores the difficulties perceived by physicians in the management of patients with COVID-19 due to the fact that the whole pattern of the patients changed from a clinical, as well as from a laboratory point of view when previous cut-offs of inflammatory markers were, in their opinion, no longer worthy to count on.	
Justifying antibiotic prescriptions	This theme explores the reasons why doctors prescribed antibiotics by approaching the clear indications for this practice, in addition to the equivocal determinants, to achieve a larger frame.	Clear indications
		When more is better

6. Times Have Changed

Before SARS-CoV-2 emerged, elevated values of inflammation markers and/or procalcitonin were linked to bacterial infection. Nowadays, when almost all patients admitted to the hospital have high levels of CRP and/or procalcitonin, together with clinical signs of pulmonary distress, doctors are tempted to associate this with a concomitant bacterial infection, thinking that maybe SARS-CoV-2 alone cannot produce biological abnormalities of such a magnitude.

> "We were used to prescribe antibiotics based on criteria regarding inflammation: CRP, ESR, and sometimes procalcitonin, and often leukocytosis, neutrophilia, and of course fever and chills. Now, due to the high prevalence of this viral infection which is associated with a marked inflammation, we tend to directly treat this inflammation, and we give much more antibiotics based only on CRP [. . .] or maybe we directly treat an elevated procalcitonin"
>
> (*Physician 1*)

However, as time went by, and more data emerged, most doctors realized that they could no longer approach the diagnosis of a bacterial infection based on biological inflammation, which is difficult to be used as a reason for "here is a bacterial infection, we have to give antibiotics." (*Physician 2*)

Besides the inflammation, many doctors felt that they could no longer use other laboratory markers of bacterial infection, such as leukocytosis and neutrophilia, nor clinical markers, such as fever and chills.

> "Those patients . . . they don't develop fever, many of them . . . or, what kind of sepsis is this, if you don't have fever, you don't have leukocytosis, you only have a CRP which is rising, and procalcitonin . . . if procalcitonin is good, at least you are somehow more comfortable [. . .] this is one question that I keep asking myself . . . either these patients with COVID-19 do not develop leukocytosis, or those patients who did not develop leukocytosis did not have a bacterial infection and we prescribed them antibiotics for nothing"
>
> (*Physician 7*)

However, in the conundrum of inflammatory marker cut-offs, procalcitonin was the one that lead to the most divided opinions, with some doctors guiding the prescriptions of antibiotics based on their value and previous thresholds, while others put it quite in the same place with the CRP levels, considering that higher cut-off values would be more appropriate, even though, before COVID-19, procalcitonin represented a strong argument in favor of bacterial infections, as it is shown below.

> "Once again, now we are resisting even when we see a procalcitonin of 1 or 2, and before, when we saw this level of procalcitonin, we were saying that it is clearly sepsis"
>
> (*Physician 7*)

> "Elevated procalcitonin. Everything that was even at the upper level of normal, I think that this was the point when I prescribed. If procalcitonin was somehow elevated, then I think that I jumped and I prescribed antibiotics".
>
> (*Physician 8*)

Left with few rapid strong arguments to diagnose a bacterial infection in an incipient phase and given the fact that previous cut-offs were no longer usable, doctors felt out of their comfort zone. Moreover, due to the enormous number of cases, some doctors were forced into treating patients with severe COVID-19, even though they were not used to treating this kind of pathology or patients with such severe respiratory distress. As consequence, they sometimes overtreated in order to feel the comfort of knowing that everything was done, while the need to cover a possible bacterial coinfection was frequently the main source of discomfort. Even though they had in mind the risks of developing an overwhelming antibiotic resistance in time, they considered that the risk of not treating a possible bacterial infection which would have explained the patients' symptoms, especially when you deal with a patient with a rapidly declining status, would have been much worse. Therefore, they often decided to do what seemed to be the best at that point, rather than keep worrying about complications which would appear after a long period of time.

> "I am always comparing with how I would feel if I were to work in a ward dealing with acute coronary syndromes . . . probably I would feel the same temptation . . . to administer any kind of medication in order to alleviate the symptoms that I am not used with, and I think that this is what everyone would do"
>
> (*Physician 10*)

> "When you are in a dilemma, you give what you consider that you should give, without any reproach, because you are in a dilemma, which means that you are outside of your comfort and expertise area, and until you build in, you have to react in a way that it is not mandatory to be 100% cortical, because you don't have the experience"
>
> (*Physician 5*)

7. Justifying Antibiotic Prescriptions

7.1. Clear Indications

When asked about the clear reasons that are decisive in favor of prescribing antibiotics, besides a positive culture, doctors exposed intricate cognitive processes involving clinical

symptoms, biological markers, and imagistic abnormalities, which go beyond a unique sine qua non factor. Therefore, most of them considered that the simultaneous appearance of consolidation on the chest computer tomography together with a shift in the patients' clinical status suggestive of bacterial infection, such as productive cough, chest pain, oxygen desaturation, or simply an alteration of the clinical status, or/and an elevation of the inflammation, was suggestive of bacterial infection; therefore, in these situations, physicians felt entitled to initiate antibiotic therapy along with active searching of the pathogen agent.

> "I would give antibiotics with all my heart when there are clinical elements that suggest bacterial coinfection [. . .] productive cough with purulent sputum from a clinical point of view . . . and from an imagistic point of view, a pattern of alveolar consolidation, in the detriment of interstitial abnormalities"
>
> (*Physician 2*)

> "An aggravation of the respiratory function, fever, usually when you don't expect for such abnormalities to appear, which means after many days since the symptoms of COVID-19 started, and all these things, of course, in the context of an elevation of the inflammation, whether it is accompanied or not by an elevated procalcitonin level"
>
> (*Physician 13*)

As it is illustrated above, no physician based their decision to prescribe antibiotics solely on one factor. They had to have more determinants, usually from the main three possible sources (clinical, biological, imagistic) to decide to administer antibiotics. Besides this mixture of determinants, another important aspect in the decision of prescribing or not prescribing antibiotics consisted in the timing of the moment when there was a shift in the clinical/paraclinical status of the patients. Thus, as it is shown below, if the abnormalities appeared soon after the onset of COVID-19 symptoms, the doctors considered that the deterioration was due to the aggravation of SARS-CoV-2 infection, rather than to bacterial overgrowth.

> "It mattered in taking the decision, when the patient came to us, because if the patients were hospitalized in the first days of the symptoms' onset, then . . . . . uuummm in the first 7–8 days, when the clinical picture is the most obvious, then I would wait to pass over this period. If the patient presented to us in the eighth or tenth day of the disease, or later, than I did not wait, because the chance for SARS-CoV-2 infection to be the explanation would be very low".
>
> (*Physician 3*)

7.2. When More Is Better

Even though physicians had in mind which were the clear indications for antibiotics prescription, many grey areas arose in practice when the feeling that more is better was legitimate in their perception, and consequently, they acted as such. In many cases, the balance between a bacterial infection versus COVID-19 aggravation represented the hardest decision to be made, considering that COVID-19 aggravation and the inflammatory storm may appear later in the disease evolution, which in many cases overlapped with a prolonged hospitalization, while the latter itself could have been a factor for bacterial coinfection or a hospital-acquired infection. Moreover, many hospitalized patients were frail, with multiple comorbidities, and received immunomodulators as a treatment for COVID-19, and for them, not treating a bacterial infection in an incipient phase could have been fatal.

> "The problem with these patients is that they come to the hospital for COVID-19, for a while they are well, and after that the CRP levels increase, and you always ask yourself . . . eventually with a degradation of the clinical status . . . and then

> the question is: is it the second phase of the disease, the cytokine storm, the hyperimmune phase, or is it a coinfection?"
>
> (*Physician 7*)

Therefore, the more is better principle arose in three settings which frequently overlapped: when the clinical status of the patient was very deteriorated, no matter the presence or the absence of previous comorbidities; when the patient had been aggressively treated with immunomodulators due to the severity of the COVID-19 disease; and when the patients were frail with multiple comorbidities, including diseases associated with immunosuppression.

> "The patient who is very severe and very fragile . . . sometimes you do not have time to wait . . . you have to give him antibiotic because you do not have much to lose at this point, and you have to save him no matter what . . . and if . . . if the antibiotic may be that saving element, and it must be prescribed early . . . I mean, you should not hesitate, you do not have time to hesitate"
>
> (*Physician 13*)

> "For example, if I want to treat a patient with immunomodulators, even if he has a colonization of the urinary tract, even if he has no complaints [. . .] if I have signs of an infection, a subclinical one, I would probably treat it, in a minimal fashion, five days a cystitis with the "easiest" or the most targeted antibiotic"
>
> (*Physician 5*)

8. Discussion

Our study revealed that doctors based their decision to prescribe antibiotics on a complex interplay of factors regarding the simultaneous appearance of consolidation on the chest-computed tomography together with a shift in the patients' clinical status suggestive of bacterial infection and/or an increase in inflammatory markers. The timing when the symptoms appeared, together with the Charlson Comorbidity Index, and the severity of the disease also played an important role in their choice. Besides these clear indications, doctors also decided to prescribe antibiotics in situations of uncertainty, when they considered that the "more is better" principle is appliable.

One of the main problems encountered during the COVID-19 pandemic regarding antibiotics prescription revolved around the fact that the clinical and paraclinical picture of the patients changed. Most of the patients had significant inflammation, while in the doctors' opinions, few of them (with confirmed bacterial infection or in sepsis) had associated markers of "traditional" bacterial infection, such as fever, leukocytosis, neutrophilia, or productive cough. Fever and productive cough did not correlate with antibiotic prescription in our study as opposed to the findings of Estrada et al. [14], which may have happened because these clinical symptoms appeared less often in practice (7.6% of the patients had productive cough, 17.2% had fever) than in previous times—inability to expectorate tracheobronchial secretions and fever blunted by corticosteroids use. Neutrophilia was strongly associated with antibiotic administration, in agreement with other results [14–16].

At first, doctors felt tempted to prescribe antibiotics based on elevated markers of inflammation, reminiscent from previous times when a high CRP value was frequently associated in clinical practice with a bacterial infection. As time went by, they realized that given the cytokine storm associated with the SARS-CoV-2 infection, this usual marker was no longer useful. Analyzing the qualitative data, only procalcitonin remained a useful argument for associated bacterial infection, its cut-off value being however debatable, with some of the doctors considering that, in COVID-19, higher diagnostic values would be more appropriate. This was further confirmed in the quantitative analysis, where high CRP values were not associated with antibiotic prescription, as opposed to procalcitonin, which showed a strong association. This may be since even though every doctor had different thresholds for bacterial infections when referring to procalcitonin, they used it to guide their prescriptions, while most of them considered elevated CRP values nonspecific,

being also associated with COVID-19 aggravation. However, previous studies reported an association not only between procalcitonin but also between higher CRP values and antibiotic prescription, which may be explained by the fact that the patients were enrolled early in the pandemic when less was known about the cytokine storm associated with COVID-19 [14–17]. Regarding procalcitonin's utility in diagnosing associated bacterial infections in patients with COVID-19, a recent study showed that a value <0.25 ng/mL has a negative predictive value of over 95% for bacteremia or bacterial pneumonia, but higher procalcitonin levels also predict COVID-19 severity in hospitalized patients [18]. Moreover, a meta-analysis also showed that elevated procalcitonin levels were associated with a nearly five-fold higher risk of developing a severe form of COVID-19, but this data needs to be cautiously interpreted since no analysis according to the presence or absence of associated bacterial infections was done [19]. Therefore, in agreement with doctors' opinions exposed in the qualitative analysis, procalcitonin is useful in guiding antibiotic treatment—low levels of procalcitonin shows that associated bacterial infections are unlikely, but high levels of procalcitonin are not diagnostic for bacterial coinfections since they may be due to COVID-19 related immune dysfunction.

Regarding the criteria for antibiotic prescription, doctors did not base their decisions solely on one reason, but rather on an interconnection of factors of which alveolar consolidation on computed tomography examination was highly predictive for antibiotic prescription. Previous qualitative studies regarding antibacterial drug use during COVID-19 were mostly developed in primary care settings where the clinical scenario along with the disease severity were completely different; therefore, we could not compare our findings regarding the complex rationale behind antibiotic prescription. However, a previous qualitative study published before the emergence of COVID-19 showed that physicians were likely to base their decisions to administer antimicrobial drugs based solely on clinical grounds, which were no longer appliable given the fact that the patient–physician interactions were severely shortened due to the risk of SARS-CoV-2 transmission [20]; this idea was also presented by Borek et al. in a qualitative study which involved general practitioners from England [21]. Regarding the presence of lung consolidation, in the Estrada et al. study [14], not only alveolar infiltrates but also interstitial infiltrates were linked to antibiotic prescriptions, while the presence of bilateral interstitial infiltrates were strongly associated with what was considered inappropriate antibiotic use in the study of Calderón-Parra et al. [16]. In our study, pulmonary infiltrates (without consolidation) were not a driver for antibiotic therapy as 90% of the patients who did not receive antibiotics had such infiltrates.

Besides clear criteria for antibiotic therapy, in qualitative analysis, all the clinicians considered that in some cases, more is better when it comes to antibiotic administration; most often, these cases were represented by an important deterioration of the patients' clinical status, iatrogenic immunosuppression (through immunomodulators for COVID-19), and patients' frailty (multiple comorbidities associated). Antibiotics overuse was previously reported to be associated with clinical uncertainty, when prescribing them was perceived to be a safer option during COVID-19 times, but also before the pandemic state [21,22]. However, the prescription of neither tocilizumab nor anakinra was associated with antibiotic therapy in quantitative analysis, probably due to a selection bias—doctors prescribed immunomodulators in patients who were unlikely to have an associated bacterial infection or in patients in whom such an infection was excluded. Therefore, immunomodulators administered on their own were not an important driver for antibiotic prescription. Although in a retrospective cohort study which took place early in the pandemic was launched the idea that antibiotic prophylaxis in tocilizumab use would be beneficial [23], we did not find previous studies to assess the association between the use of potent immunomodulators and antibiotic use.

Both the severity of the disease and the Charlson Comorbidity Index were associated with antibiotic prescription, which was further confirmed in the qualitative approach; physicians considered that in some situations of uncertainty—severe deterioration of the patients' clinical status, treatment with immunomodulators, and the presence of

multiple comorbidities—antibiotic prescriptions were justifiable, in order to avoid an unfavorable outcome.

The strength of this study resides in its mixed-methods design with the quantitative part being approached through a prospective, multicenter study. Given the fact that our aim was not to assess the degree of in-hospital implemented antimicrobial stewardship, we did not perform analyses regarding the appropriate vs. inappropriate antibiotic administration, but we evaluated which factors were the determinants in deciding whether to prescribe or not antibacterial drugs. One of the limitations of the study resides in the fact that we involved patients (quantitative study) and physicians (qualitative study) from only three specialties: internal medicine, pneumology, and infectious diseases, having in mind the fact that other specialties may have had other practices regarding antibiotic prescription. In our cohort, the number of patients enrolled from the infectious diseases departments was relatively small, and therefore, we could not perform a subgroup analysis.

Overall, we identified that physicians chose to prescribe antibiotics also in situations of uncertainty (in patients with a severe form of the disease or with multiple associated comorbidities), while in their opinion, the simultaneous appearance of abnormalities in the patients' clinical status, biological markers, and pulmonary-computed tomography represented a clear indication for in-hospital antibiotic use during the COVID-19 pandemic. Given the fact that antibiotic overuse in viral respiratory tract infections represents a common problem and still poses a challenge, these kinds of studies are important to be conducted so that the key arguments in doctors' views are identified and understood, in order to improve further antibiotic prescriptions in viral infections through targeted antimicrobial stewardship programs. Therefore, periodic in-hospital trainings regarding the peculiarities of patients with viral infections with and without associated bacterial infections (imagistic abnormalities, the role of inflammatory markers, rate of bacterial coinfections, criteria of certainty when it comes to antibacterial drugs administration) should be done to reduce treating out of the fear of missing infections (FOMI) and its inherent consequences.

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Review

Current Treatment Options for COVID-19 Associated Mucormycosis: Present Status and Future Perspectives

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Abstract: Mucormycosis has become increasingly associated with COVID-19, leading to the use of the term "COVID-19 associated mucormycosis (CAM)". Treatment of CAM is challenging due to factors such as resistance to many antifungals and underlying co-morbidities. India is particularly at risk for this disease due to the large number of patients with COVID-19 carrying comorbidities that predispose them to the development of mucormycosis. Additionally, mucormycosis treatment is complicated due to the atypical symptoms and delayed presentation after the resolution of COVID-19. Since this disease is associated with increased morbidity and mortality, early identification and diagnosis are desirable to initiate a suitable combination of therapies and control the disease. At present, the first-line treatment involves Amphotericin B and surgical debridement. To overcome limitations associated with surgery (invasive, multiple procedures required) and amphotericin B (toxicity, extended duration and limited clinical success), additional therapies can be utilized as adjuncts or alternatives to reduce treatment duration and improve prognosis. This review discusses the challenges associated with treating CAM and the critical aspects for controlling this invasive fungal infection—early diagnosis and initiation of therapy, reversal of risk factors, and adoption of a multipronged treatment strategy. It also details the various therapeutic options (in vitro, in vivo and human case reports) that have been used for the treatment of CAM.

Keywords: mucormycosis; COVID-19; fungal infection; risk factors; diagnosis; treatment

1. Introduction

Mucormycosis is a life-threatening invasive fungal infection (IFI), which, although once considered rare, has become increasingly prevalent in patients affected by SARS-CoV-2 [1]. The fungi responsible for mucormycosis belong to the order *Mucorales* and include genera such as *Rhizopus*, *Rhizomucor*, *Mucor*, *Lichtheimia*, *Cunninghamella* and *Saksenaea*. These fungi

are commonly present in the environment. Although they are well recognized to cause opportunistic infections in immunocompromised patients, 19% of mucormycosis has been reported in immunocompetent patients [2,3]. A main reason behind recent mucormycosis infections is COVID-19 [4].

Depending on varying factors, mucormycosis infection is classified into five major types: rhino-orbital/rhino-cerebral/rhino-orbital cerebral mucormycosis (ROM/RCM/ROCM), pulmonary mucormycosis, cutaneous mucormycosis, disseminated mucormycosis and gastric mucormycosis. Various rare forms of mucormycosis are osteomyelitis, renal, peritonitis and cardiac [5]. This review focuses on the different types of mucormycosis, wherein ROCM/ROM/RCM mucormycosis commonly reported in COVID-19 is briefly discussed [6]. ROCM/ROM/RCM is caused by the colonization and spread through inhalation of fungal spores in the nasal pathways and surrounding regions [7]. Like RCM, pulmonary mucormycosis is also caused by the inhalation of fungal spores [8]. Cutaneous mucormycosis is an invasive form of infection which occurs through skin abrasions. It has been reported to be contracted through intravascular devices in a heart transplant patient affected by COVID-19 [9]. Intake of food contaminated by fungal spores causes gastrointestinal mucormycosis, which is usually rare in immunocompetent patients but has been reported in patients associated with COVID-19 infection [10,11]. This condition might also typically have a poor prognosis, especially if it disseminates to the heart, usually diagnosed during an autopsy [12]. Renal mucormycosis is commonly observed in COVID-19 patients with kidney transplants and is often associated with poor prognosis [13–15]. Mucormycosis peritonitis has been reported in patients undergoing dialysis [16]. Maxillary osteomyelitis associated with mucormycosis is quite common, resulting in pain, swelling, and bone exposure. Disseminated mucormycosis is a non-specific form that is widespread in the body due to the angio-invasive nature of the fungi [13].

Since early 2021, IFIs such as COVID-19-associated pulmonary aspergillosis (CAPA) and COVID-19-associated mucormycosis (CAM) have been increasingly found. CAPA, also called white fungus infection, primarily affects the lungs, and severely impacts the kidney, mouth, skin and brain. CAM, also called black fungus infection, primarily affects the nose and sinuses associated with COVID-19 but can also affect other areas depending on the sub-type [14]. Although more cases of CAPA were reported initially, the number of instances of CAM has progressively increased after the pandemic. It was reported by Pal et al., 2021 that the most significant number of mucormycosis infections were from India, which might occur due to the high prevalence of diabetes mellitus (DM) [1]. Mucormycosis is usually detected 13–18 days after development of COVID-19. However, many cases of CAM have been reported after the complete resolution of COVID-19 [15]. The high morbidity and mortality associated with CAM necessitate early treatment initiation [17]. This review focuses on the mechanisms of pathogenesis, risk factors, and various strategies used to treat CAM.

2. Mechanisms of Pathogenesis

Mucormycosis invasion occurs through glucose-regulated proteins (GRPs), which are molecular chaperones of the Hsp70 family (70 KDa Heat Shock Proteins) [18]. Although these are present in the endoplasmic reticulum (ER) under normal circumstances, ER stress conditions such as DKA, and the associated changes in tissue microenvironment (glucose, iron and ketone bodies), result in overexpression of GRPs in different compartments and the cell surfaces [8]. GRP78 is an essential receptor for adhesion and invasion of fungal hyphae and the resultant injury of endothelial cells [19,20]. The interaction with fungi is mediated by the fungal ligand spore-coating homolog protein (CotH) in *Rhizopus*, commonly CotH3 for ROCM. In pulmonary mucormycosis, invasion and infection are facilitated by fungal CotH7 with integrin-β1 (with heterodimer formation with integrin-α3) [20], which enables the superficial entry into the nasal epithelium. Further invasion involves attachment to the collagen IV and laminin in the extracellular matrix of the basement membrane of the endothelial cells [16]. Mucoricin, a ricin-like toxin produced by the fungi, may also aid

this invasion and virulence [21,22]. Apart from adhesion, endocytosis is also responsible for causing damage to the host cells. Platelet-derived growth factor receptor (PDGFR) is involved in endocytosis and angioinvasion, which results in the dissemination of the infection and necrosis [23]. The mechanisms are discussed further along with risk factors to highlight the role of each element in causing disease.

3. Challenges in Control of Mucormycosis

The atypical clinical presentation of mucormycosis leads to increased disease spread, and hence early diagnosis is crucial and is the main target of current research. Direct examination, culture, and histopathology are the cornerstones of diagnosing mucormycosis, but they are time-consuming and lack sensitivity. Newer molecular diagnostic techniques, such as in situ hybridization and Polymerase Chain Reaction (PCR), offer an alternative that may lead to earlier diagnosis and prompt initiation of treatment [15]. Since mucormycosis is encountered during different phases of COVID-19, or even after recovery, high-risk patients should have regular follow ups [1].

Treatment of CAM is also complicated because early initiation of therapy is required to control the disease, but it should also be ensured that any empirical treatments for COVID-19 do not amplify the underlying co-morbidities, thus increasing the severity of the disease (e.g., steroid therapy causes immunosuppression, thus aggravating the disease) [24]. Additionally, mortality continues to be nearly 50% even after treatment [25]. Furthermore, since rural areas of India have limited access to health care facilities, this further adds to compromised treatment and increases mortality rates [15]. One of the most critical challenges is that a complete causal relationship between COVID-19 and mucormycosis is yet to be uncovered [26]. Hypotheses and possible associations between these two infections are discussed below.

4. Association of COVID-19 with Risk Factors of Mucormycosis and Their Role in Infection

The probability of acquiring mucormycosis is associated with various risk factors, of which the most important ones are DM (with or without ketoacidosis) and conditions causing immunocompromised status [27]. The primary risk factor affecting a population may also vary with geographical location. For example, in countries such as India, Iran and Mexico, the major pre-existing risk factor is DM, while primarily hematological malignancies are the main risk factor in Europe [5]. The predisposing condition may also determine the type of mucormycosis caused. Hematological malignancies and neutropenia are commonly associated with pulmonary mucormycosis, while DM is often related to rhinomaxillary and rhinocerebral disease [5,17,28]. Cutaneous mucormycosis is often associated with trauma or burns [5,9]. COVID-19, with or without immunosuppressive therapies, may act via various pathways to have a synergistic effect in creating an environment favorable for the development of CAM. Therefore, severe COVID-19 is considered a risk factor for mucormycosis. This section analyses CAM based on the link between COVID-19 and the various risk factors for mucormycosis. Additionally, the synergistic roles of these risk factors are explored.

4.1. Diabetes Mellitus and Diabetic Ketoacidosis

One of mucormycosis's primary and most common risk factors is uncontrolled DM (especially with ketoacidosis). DM increases the severity of SARS-CoV-2 and the risk of mucormycosis [9], especially RCM. Mucormycosis seen in diabetic patients has clinical manifestations, including cranial nerve palsy, diplopia, mid-facial pain, proptosis, periorbital oedema, apex orbital syndrome, and palatal ulcers [7]. COVID-19 is responsible for an acute cortisol stress response, which may raise serum cortisol levels and hyperglycemia in both persons with and without DM [29].

Diabetes may be pre-existing or associated with COVID-19 infection (corticosteroid therapy for COVID-19 or other infectious diseases predisposes patients to mucormyco-

sis) [27]. Diabetes or a hyperglycemic state is often associated with an inflammatory condition responsible for constant recruitment and activation of immune cells, which further exacerbates the inflammatory phenotype by increased secretion of proinflammatory cytokines. In these circumstances, antiviral immunity activation in response to SARS-CoV-2 infection also intensifies inflammation, which increases the chances of mucormycosis and other secondary infections [27]. DM promotes the growth and proliferation of fungal pathogens by affecting the immune system, affecting phagocytosis, chemotactic activity and transendothelial migration of neutrophils [30].

The virus affects angiotensin-converting enzyme 2 (ACE2) producing cells (including beta cells of the pancreas), leading to the decreased breakdown of angiotensin II. This causes insulin resistance and upregulation of the sodium and hydrogen exchanger (NHE). NHE can increase damage to the pancreas due to its role in insulin release [31]. NHE affects Na+ and Ca2+ transport, which leads to hypoxia [32]. This, along with COVID-19 associated cell lysis, leads to increased lactate levels, insulin resistance and endothelial damage. COVID-19 also causes lactic acidosis (accumulation of lactic acid), which further increases the activity of the NHE pump and increases the blood glucose level by gluconeogenesis. This also increases the serum iron concentration, which acts as a nutrition source for the growth of fungi [30].

Fungi of *Mucorales* are present generally in the environment [33]. They are opportunistic pathogens because normal human serum (at physiological pH range) can provide nutritional immunity against fungal invasion due to the iron-binding properties of transferrin and ferritin. This prevents fungi from getting access to iron for its functions [34]. However, COVID-19 may also cause diabetic ketoacidosis. Under the acidic conditions of diabetic ketoacidosis (DKA) (pH 4), this iron-binding ability reduces due to glycosylation of iron sequestering proteins, and so iron is no longer bound and utilized by the fungus for its disease pathogenesis [35].

Further, the favorable environment for fungal growth (high glucose levels, acidic conditions, ketone bodies such as β-hydroxy butyrate [BHB] and resultant free iron) created by DKA is responsible for increased expression of glucose-regulator protein 78 (GRP-78) on the surface of endothelium cells [8]. This interaction traps the inhaled spores in the nasal cavity, causing ROCM [20]. It is also involved in the entry of the SARS-CoV-2 and has been proposed as a potential drug target for targeting the virus [36,37]. As a result, invasion and injury of endothelial cells by *Rhizopus* is increased and tissue necrosis is observed [38]. DKA also causes immunosuppression by affecting T-lymphocyte induction, interferon-gamma and phagocytosis [8]. Additionally, administration of steroids in COVID 19 patients with pre-existing diabetes can affect phagocytosis by White Blood Cells and the destruction of pathogens by macrophages at various stages, making them more susceptible to *Mucorales* infections [38].

4.2. Immunosuppression

Prolonged administration of corticosteroid therapy or immunomodulatory drugs to patients with COVID-19 and pre-existing comorbidities can increase their risk of developing CAM. It was found that immunocompromised patients who crossed a threshold of 600 mg of prednisone (cumulative dose) or 2–7 g methyl prednisone (preceding month alone) are at higher risk of mucormycosis infection. In a study conducted by Patel et al. 2021, it was found that for the majority of the patients, the cumulative glucocorticoid dose administered vastly exceeded the recommended dosage. However, shorter courses of corticosteroid treatment of even 5–14 days have been found to predispose diabetic patients to mucormycosis [38,39]. Additionally, dexamethasone, a WHO-recommended corticosteroid treatment for severe or critically ill patients with COVID-19, has been associated with higher susceptibility to IFIs. These immunomodulatory and corticosteroid treatments and COVID-19 may affect phagocytosis and other immune responses [27]. Although steroid treatment in DM patients increases the risk of them developing CAM, the literature supports that patients

without DM have also developed CAM after steroid use. Therefore, it is recommended that steroid therapy be avoided, especially in patients who exhibit mild COVID-19 [40].

It has been hypothesized that COVID-19-mediated ACE2 dysregulation creates a cascade that results in an environment suitable for fungal growth through its effects on the pancreas, lungs, colon, ileum, esophagus, cardiovascular and cardiovascular tissues [30]. ACE2 is ubiquitous on the lymphocyte surface and is likely involved in lymphocyte damage in COVID-19 infection [41]. COVID-19 is believed to cause immunosuppression due to lymphocyte damage by apoptosis due to the cytokine storm (which involves elevated levels of various proinflammatory cytokines such as several interleukins and TNF-α) and the resultant lymphoid tissue atrophy [30,42]. This cytokine storm also results in lactic acidosis, which has a detrimental effect on the proliferation of lymphocytes [43]. Together, these factors cause a reduction in lymphocytes (lymphocytopenia) [31]. SARS-CoV-2 infection lowers the levels of CD4 and CD8 T-cells. It also affects the responses of lymphocytes Th1 and Th2 (T helper type 1 and 2 cells) [44]. As a result, COVID-19 patients with acute respiratory distress syndrome (ARDS) exhibit immune system alteration and increased susceptibility to IFIs such as mucormycosis. Given the potential impact on the immune system, COVID-19 treatment with immunomodulatory drugs, such as IL-6 inhibitors, should be reserved for selected patients according to existing guidelines [40].

COVID-19 is also associated with a reduction in phagocytosis, thrombosis and endothelialitis [38]. Endothelial adhesion and penetration are crucial for mucormycosis entry and infection. The increased IL-6 levels in response to COVID-19 and acidosis also result in ferritin production, leading to intracellular iron accumulation, which damages the tissue. This tissue damage is responsible for releasing iron into the bloodstream, enabling fungus growth [45].

4.3. Nosocomial Sources

Mucormycosis may also be associated with nosocomial sources, especially during prolonged hospitalization [46]. Non-sterile equipment in hospitals is the main disseminator of infections among immunocompromised patients. Such equipment includes unsterilized/non-sterile bandages, nitroglycerin patches, ostomy bags, hospital linens, adhesive tape, wooden tongue depressors and even consumables such as probiotics, prepackaged food and allopurinol tablets [5,47,48]. Medical apparatus and devices inserted into the body can allow direct access of fungal pathogens to infect the body. This includes intravascular devices such as IV catheters, lancets for insulin measurement, tubes inserted into the body, intubation, injections, and dental and surgical procedures [49]. A similar mode of infection is seen in intravenous drug abusers [38]. Prolonged ICU treatment can also increase the risk of mucormycosis, especially in patients under mechanical ventilation [50]. Environmental factors such as fungal pathogens in the air, water or surfaces in a hospital may also be responsible for hospital-associated mucormycosis. One such instance is the presence of oxygen humidifiers in hospitals which can spread potentially contaminated water, resulting in the significant spread of the disease [1]. Additionally, problematic plumbing and ventilation can augment the spread of infection among patients and lead to a community outbreak [5].

In the case of a heart transplant patient who did not demonstrate any of the usual risk factors associated with CAM, it was suggested that COVID-19 was responsible for lymphocytopenia and the resultant immunosuppression, which led to fungal infection [9]. The extent of respiratory pathology or pulmonary damage has been correlated with the nature of the risk of contracting CAM [51]. Intubation or mechanical (invasive) ventilation in the intensive care unit (ICU) for COVID-19 patients with ARDS for prolonged periods is a commonly observed risk factor for acquiring mucormycosis [52].

4.4. Other Factors

In general, treatment for COVID-19 with various antibiotics and immunosuppressive therapies such as monoclonal antibodies and steroids can cause dysbiosis of the human

microbiome and damage epithelial linings, which aids the development of IFIs. One such treatment for COVID-19 is zinc, since it is known to have antiviral effects [53]. However, extensive use of zinc is significantly associated with occurrence of CAM since it promotes the growth of pathogenic fungi, without much benefit in treating COVID-19 [54,55]. Protracted treatment with antifungals for pre-existing fungal infections and a history of IFIs also increase the patient's chances of being infected by *Mucorales* fungi [27]. Additionally, the renal tropism of the COVID-19 virus may also be responsible for kidney injury. Deferoxamine, administered to treat renal failure, is involved in iron sequestration by the *Mucorales* fungi, leading to mucormycosis [27]. In addition to all these aspects, in some cases, mucormycosis was observed even in COVID-19 patients without underlying predisposing factors, suggesting that the infection was responsible for creating a microenvironment favorable for the fungal population [56].

5. Diagnosis

Early diagnosis and intensive, multidisciplinary treatment and management of the disease are critical for a better prognosis. Intracranial extension was associated with a poor prognosis [57]. Hence early diagnosis is essential for better outcomes. Apart from clinical examination, imaging, histopathology, and culture are adjuncts for diagnosis.

5.1. Clinical Examination

Since early diagnosis is essential for a higher chance of patient survival, clinical examination plays a vital role in identifying clinical manifestations of patients with COVID-19 at moderate to increased risk of developing mucormycosis. This involves ocular examination as well as examination for sinus tenderness. The ocular examination involves testing for visual acuity, pupil and ocular motility, extraocular abnormalities, and examination of the fundus and biomicroscopy [52,57]. Abnormalities such as ophthalmoplegia, proptosis, blepharoptosis, affected visual acuity and perception of light, oedema and necrosis have been commonly observed in mucormycosis patients [58–60]. Intra oral examination should be performed to evaluate the presence of tooth mobility, swelling, tenderness and bone exposure [61].

5.2. Imaging

Imaging may not always be specific or diagnostic and the presentation may vary with the severity of mucormycosis. Computerized Tomography scan (CT), Magnetic Resonance Imaging (MRI, with/without contrast) and endoscopy are the standard imaging modalities used to assess the extent of involvement in mucormycosis. Staging is usually done based on sinus and cerebral involvement. Radiological imaging usually can be done by CT or MRI, with or without contrast. CT and MRI have been used to ascertain the extent of the fungal invasion and intracranial extension and, thus, the disease progression of mucormycosis. For this purpose, brain MRI is required as it helps ascertain brain, orbit and sinus involvement. MRI of orbits or paranasal sinuses may also be used to diagnose mucormycosis [62,63]. MRI has been found to detect the extent of the participation in mucormycosis with a higher degree of sensitivity when compared to CT. The most distinctive feature of mucormycosis visualized by an MRI is a peri-sinus invasion [28].

CT imaging may be performed for the paranasal sinus, nose, orbits, brain or chest for diagnosis. Bone destruction is generally observed using CT imaging of the paranasal sinus and brain. The presence of mucormycosis may be identified in CT at early stages using features such as a reverse halo often seen in the periphery of the lung. This might also be visualized as central necrosis and an air crescent sign [13,64]. Diagnostic features such as opacifications in the paranasal sinuses and orbits, optic nerve or mucosal thickening, fluid collection and inflammation can also be seen using CT.

Endoscopy can be performed alone or in combination with other procedures for diagnostic purposes, and may be rhinoscopy, sinonasal endoscopy or bronchoscopy for ROCM. Bronchoscopy detects tissue masses that obstruct the bronchus [47]. Further

investigations are required to determine if this is due to fungi or a tumor. Endoscopy usually detects pus, blackish necrotic tissues, lesions and destroyed or damaged tissues. Alternatively, a minimally invasive procedure, called functional endoscopic sinus surgery, can be used. Esophago-gastroduodenoscopy can detect uncharacteristic necrotic ulcers (exudate), especially in COVID-19 patients, to diagnose GI mucormycosis [11].

5.3. Histopathology

Histopathology is the best approach for diagnosing mucormycosis due to its sensitivity and specificity [13]. Histopathological examination is conducted on samples from the palate, nasal samples, gastric ulcers, skin lesions and biopsy during endoscopy, and surgical debridement and post-operative samples. It usually confirms the presence and diagnosis of mucormycosis. Hematoxylin and eosin (H&E), Periodic acid-Schiff (PAS) and Gomori methenamine silver (GMS) are histological stains used for the identification of *Mucorales* structures [58]. Pauci-septate or aseptate, irregular, broad, filamentous hyphae branched at right angles and spores are typical features of mucormycosis under biopsy. Biopsy may also reveal necrosis, ulcers, granulation, inflammation, exudates, angioinvasion and vasculitis [11,47,58,60].

5.4. Culture

The microscopic examination of the exact fungi and the fungal hyphae can be done using culture. A nasal swab is usually used for a sample collection from suspected mucormycosis patients. This sample is viewed under a microscope by preparing a direct smear with 10% KOH to detect fungal colonies and hyaline mycelium [24,38]. However, mucormycosis may not always give rise to growth in culture, and may provide a false-negative result. Additionally, the layered appearance of the fungal ball may cause it to be misdiagnosed as allergic rhinosinusitis in low power microscopes, which can be avoided using high power microscopes [52]. Fluorescence brighteners can also be used to distinguish the colonies. Alternatively, samples such as tracheal aspirate, bronchial aspirate, bronchoalveolar lavage fluid (BALF), sputum, skin lesions and operative samples can also be collected and analyzed [4,65]. Fungi in these samples may be grown on Sabouraud Dextrose Agar (SDA) at 25–37 °C. The fungal structures can be visualized by staining using lactophenol cotton blue. Culturing on SDA can also be a confirmatory test [60].

Fungal colonies are usually detected based on morphological features such as color (cottony black, white or grey), but more specific tests such as DNA-sequencing can also be carried out. This may involve sequencing rRNA or 18S, 28S, internal transcribed spacer (ITS), and other barcode genes. MALDI-TOF spectrometry can also be used for confirmatory tests. Owing to fungal colonization, fungal DNA may be detected in various clinical samples such as tissue and serum. However, this approach requires further standardization [65].

Other non-invasive diagnostic techniques include quantitative multiplex polymerase chain reaction (qPCR) of blood serum targeting 18S rRNA of *Mucorales* fungi. qPCR-based detection designed by Million et al., 2006 was found to aid in early diagnosis by detecting *Mucorales* DNA at least three days before diagnosis of mucormycosis in over 90% of the study patients [66,67]. Commonly observed *Mucorales* genera such as *Mucor*, *Rhizopus*, *Lichtheimia*, and *Rhizomucor* have been detected using in-house assays. Since the use of these non-invasive methods for detection aid in early diagnosis and improved survival rate, qPCR result is also considered in addition to the reverse halo in the CT for diagnosis [13]. DNA can also be manually extracted and amplified using semi-nested PCR with primers specific to *Mucorales* and the resultant amplicon can be sequenced [29]. Additionally, MucorGenius®, developed by Pathonostics (Maastricht, The Netherlands), is an easy-to-use multiplex PCR assay for detecting *Cunninghamella* spp. in addition to the above clinically relevant *Mucorales* in BAL and serum [27]. Alternatively, other molecular methods such as Restriction fragment length polymorphism (RFLP) and melt curve analysis

of PCR products enable earlier diagnosis with 70 to 100% sensitivity, making them valuable diagnostic tools [68].

Biomarkers such as *Mucorales* specific antigens have not been found in the blood serum of mucormycosis patients. So, unlike most IFIs, antigen tests such as the galactomannan test and detection of (1,3)-b-D-glucan (BDG) are not used for the detection of mucormycosis [29]. Moreover, *Mucorales* fungi are not detected in the cerebrospinal fluid culture of ROCM patients. However, T-cells such as CD4+ and CD8+ are seen explicitly in invasive mucormycosis and have been suggested as a possible non-invasive diagnostic test for mucormycosis. These T-cells may be detected using enzyme-linked immunospot (also known as ELISpot) [68]. Once the pathogen is identified, antimycotic susceptibility testing (using reference methods such as CLSI (Clinical and Laboratory Standards Institute) and EUCAST (European Committee for Antimicrobial Susceptibility Testing) or commercial tests such as Etest) is carried out so that the physician can determine the course of treatment [69].

6. Current Recommended Strategies for Treatment of CAM

CAM displays a high degree of angio-invasiveness. As a result, a multi-pronged approach is required to control the disease and prevent a recurrence. The treatment strategy for CAM is similar to that of mucormycosis. It primarily involves three aspects: addressing risk factors and co-morbidities, surgical debridement of infected tissue and administration of antifungals to control the spread of infection [4,70]. Adjunctive therapies may also be utilized depending on individual patient presentation and history. However, an early diagnosis is the most critical aspect of treatment.

6.1. Reversal of Risk Factors

Reversal of risk factors involves reversing hyperglycemic, immunosuppressed states and other risk factors that perpetuate mucormycosis in patients with COVID-19. The immunosuppressed condition may be changed by tapering or discontinuing immunosuppressants such as corticosteroids, antimetabolites, and calcineurin inhibitors. In the case of CAM-affected transplant patients, this might not be possible, and so the patient is treated with corticosteroid monotherapy and cessation of all other drugs [44]. Glucose levels must also be strictly controlled using insulin therapy and antidiabetic drugs, while ketoacidosis must be promptly treated [68]. Neutropenia management was found to have less severe implications in mucormycosis when compared to DM and corticosteroid therapy. These co-morbidities must be kept under control even after discharge to prevent recurrence [57].

6.2. Surgical Debridement

The surgical part of the treatment involves otorhinolaryngology, ophthalmology, neurosurgery, oral and maxillofacial surgery [5]. Hoenigl et al., 2021 demonstrated that surgical intervention and systemic antifungal therapy were associated with improved outcomes compared to antifungal therapy alone for patients with COVID-19 affected by rhino-orbital cerebral mucormycosis without central nervous system (CNS) involvement [27]. Due to the angio-invasive nature of mucormycosis, surgical debridement is an essential part of the treatment regime. It is usually performed using endoscopy or functional endoscopic sinus surgery. As for mucormycosis, sinus debridement must be performed repeatedly, intensively, and regularly to control CAM [7,65]. It should be widespread and completed at the earliest, removing all black, necrotic tissues for improved prognosis. Usually, surgical debridement is easier and more useful for ROCM and soft tissue infection than for pulmonary mucormycosis. It is not of much use for mucormycosis infections, which are disseminated in the blood or are found in inaccessible regions. For pulmonary mucormycosis, the thoracic cavity may be debrided, and in more critical cases, lung transplantation may be required [13]. For extreme, threatening cases, orbital exenteration is a last-resort technique for patient survival. This includes patients who did not respond well to the systemic antifungal medication and developed symptoms such as lack of light sensitivity, necrosis of the orbits and total ophthalmoplegia [4,57]. Following surgical debridement/orbital

exenteration, the tissues are sent for histopathological and microbiological examinations to ensure that clear margins have been obtained. In the absence of clear margins, further debridement may be required [63]. Following surgical treatment, facial reconstruction or prosthetic rehabilitation might be necessary, especially for patients with orbital exenteration, to improve their quality of life [71]. As this surgery is associated with the spread of infectious aerosol particles, appropriate personal protective equipment (PPE) and precautions must be used by surgeons, while debriding CAM-infected tissue [72].

6.3. Systemic Antifungal Therapy

Since *Mucorales* are resistant to many antifungals, the current first-line therapy against mucormycosis involves polyenes such as intravenous liposomal Amphotericin B (LAmB) (polyene). In contrast, salvage therapy includes IV posaconazole and isavuconazole (triazoles). However, systemic antifungal therapy is considered an adjunct to surgical debridement [11]. Cytokines can also be administered along with antifungal drugs for improved antifungal effects [68].

Amphotericin B deoxycholate and Amphotericin lipid complex (ABLC) have also been used. LAmB is preferred due to its reduced nephrotoxicity (especially at higher doses), improved CNS penetration and results in a murine model [13]. Amphotericin B deoxycholate is highly toxic, causing cholestasis and renal failure [47]. Consequently, if Amphotericin B is administered, monitoring kidney function is crucial. Amphotericin B acts on ergosterol, affecting the ion balance of cells, variations in membrane permeability due to oxidation and increased phagocytosis (Figure 1) [73]. Amphotericin B administered in cases of CAM varies from 3 mg/kg/day to 5 mg/kg/day or even 10 mg/kg/day in some cases, depending on the condition and co-morbidities of the patient [5,7,13,56–58]. Administration may be oral, intravenous or topical. Salehi et al., 2020 proposed the combination of LAmB, posaconazole and endoscopic surgical debridement (without craniotomy) as a treatment for ROCM patients who are not eligible for or willing to undertake extensive surgery [74]. Intranasal delivery of Amphotericin B (using nebulisation) in combination with systemic LAmB administration was favoured by Raj et al. 1998 [75]. Amphotericin B susceptibility also varies between different species of *Mucorales*. The duration of first-line treatment must be adjusted as per the co-morbidities and response of the patient, assessed by diagnostic tests. As amphotericin B is a fungistatic agent, the treatment duration is protracted compared to fungicidal agents. Polyenes such as LAmB have also been combined with echinocandins (which have low activity when used as monotherapy) such as caspofungin or micafungin and iron chelators such as deferasirox that control angioinvasion and pathogenesis and improve survival [13]. These combination therapies fall under second-line treatment options.

A double-blind placebo-controlled study by Spellberg et al., 2012 found that deferasirox was associated with higher mortality and lower success rate. Still, they could not draw generalized conclusions due to imbalances in the populations of deferasirox and placebo arms [76]. Amphotericin-B/LAmB/ABLC combinations have been tested with various drugs to treat mucormycosis with varying effectiveness. The combinations tested against mucormycosis and CAM are listed in Tables 1 and 2. Posaconazole is active in vitro and in vivo (murine models) against various *Mucorales* fungi but demonstrated poor activity against *M. circinelloides*-infected mice [69]. It prevents fungal cell wall synthesis by inhibiting ergosterol biosynthesis through its action on CYP51, the fungal cytochrome P450 lanosterol 14-alpha-demethylase involved in ergosterol biosynthesis conversion lanosterol to ergosterol. This inhibition reduces ergosterol levels, thereby affecting the fungal cell membrane, causing the death of the fungus. Mutation of this gene can cause resistance [13,77]. It is used for salvage therapy and prophylaxis against Mucormycosis in patients with Graft-vs-host disease and high-risk factors [43]. It has also been used as part of the first-line treatment for some CAM patients, especially patients for whom amphotericin B cannot be used, or in cases where the infection has been controlled by initial Amphotericin B treatment. However, as Mucormycosis infections occur despite posaconazole prophylaxis,

it is not the preferred drug for first-line treatment. It may be administered intravenously in the form of a delayed-release tablet or even as a syrup [65,78].

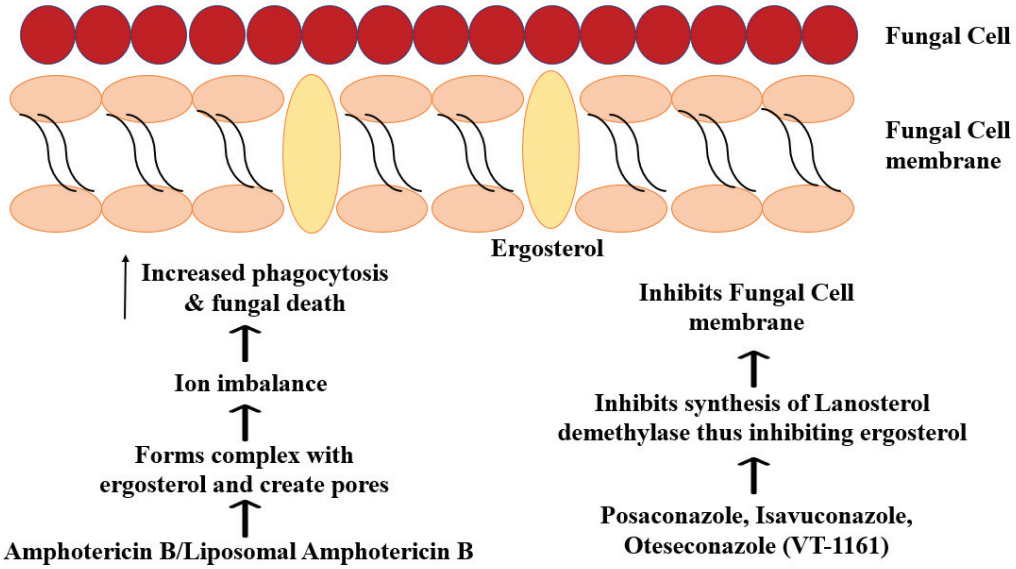

Figure 1. Drug action of Amphotericin B and azoles in the fungal cell membrane.

Isavuconazole, administered intravenously or orally, is an extended-spectrum antifungal, which is the reason for its use in the treatment of invasive mucormycosis [63]. It is used as a second-line drug for salvage therapy for CAM patients [4,63,65]. Due to the low hydrophilicity of isavuconazole, it is administered as a hydrophilic prodrug, isavuconazonium sulphate, which is converted to isavuconazole by esterase-mediated hydrolysis. As a result, unlike other azoles, it does not require cyclodextrin (likely to cause nephrotoxicity) to ensure drug solubility. So, it has a good safety profile in addition to being absorbed easily and having linear pharmacokinetics. It acts by inhibiting the synthesis of the fungal cell membrane. Like the other azoles, isavuconazole accomplishes this by inhibiting CYP51 of the CYP superfamily (cytochrome P450 monooxygenase).

Itraconazole has also been limited activity and therapeutic effect against mucormycosis, acting primarily against *Saksenaea*, *Lichtheimia* and *Rhizomucor* [79,80]. Fluconazole and voriconazole are not used to treat mucormycosis due to lack of activity and low activity, respectively, with mucormycosis arising despite voriconazole treatment in some cases [81]. The effects achieved in these combinations for complete remission in CAM are listed in Tables 1 and 2.

Table 1. Drug Combinations used against different types of mucormycosis.

Serial Number	Combination/ Regimen	Type of Study	Type of Mucormycosis	Organism	Diagnostic Tests	Risk Factors (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis (and Other Antifungals)	Other Concomitant Treatment (If Any)	Effect and/or Outcome	Addl Details	Reference
1	LAmB + CAS + SD	Case report	RCM	*Mucor*	CT, Clinical Diagnosis, Histopathology	1. Acute Myeloid Leukemia 2. Chemotherapy 3. Neutropenia	1. Liposomal Amphotericin B 2. Liposomal Ampotericin B + Caspofungin (24 days) 3. Surgical Debridement 4. Caspofungin (45 days)	1. Cytarabine 2. Idarubicin 3. Mitoxantrone 4. Broad-spectrum antibiotics 5. G-CSF 6. Potassium supplements	No infection after 3 months	Addition of Caspofungin was associated with improvement in patient's conditions (LAmB monotherapy had no response)	[82]
2	(LAmB → ABLC) + CAS + SD	Case report	Oromandibular	*Rhizopus oryzae*	Clinical Suspicion, CT, Histopathology	1. Diabetes mellitus 2. Acute Myeloid Leukemia 3. Chemotherapy	1. AmB-deoxycholate 2. Fluconazole (stopped upon suspicion of mucormycosis) 3. Liposomal Amphotericin B + Caspofungin (56 days, maintained even after surgery) 4. Surgical Debridement 5. ABLC (5 Weeks)	1. Idarubicin 2. Cytarabine 3. Tobramycin 4. Colimycin 5. Morphine 6. Imipenem 7. Amikacin 8. Vancomycin	Alive, no recurrence at 6-year follow-up		[83]
3	LAmB + MCF + SD	Case report	ROM	*Rhizopus oryzae*	CT, Histopathology	1. Diabetes mellitus 2. Hemodialysis for chronic renal failure	1. Insulin therapy 2. Liposomal Amphotericin B 3. Surgical Debridement 4. Liposomal Amphotericin B + Micafungin (Oral) (2 + 4 weeks) 5. Amphotericin B (Sinus irrigation)	1. Meropenem	No recurrence seen in 1 year follow-up		[84]
4	HBO + LAmB + DEF + S	Case report	Hepatosplenic	*Candida zeylanoides* from blood cultures	Histopathology	1. Febrile neutropenia 2. Minimally differentiated AML 3. Chemotherapy	1. Voriconazole (9 days) 2. Voriconazole + Caspofungin 3. Liposomal Amphotericin B + Deferasirox 4. Hyperbaric Oxygen Therapy (60 sessions) + LAmB (21 days) + Deferasirox (Throughout)	1. Cefepime → Meropenem 2. Vancomycin 3. Consolidation therapy—high-dose cytarabine	CT unremarkable after first consolidation therapy		[85]
5	HBO + LAmB + PSZ + CAS + SD	Case report	ROM	*Rhizopus*	CT scan, Culture	1. Acute Lymphoid Leukemia (ALL) 2. Chemotherapy	1. Liposomal Amphotericin B 2. Sinus debridement 3. Liposomal Amphotericin B + Caspofungin + Posaconazole 4. Hyperbaric Oxygen Therapy (19 sessions) (Caspofungin stopped after 1 week of HBO, Amphotericin B continued for 2 months) 5. Discharged with oral posaconazole (4 months)	1. Ceftazidime 2. Vancomycin 3. Consolidation chemotherapy	Favourable		[86]

Table 1. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of Mucormycosis	Organism	Diagnostic Tests	Risk Factors (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis (and Other Antifungals)	Other Concomitant Treatment (If Any)	Effect and/or Outcome	Addl Details	Reference
6	IFN-γ + NVB	Case report	Gastric		Histopathology	1. Immunosuppression	1. Liposomal Amphotericin B + Posaconazole 2. Gastrectomy 3. Splenectomy 4. Immunoadjuvant therapy 5. Nivolumab (1 dose)		Immunosuppression reversed. Patient discharged at 80 days		[87]
7	DAmB + LAmB + SD + VAC	Case report	Skin and Soft tissue	*Rhizopus*	Histopathology, Culture	1. Bilineal leukemia (ALL and AML) 2. Chemotherapy	1. Fluconazole (discontinued on diagnosis of mucormycosis) 2. Liposomal Amphotericin B (8 weeks) 3. Surgical Debridment 4. Vacuum-assisted closure (VAC) therapy 5. Deoxycholate amphotericin B (Topical) (3 weeks)	1. Chemotherapy for AML (cytosine arabinoside, daunorubicin, and etoposide) 2. Chemotherapy for ALL (cytosine arabinoside and L -asparaginase) 3. Trimethoprim-sulfamethoxazole 4. Gentamicin 5. Vancomycin 6. Salvage chemotherapy (vinorelbine, thiotepa, gemcitabine, topotecan and dexamethasone) 7. Alternative salvage chemotherapy (6-mercaptopurine, imatinib and methotrexate) 8. Palliative chemotherapy—vincristine	Mucormycosis controlled; no recurrence. Patient died of unrelated causes		[88]
8	LAmB (i.v.) + SD + AMB (N)	Case report	Sinonasal	*Absidia corymbis-era* (Now *Lichtheimia corymb-ifera*)	Histopathology	1. Acute promyelocytic leukemia 2. Chemotherapy	1. Liposomal Amphotericin B (intravenous) 2. Amphotericin B (nebulisation)		Alive, no recurrence at 6-year follow-up		[75]

Table 1. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of Mucormycosis	Organism	Diagnostic Tests	Risk Factors (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis (and Other Antifungals)	Other Concomitant Treatment (If Any)	Effect and/or Outcome	Addl Details	Reference
9	ABLC + (PSZ → ISZ) + CAS + SD	Case report	Disseminated	*Cunninghamella*	Clinical suspicion, Microscopic examination, Immunohistochemistry, PCR, Sanger sequencing, CT	1. Acute Lymphoid Leukemia 2. Chemotherapy 3. Neutropenia	1. Voriconazole (discontinued later) + Granulocyte colony-stimulating factor (G-CSF) 2. Amphotericin B Lipid Complex + Caspofungin 3. Posaconazole (3 days) 4. Isavuconazole (101 days, initially combination therapy, later monotherapy)	1. Cefepime 2. Vancomycin 3. Clarithromycin	Patient observed to be well at 10-month check		[89]
10	DAmB + MCF + PSZ	Case report	Disseminated	*Rhizopus*	Histopathology	1. Preterm birth 2. Mother underwent chemotherapy before delivery	1. Amphotericin B Deoxycholate + Caspofungin 2. Amphotericin B Deoxycholate + Caspofungin + Posaconazole 3. Micafungin discontinued subsequently (AMB—7 weeks; CAS—4 weeks, PSZ—3 weeks)	1. Ampicillin + Gentamicin 2. Vancomycin + Gentamicin 3. Ampicillin + Gentamicin + Metronidazole			[90]
11	AMB + CAS + SD	Case report	RCM	*Rhizopus arrhizus*	Histopathology, Molecular identification	1. Diabetes mellitus	1. Amphotericin B (60 days) 2. Amphotericin B + Caspofungin (4 weeks)	1. Targocid 2. Cefaxone 3. Flagyl	No recurrence in over 4 years	Caspofungin inclusion was associated with rapid improvement in symptoms	[91]
12	LAmB + PSZ + CAS + SD	Case report	Disseminated	*Absidia corymbisera* (Now *Lichtheimia corymbifera*)	Microscopic examination	1. Chemotherapy 2. Osteosarcoma 3. Brief neutropenia 4. Malnutrition	1. Surgical debridement—Multiple 2. Liposomal amphotericin B + Posaconazole 3. Liposomal amphotericin B + Posaconazole + Caspofungin (1 month) 4. Liposomal amphotericin B + Posaconazole (3 months)	1. High-dose methotrexate and etoposide-ifosfamide	Culture negative after triple combination therapy		[92]

Table 1. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of Mucormycosis	Organism	Diagnostic Tests	Risk Factors (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis (and Other Antifungals)	Other Concomitant Treatment (If Any)	Effect and/or Outcome	Addl Details	Reference
13	(LAmB → PSZ) + S	Case report	Disseminated mixed invasive	*Rhizopus*	Histopathology	1. Pancytopenia	1. Fluconazole (discontinued eventually) 2. Liposomal Amphotericin B (discontinued on Day 100) 3. Surgical removal of fungal abcesses 4. Splenectomy 5. Nephrectomy (partial) 6. Lower lobe wedge resection (left) 7. Posaconazole (6 months, initiated on Day 100)	1. Immunosuppressant therapy (rabbit anti-thymocyte globulin, methylprednisolone, G-CSF) 2. Imipenem–cilastatin 3. Vancomycin 4. Hematopoietic Stem Cell Transplantation 5. Cyclophosphamide 6. Rabbit anti-thymocyte globulin 7. Cyclosporin 8. Methotrexate	No residual abscess seen at 30-month follow-up MRI		[93]
14	LAmB + PSZ + SD + S	Case report	Disseminated Cutaneous	*Rhizomucor pussilus*	Histopathology	1. Acute Lymphoblastic Leukemia 2. Neutropenia 3. Chemotherapy 4. Steroid Therapy	1. Surgical Debridement 2. Lung resection 3. Liposomal Amphotericin B + Posaconazole (12 weeks)	1. Cefoperazone-sulbactam 2. Amikacin 3. Induction chemotherapy	Complete remission		[94]
15	(LAmB + CAS + VOR) → (LAmB + PSZ + TER + SD + LAmB (N) + ABLC (i.pl))	Case report	Disseminated	*Cunninghamella berthol-letiae*	Histopathology, PCR	1. Acute Lymphoblastic Leukemia 2. Pancytopenia	1. Liposomal Amphotericin B 2. Voriconazole (Discontinued subsequently) 3. Caspofungin (Discontinued subsequently) 4. Posaconazole + Terbinafine 5. Surgical Debridement 6. Liposomal Amphotericin B (Nebulisation) 7. Amphotericin B Lipid Complex (Intrapleural)	1. Broad spectrum antibiotics 2. Chemotherapy	No recurrence at 30 month follow up		[95]
	LAmB + TER + PSZ	Case report	Disseminated	*Cunninghamella berthol-letiae*	PCR, Culture	1. Acute Lymphoblastic Leukemia 2. Allogenic Stem Cell Transplant 3. Steroid Therapy 4. Diabetes mellitus 5. Iron overload	1. Voriconazole (Discontinued later) 2. Liposomal Amphotericin B 3. Liposomal Amphotericin B + Terbinafine + Posaconazole	1. Methylprednisolone 2. Etanercept 3. Mycophenolate mofetil 4. granulocyte-monocyte colony-stimulating factor (GM-CSF) 5. Simvastatin 6. Deferasirox	Patient died 3 years later (cause not mentioned)		[95]

Table 1. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of Mucormycosis	Organism	Diagnostic Tests	Risk Factors (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis (and Other Antifungals)	Other Concomitant Treatment (If Any)	Effect and/or Outcome	Addl Details	Reference
16	LAmB + PSZ	Case report	Disseminated	*Rhizopus microsporus*	Culture, Clinical suspicion,	1. AML	1. Voriconazole (Discontinued later) 2. Caspofungin (Discontinued later) 3. Liposomal Amphotericin B + Posaconazole (5 months) 4. Allogenic HSCT 5. Posaconazole 6. Surgery	1. Broad spectrum antibiotics 2. Antithymocyte globulin + Tacrolimus + Etanercept	No residual fungal lesions at 18 months		[96]
17	LAmB + PSZ + DEF	Case report	Hepatic	*Rhizomucor pusillus*	Microscopic examination, Histopathology	1. AML 2. Chemotherapy 3. Neutropenia 4. HSCT	1. Liposomal Amphotericin B 2. Liposomal Amphotericin B + Posaconazole 3. Surgical Debridement 4. Discharged with posaconazole 5. Deferasirox		Favourable		[97]
18	LAmB + CAS + SD	Case report	RCM	*Rhizopus oryzae*	Histopathology	1. Diabetes mellitus	1. Liposomal Amphotericin B 2. Liposomal Amphotericin B + Caspofungin 3. Liposomal Amphotericin B (Discharge, 2nd hospitalization)	1. Maxillectomy 2. Endoscopic decompression of orbita 3. Functional endoscopic sinus surgery 4. Meropenem 5. Ciprofloxacin	Recurrence due to patient non-compliance. Patient expired due to sepsis		[98]
19	DAmB + RIF	Case report		*Rhizopus oryzae*	Bronchoscopy, Culture	1. Diabetic Ketoacidosis	1. Rifampicin + Amphotericin B		Culture and histopathology negative after 8 weeks. Died of unrelated causes 3 years later.		[99]
20	AMB + (PSZ → AFG)	Case report	Hepatic	*Mucor* spp.	Histopathology, Immunochemical testing	1. AML 2. Neutropenia 3. Chemotherapy	1. Amphotericin B (10 days) 2. Amphotericin B + Posaconazole (2 months) 3. Amphotericin B + Anidulafungin	1. Chemotherapy-azacitadine 2. Moxifloxacin 3. Valacyclovir 4. Voriconazole 5. Levofloxacin 6. Metronidazole	Liver lesions improved. Patient expired due to complications		[100]
21	AMB/LAmB + CAS	Retrospective study	ROCM, ROM (Coexisting pulmonary, cutaneous)	*Rhizopus* spp., *Rhizomucor* spp.	CT, MRI	1. Diabetes mellitus 2. Neutropenia 3. Steroid therapy 4. Cancer 5. Transplant	1. Caspofungin + Polyene (ABLC/LAmB) 2. Surgical Debridement		1 Patient who received combination therapy expired within 30 days		[101]

AMB—Amphotericin B; PSZ—Posaconazole; AFG—Anidulafungin; RIF—Rifampin; TER—Terbinafine; CAS—Caspofungin; FLU—Fluconazole; ABLC—Amphotericin B Lipid Complex; LAmB—Liposomal Amphotericin B; MCF—Micafungin; DEF—Deferasirox; DAmB—Deoxycholate Amphotericin B; SD—Surgical Debridement; S—Surgery; IFN-γ—Interferon-γ; NVB—Nivolumab; VAC—Wound Vacuum Assisted Closure; G-CSF—Granulocyte-Colony Stimulating Factor; HSCT—Hematopoietic stem cell transplantation.

Table 2. Combinational drug therapy used in the treatment of CAM.

Serial Number	Combination/ Regimen	Type of Study	Type of CAM	Organism	Diagnostic Tests	Risk Factors Other Than COVID-19 (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis	Other concomitant Treatment (If Any)	Effect and/or Outcome	Reference
1	AMB + (ISZ → PSZ) + TCR + HBO + SD + Maxillectomy	Case report	Rhinosinusal	*Rhizopus oryzae*	Endoscopy, Culture, Palate Biopsy	1. Kidney Transplant 2. Immunosuppression 3. Prolonged history of isavuconazole use and IFIs 4. Diabetes mellitus (No DKA) 5. Steroid therapy	1. Treatment with Amphotericin B and azole (initially Isavuconazole, later posaconazole to avoid resistance) for 5 months 2. Surgical Debridement—7 times 3. Total Maxillectomy 4. Reduction of steroid (prednisone) dosage 5. Tacrolimus (Before diagnosis of CAM and during CAM treatment) 6. Hyperbaric Chamber Therapy	Azithromycin Ceftriaxone Dexamethasone Piperacillin/Tazobactam	No recurrence of infection after 5 months	[44]
2	Fasciotomy + SD + LAmB + ISZ	Case report	Musculoskeletal	*Lichtheimia ramosa*	Culture	1. Immunosuppression (Steroid Therapy—prednisone, mycophenolate and tacrolimus) 2. Kidney transplant (graft dysfunction)	1. Liposomal Amphotericin B + Isavuconazole (24 days) 2. Isavuconazole for 3 months 3. Surgical Debridement—3 times 4. Fasciotomy	1. Immunosuppressants (IS): prednisone, mycophenolate and TCR 2. Hydroxychloroquine 3. Azithromycin 4. Lopinavir/Ritonavir 5. Heparin 6. Tocilizumab (400 mg)	Favourable	[44]
3	FLU + AMB + SD	Case report	Sino-orbital	*Rhizopus oryzae*	Culture Histopathology MRI	None	1. Surgical Debridement—2 times 2. Fluconazole 3. Amphotericin B (injection and lavage) 4. Discharged with prescription for continuation of Amphotericin B and Fluconazole	1. Remdesivir 2. Methylprednisolone 3. Dexamethasone 4. Piptaz 5. Metronidazole 6. Tobramycin 7. Nepalact TDS 8. Monocef	Favourable at 2 month review	[59]
4	LAmB + PSZ + Sinus debridement without craniotomy	Case report	ROCM	Not Mentioned	MRI CT Culture of biopsy sample	1. B-cell lymphoma 2. Chemotherapy 3. Neutropenia	1. Liposomal Amphotericin B 2. Liposomal Amphotericin B + Posaconazole for 4 weeks 3. Surgical Debridement—Multiple	1. R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) 2. CODOX-M/IVAC (cyclophosphamide, vincristine, doxorubicin, high-dose methotrexate/ifosfamide, etoposide, and high-dose cytarabine) 3. Meropenem 4. Vancomycin	Patient discharged after 12 weeks. No recurrence for upto patient's death, 3 months after discharge (unrelated to ROCM)	[74]

Table 2. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of CAM	Organism	Diagnostic Tests	Risk Factors Other Than COVID-19 (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis	Other concomitant Treatment (If Any)	Effect and/or Outcome	Reference
5	Amphotericin B + Azoles	Multicenter Epi-demio-logic Study	ROM, ROCM, Pulmonary, Renal, Dissemi-nated, Others	*Aspergillus* and *Mucorales*	Microscopy Culture Histopathology	1. Steroid Therapy 2. Diabetes mellitus	Amphotericin B + Posaconazole (Concurrent or sequential)	1. Glucocorticoid drugs 2. Tocilizumab	The survival rates of sequential combination therapy were found to be better at 6 and 12 weeks compared to concurrent and single antifungal therapy	[39]
6	AMB + PSZ	Descriptive multi-centre study (Cross-sectional)	Orbital	Not Men-tioned	Not Mentioned	1. Diabetes mellitus 2. Steroid Therapy 3. Neutropenia	1. Amphotericin B 2. Posaconazole (2 weeks) 3. Orbital exenteration		Alive	[102]
	AMB + PSZ + SD		ROM			1. Diabetes mellitus	1. Amphotericin B 2. Posaconazole (2 weeks) 3. Surgical Debridement	Dexamethasone	Alive	
	AMB + PSZ + CAS + SD		ROM			1. Diabetes mellitus 2. Steroid Therapy	1. Amphotericin B 2. Posaconazole (2 weeks) 3. Caspofungin (2 weeks) 4. Surgical Debridement	Dexamethasone	Alive	
	AMB + CAS + SD		ROM			1. Diabetes mellitus	1. Amphotericin B 2. Caspofungin (2 weeks) 3. Surgical Debridement		Alive	
	AMB + PSZ + SD		Sino-orbital			1. Diabetes mellitus 2. Steroid Therapy	1. Amphotericin B 2. Posaconazole (2 weeks) 3. Surgical Debridement	Dexamethasone	Alive	
	AMB + CAS + SD		Sinonasal			1. Acute Myeloid Leukemia 2. Chemotherapy 3. Neutropenia	1. Amphotericin B 2. Caspofungin (2 weeks) 3. Surgical Debridement	Dexamethasone	Alive	
7	AMB + Azoles	Review (Statisti-cal Analy-sis)	ROM, ROCM, Pulmonary, Cutaneous, Gastroin-testinal, Dissemi-nated, Others	*Rhizopus arrhizus*, *Rhizopus mi-crosporus*, *Rhizopus* spp., *Lichtheimia* spp. And *Mucor* spp.	-	1. Glucocorticoid usage 2. Diabetes mellitus 3. Solid Organ Transplant 4. Immunosuppressive therapies	Amphotericin B + Azole (Isavuconazole or Posaconazole) (Sequential) Details of surgical debridement in combination with antifungal treatment not provided.	Details for individual cases unknown	Details for individual cases unknown	[103]

Table 2. *Cont.*

Serial Number	Combination/ Regimen	Type of Study	Type of CAM	Organism	Diagnostic Tests	Risk Factors Other Than COVID-19 (If Applicable)	Details of Combination/Regimen for Treatment of Mucormycosis	Other concomitant Treatment (If Any)	Effect and/or Outcome	Reference
8	LAmB + VRZ + PSZ + SD	Retrospective Inter-ven-tional study	ROCM	Not Men-tioned	Histopathology, Imaging	1. Diabetes mellitus 2. Steroid Therapy	1. liposomal Amphotericin-B+ Voriconazole 2. Posaconazole 3. Orbital exenteration 4. Surgical Debridement	cefoperazone + sulbactam		[25]
	LAmB + PSZ + SD		ROCM		Histopathology, Culture	1.Diabetes mellitus 2. Steroid Therapy	1. Liposomal Amphotericin-B 2. Posaconazole 3. Surgical Debridement	1. Methylprednisolone 2. Prednisolone		
	LAmB + PSZ + SD		ROCM		Diagnosed as possible *Mucor* based on clinical evidence and imaging	1.Diabetes mellitus 2. Steroid Therapy	1. Liposomal Amphotericin-B 2. Posaconazole 3.Surgical Debridement	1.Dexamethasone 2.Prednisolone 3. Gabapentin		
	LAmB + PSZ + SD		ROCM		Histopathology, Culture	1.Diabetes mellitus 2.Steroid Therapy 3.Existing Antifungal Therapy	1. Liposomal Amphotericin B 2.Posaconazole 3.Surgical Debridement	1. Prednisolone		
	LAmB + PSZ + SD		ROCM		Histopathology, Culture	1.Diabetes mellitus 2.Steroid Therapy 3.Existing Antifungal Therapy	1. Liposomal Amphotericin B 2. Posaconazole 3. Orbital enteration 4. Surgical Debridement	1. Dexamethasone		
9	AMB + PSZ	Case report	ROM	Not Men-tioned	Histopathology	1. Diabetes mellitus	1. Insulin injections to control hyperglycemia 2.Surgical Debridement 3.Amphotericin B 4. Posaconazole	1. Remdesivir 2. Levofloxacin 3. Dexamethasone 4. Vancomycin 5. Piperacillin-Tazobactam	Patient alive and stable at 2-month and 7-month follow up check	[58]
10	LAmB + CAS + PSZ	Case report	ROM	*Rhizopus* spp.	Histopathology CT Culture	1. Hyperglycemia	1. Liposomal Amphotericin B (4 days) 2. (Liposomal Amphotericin B → Posaconazole) + Caspofungin 3. Glucose Management 4. Surgical Debridement	1. Remdesivir 2. Vancomycin 3. Cefepime 4. Dexamethasone	Patient died due to COVID-19 associated ARDS	[28]
11	AMB + ISZ + MCF	Case report	ROCM		Mucormycosis suspicion based on MRI	1. Diabetes mellitus 2. Diabetic Ketoacidosis 3. Steroid Therapy	1. Amphotericin B 2. Isavuconazole 3. Micafungin	1. Remdesivir	Patient expired on Day 4 due to poor prognosis and rapid decline	[104]
	AMB + ISZ	Case report	ROCM	*Rhizopus*	CT Culture	1. Diabetic Ketoacidosis	1. Amphotericin B (3 weeks) 2. Amphotericin B + Isavuconazole (10 days)	1. Remdesivir	Patient expired	

7. Successful Drugs and Combinational Therapies against CAM

7.1. Hyperbaric Therapy

In some patients, hyperbaric oxygen therapy (HBOT) or hyperbaric chamber therapy is used as adjunctive therapy to other conventional therapies to improve survival rates. The humidifiers for oxygen therapy must use sterile distilled water. HBOT involves the patient's exposure to 100% oxygen at pressures above one atmosphere (usually 2–2.5 atmospheres) for multiple treatments. This increases the oxygen transport capacity of the blood by increasing the alveolar partial pressure of oxygen, thereby causing revascularization and tissue oxygenation, thus reversing hypoxia. Theoretically, this could increase the oxygen concentration to a fungicidal level. However, hyperbaric oxygen is usually found to be fungistatic [105]. On the other hand, this is also frequently associated with oxygen toxicity due to free radical generation [106]. It also corrects lactic acidosis, which is a risk factor for mucormycosis, and, as a result, increases the activity of Amphotericin B. Furthermore, it also acts by boosting the immune response and reduces the area to be debrided, and hence is recommended to be used along with surgical debridement [107]. It is recommended for diabetic patients [108]. HBOT was part of a successful treatment regimen with antifungal treatment and surgical debridement to control CAM in a kidney transplant patient [44].

7.2. Immunosuppressants Used for Transplant Patients

These includes drugs such as calcineurin inhibitors (CNIs) and CNI alternatives such as sirolimus [109]. CNIs act against a conserved virulence factor, calcineurin, which is responsible for the hyphal growth of fungi. Calcineurin is central to virulence, morphogenesis and physiological processes. It is a serine/threonine phosphatase, which depends on a calcium-bound calmodulin binding to it for activation of phosphatase activity. Calcineurin inhibitors, which include drugs such as tacrolimus, act by reducing the virulence of mucormycosis, shifting from hyphal growth to yeast growth (lower virulence). CNI resistance occurs due to mutations in fkbA gene (which encodes for FKBP12, which binds to FK506 (sirolimus)), mutations in its binding sites (calcineurin catalytic A subunit or regulatory B subunit (cnbR)), and a mutation in both cnbR and bycA, which codes for an amino acid permease that regulates PKA activation. (Figure 2B) [110]. An epigenetic mechanism can induce transient or unstable resistance by RNA interference (RNAi) [111]. However, they primarily are used in combination studies as they increase the activity of other antifungals and demonstrate lower activity on their own. However, organ transplant patients treated with CNI as immunosuppressants showed reduced susceptibility to mucormycosis than those who did not receive CNI treatment. These combination studies have shown promising in vitro and in vivo results, but human trials are required [112].

Tacrolimus is a CNI used for transplant patients affected with Mucormycosis or CAM [44]. A study by Lewis et al., 2013 in mice showed that tacrolimus monotherapy prolonged survival while combination therapy was associated with close to complete resolution of lesions and symptoms [113]. Synergistic interactions were also observed in vitro at permissible human plasma concentrations. Notably, Tacrolimus was also a significant protective effect against mucormycosis in solid organ transplant patients [114].

Rapamycin (sirolimus) is an immunosuppressant drug that demonstrated in vitro and in vivo activity against *M. circinelloides* with improved survival rates (*Galleria mellonella* model) in a study conducted by Bastidas et al. 2012 [115]. They identified the drug targets as *M. circinelloides* homologs of FKBP12 (FK506-binding protein) and Tor (Target of Rapamycin) proteins. FKBP12 was critical for the inhibition of Tor (Figure 2C). FKBP12-Rapamycin inhibits Tor, which is involved in several cellular pathways dependent on nutrients. As a result, Tor inhibition causes nutrient starvation responses in the cell, leading to cell cycle arrest and autophagy. Although immunosuppressive therapies are usually tapered during mucormycosis patients, they are generally required for transplant patients. Consequently, rapamycin immunosuppressive therapy might help control mucormycosis in such patients. They suggested that the antifungal effects of rapamycin could be exploited with reduced or no immunosuppressive effects through combination therapy, modified

delivery strategies such as lipid formulations, local delivery, topical applications or the use of non-immunosuppressive analogues of rapamycin [116,117].

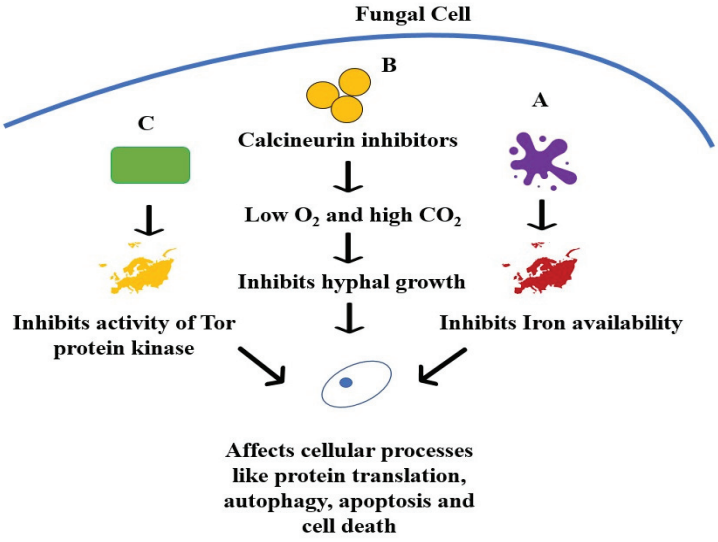

Figure 2. Drug action mechanism of (**A**) Deferasirox, (**B**) Calcineurin Inhibitors and (**C**) Rapamycin on the fungal cell.

7.3. Iron and Zinc Chelators

Iron is critical for the survival of *Mucorales* fungi. Consequently, sequestration of iron can be a strategy used to treat mucormycosis. Deferasirox is an iron chelator administered orally and may be fungistatic or fungicidal. It acts by affecting the iron availability to the pathogen, generating an iron-starvation response which terminates in metacaspase dependent apoptosis and cell death (Figure 2A). It was observed to have good activity in vitro and mouse models, increasing the survival period of mice. It demonstrated an activity comparable to that of LAmB in DKA mice and combination therapy demonstrated a longer survival time, but it did not lower the fungal burden consistently [59].

Additionally, Ibrahim et al., 2007 demonstrated that deferasirox showed higher activity against diabetic mice than in eutropenic mice, and that the activity was time-dependent rather than concentration-dependent [47], although the same combination was associated with higher mortality in clinical trials. The results might have been affected due to the small sample size (20 patients) and confounding factors such as variations in previous antifungal treatment and pre-existing conditions.

Zinc is a promoter of fungal growth, as demonstrated in an in vitro study of *Rhizopus arrhizus* strains isolated from CAM patients. This is due to its role in reducing the economic coefficient of the organism and facilitating the growth promoting activities of other micronutrients. However, the role of zinc in growth varies from strain to strain [55]. A study by Leonardelli recommended a combination of posaconazole with clioquinol, a zinc chelator, as it was found to be synergistic, especially against *Rhizopus microsporus*. Other combinations were also found to have synergistic activity, but varied from strain to strain [118].

7.4. Echinocandins

Echinocandins are combined with Amphotericin B to treat mucormycosis for synergistic effects. They inhibit cell wall synthesis in fungi by affecting the synthesis of BDG. The nature of the synergy remains unknown. These synergistic effects are observed with

echinocandins such as caspofungin, micafungin and anidulafungin. They are used for the treatment of ROCM [119].

8. New or Repurposed Drugs

8.1. Drugs Used in Monotherapies

8.1.1. VT-1161

VT-1161 is an investigational drug active in vitro against *Mucorales* species such as *R. oryzae*, *R. arrhizus*, *Lichtheimia* and *Cunninghamella*. It is a metalloenzyme inhibitor targeting the fungal CYP51 (such as isavuconazole), thus affecting cell membrane synthesis (Figure 1). VT1161 treatment performed favourably compared to posaconazole and LAmB, while prophylaxis by VT1161 was favourable compared to Posaconazole [120]. However, it was observed to have higher MICs than these existing therapies. VT1161 treatment and prophylaxis were also associated with increased survival and fungal burden reduction in neutropenic mice [121]. VT1161 has lower toxicity and better pharmacokinetics when compared to existing therapies such as azoles and polyenes. It also causes fewer off-target effects as it is selective to fungal CYP51 rather than CYP450 in humans. Further studies are required to evaluate the impact of this drug in experimental and therapeutic models.

8.1.2. Manogepix

Manogepix is a broad-spectrum antifungal agent that inhibits the conserved fungal protein Gwt1, affecting the trafficking and anchorage of mannoproteins to the cell membrane and outer cell wall. PIGW, the nearest ortholog in mammals, is not affected by Manogepix. Since mannoproteins are essential for fungi's structural integrity and pathogenicity, Manogepix-mediated inhibition of mannoproteins can have various physiological and pleiotropic effects on growth and virulence (Figure 3A). It is more effective and has lower MICs and MECs (Minimum Inhibitory Concentration and Minimum Effective Concentration, respectively) for treatment of *Candida* and *Aspergillus*, and it usually exhibits higher MECs with *Mucorales*. However, it was demonstrated to be effective in two murine models of mucormycosis with low MECs, suggesting that using it for clinical treatment exists and must be explored further [122].

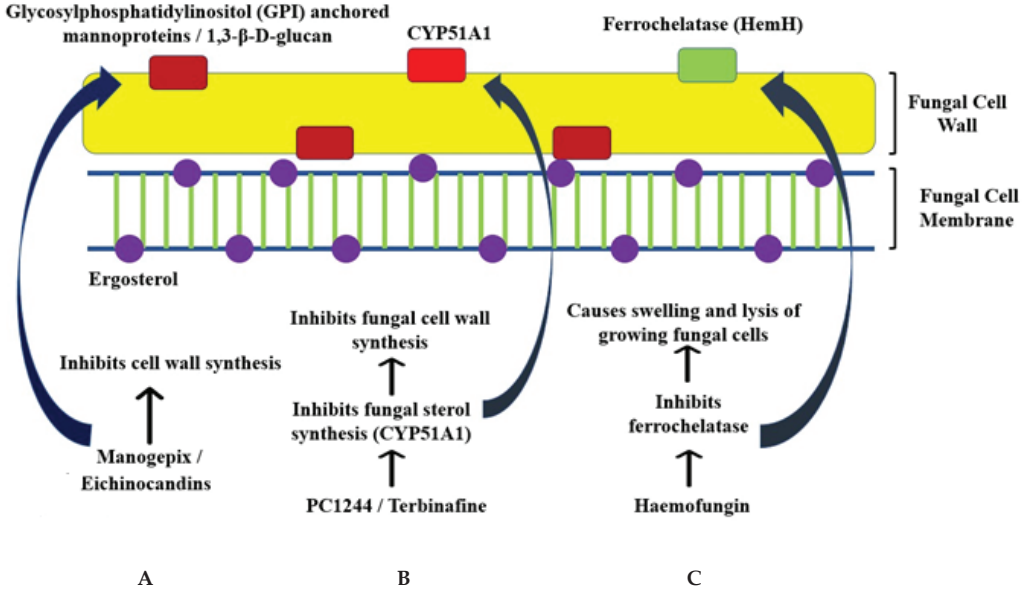

Figure 3. (**A**) Manogepix-mediated inhibition of mannoproteins. (**B**) PC1244-mediated inhibition CYP51A1. (**C**) Haemofungin-mediated inhibition of ferrochelatase.

8.1.3. Fosmanogepix (APX001)

Fosmanogepix is the prodrug form of Manogepix. Systemic phosphatases convert it to the active form of the drug, Manogepix. This pro-drug form is required due to the low solubility of Manogepix in water, making a delivery in an intravenous state complex [123]. It is now a first-in-class drug for the treatment of invasive mucormycosis. It demonstrated good activity, increase in survival and good tissue clearance in mouse models of invasive pulmonary mucormycosis. Fosmanogepix activity was comparable to isavuconazole [124] and found to have good pharmacokinetic properties, high bioavailability, widespread tissue distribution, and suitability for once-daily dosing in both oral and intravenous administration. It also has favourable interactions with other drugs and has no food effect. Consequently, it is currently in Phase 2 of clinical trials to treat infections caused by *Candida*, *Aspergillus* and rare moulds [125].

8.1.4. Haemofungin

Haemofungin is an antifungal compound identified to affect cell wall synthesis leading to swelling and death. It targets HemH/ferrochelatase, thus preventing the final step of haem biosynthesis, leading to the accumulation of toxic intermediates, which also cause death (Figure 3C). It is active in vitro and in vivo (*Drosophila* model). It exhibited an inhibitory effect against various fungi apart from *Rhizopus* and is non-toxic. Although the targets of haemofungin were highly similar to the corresponding human protein, the authors suggest that this can be overcome, as the azoles currently in use as antifungals share 40% identity with a human protein [126].

8.1.5. PC1244

PC1244 is a broad-spectrum antifungal active against various species of fungi, including *Mucorales* like *Rhizopus oryzae*, *Rhizomucor pusillus*, *Mucor circinelloides* and *Lichtheimia corymbifera*. It was found to have good activity in vitro against these fungi, where it demonstrated lower MICs compared to voriconazole and posaconazole. Additionally, it also shows rapid cellular permeation and persistence of action. The latter was observed when administered before inoculation in *Aspergillus fumigatus*, suggesting that it can be used for prophylaxis. It is proposed to act by inhibiting cell wall synthesis through inhibition of fungal sterol 14α-demethylase (CYP51A1) (Figure 3B). This study majorly focused on *A. fumigatus*. Further studies on *Mucorales* are required [127].

8.1.6. EGFR Inhibitors

The host epidermal growth factor receptor (EGFR) is phosphorylated, activated, and colocalized with Mucorales fungi during infection. EGFR activation is critical for fungal invasion. As a result, network analysis identified EGFR as a potential drug target. Gefitinib (a drug) and Cetuximab (an antibody) are inhibitors of EGFR which were associated with lowered ability to invade fungi and more prolonged survival in mice with pulmonary mucormycosis. The response of EGFR to fungal infections is also reduced by gefitinib treatment [128].

8.2. Potential adjunct Drugs for Treatment of CAM

Various drugs have exhibited different interactions with existing medications to treat mucormycosis and therefore could potentially be used as combination therapies for CAM. These drugs and their activities have been described in detail.

8.2.1. Colistin

A study conducted by Ben-Ami et al., 2010 found that colistin had modest activity against mucormycosis [129]. It was demonstrated to act by affecting the cytoplasmic membrane by bleb formation adjacent to it and vacuolar membranes resulting in increased size and number of vacuoles. This collectively led to leakage of intracellular material, which is responsible for the fungicidal effect of colistin. When colistimethate was used in a murine

model of pulmonary mucormycosis, the intranasal route (prophylaxis) was found to significantly impact the survival of mice compared to the intraperitoneal route (treatment), due to the possibility of attaining fungicidal concentrations in the lungs. However, colistin therapy alone was found to lead to regrowth, which was suppressed by using concentrations of Amphotericin B lower than the MIC. Hence, the authors proposed colistin as adjunctive therapy for mucormycosis.

8.2.2. HDAC Inhibitors

Pfaller et al., 2009 studied the effects of MGCD290, a Hos2 fungal histone deacetylase (HDAC) inhibitor, as monotherapy and in combination with triazoles [130]. Monotherapy had modest MICs, while synergistic activity was observed against most *Mucor* and *Rhizopus* fungi. Combination therapy was associated with synergy even in azoles to which these fungi are innately resistant (such as fluconazole). These effects are due to the suppression of Hos2 transcriptional complexes associated with resistance toward azoles.

8.2.3. Miltefosine

Miltefosine, a membrane phosphatidylcholine analogue, was tested for activity against fungal pathogens as a monotherapy and in combination with voriconazole or posaconazole. The monotherapy exhibited high MICs, but in vitro synergy was observed with both azoles, as demonstrated by lowered MICs. Although it is known that Miltefosine targets fungal phospholipase B1 enzymes, the mechanism of synergy is unknown. Further in vivo studies are required [131].

8.2.4. Statins

Lovastatin was found to be active against mammalian and fungal cells by generating apoptosis-like responses. In mouse models, it was found to act by inhibiting prenylation of signaling molecules such as Ras. In fungi, it led to morphology that resembled apoptotic cells, DNA degradation and loss of cell viability. However, it was ineffective in the spherical stage of fungal growth, possibly due to differences in metabolism from polarized growth [132]. It was found to improve the activity of voriconazole against *Rhizopus* and *Mucor* spp. in vitro. Synergy was observed with voriconazole against mucormycosis-infected models of *Drosophila*. However, studying the pharmacodynamics and pharmacokinetics of orally absorbed drugs is complex in *Drosophila* [133].

A study by Naeimi Eshkaleti et al., 2019 demonstrated that the combinations of Atorvastatin (synthetic statin) and Lovastatin (natural statin) with Amphotericin B led to a reduction of Amphotericin B MICs against *R. oryzae* [134]. Atorvastatin was found to cause a greater decrease of Amphotericin B MICs than Lovastatin. Statins and Amphotericin B are generally effective at higher concentrations, but these higher concentrations are also toxic to humans. A Statin-Amphotericin B combination reduces the harmful effects of both, improving activity.

8.2.5. Rifampin

In combination with Amphotericin B, Rifampin demonstrated synergy against *Rhizopus* species in vitro. No significant effect was observed with Rifampin alone. This synergy was also observed in a patient with *Rhizopus* pneumonia. It is proposed to act by increasing cell permeability to Rifampin due to Amphotericin B binding with ergosterol. Rifampin entry results in DNA-dependent RNA polymerases inhibition, inhibiting fungal growth [135].

8.2.6. Terbinafine

Terbinafine is an antifungal that inhibits fungal sterol synthesis, thus affecting ergosterol synthesis and cell wall synthesis. Terbinafine exhibited synergistic and additive effects against *Rhizopus*, *Rhizomucor* and *Mucor* species combined with amphotericin B and voriconazole [135]. The efficacy of terbinafine in animal models was poor [69].

8.2.7. Quinolones

Quinolones are a class of bactericidal drugs that inhibit bacterial DNA replication by interfering with topoisomerase activity. Sugar and Liu, 2000 tested the effect of the Quinolone-Amphotericin B combination on pulmonary mucormycosis in a mouse model. The combination of fluconazole and trovafloxacin (a quinolone) was found to have improved median survival time (MST) compared to control and fluconazole monotherapy. Varieties of Amphotericin B-trovafloxacin and Amphotericin B-trovafloxacin-fluconazole were associated with longer MST than all other treatments (control, monotherapies, fluconazole-trovafloxacin combination therapy). Still, there was no significant difference in MSTs between these two treatments. Similar MST was also observed when the mice were administered fluconazole-ciprofloxacin treatment [136].

8.3. Immunomodulating Strategies

8.3.1. Anti-CotH3 Antibodies

Anti-CotH3 binds to the receptor GRP78 and facilitates invasion. A predicted highly immunogenic and conserved domain present in the GRP78 binding domain of CotH3 was targeted using polyclonal antibodies. This was found to prevent invasion, angioinvasion and dissemination to the brain in DKA and neutropenic mice. It acts by multiple mechanisms, including increased phagocytic recruitment, higher phagolysosome acidification and increased ROS (Reactive Oxygen Species) production. Opsonophagocytosis helps in reducing the fungal burden. It might have a role in improving the fungicidal role of macrophages, further favouring its use in neutropenic patients [137].

8.3.2. Anti-GRP78 Antibodies

Liu et al., 2010 demonstrated that blocking GRP78 using antibodies effectively prevented infection in mice with DKA [19]. Mucormycosis is an endothelial receptor critical for mucormycosis invasion. This method was not found to be effective in *Candida* or *Aspergillus*. This suggested the relevance of blocking GRP78 to treat mucormycosis.

8.3.3. Cytokine Administration

This includes interferon-γ (IFN-γ), granulocyte-colony stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor and macrophage-colony stimulating factor (M-CSF). G-CSF and IFN-γ, in combination with GM-CSF, favour immune response by polymorphonuclear leukocytes (PMNs) and M-CSF promote the destructive activity of monocytes and macrophages. These are active against various invasive fungi in vitro and humans. Some combinations of cytokines act synergistically. IFN-γ is active against the broadest range of organisms [138]. IFN-γ, in variety with Nivolumab, has also helped reverse the effects of mucormycosis infection, which was unresponsive to existing therapy. G-CSF and GM-CSF have not been associated with reduced mortality but have suggested promoting shortened duration of neutropenia, lower antibiotic usage, and faster recovery. M-CSF has not been FDA-approved for administration to patients [139].

8.4. Other Therapies

8.4.1. Photodynamic Therapy

Antimicrobial photodynamic therapy (aPDT) involves using a photosensitizer (PS), which sensitizes pathogenic fungi to the wavelength of light produced by an LED, resulting in a phototoxic reaction that produces reactive oxygen species, killing the fungi. This has been found to be useful for many pathogenic fungi, including *Rhizopus*. Pre-treatment with LED and methylene blue was observed to lower the MICs of existing antifungals used for mucormycosis treatment, such as itraconazole, posaconazole and amphotericin B. It is proposed as an alternative or adjunctive to surgical debridement owing to its high tissue transmission, localization to tissues with PS accumulation, non-invasiveness, low cost and convenience. Additionally, it can lower antifungal dosage and side effects, thus increasing patient compliance [140].

8.4.2. Hyperthermia

Shirazi et al., 2013 conducted an in vitro study on the effects of hyperthermia on the activity of CNIs (tacrolimus) and triazoles (itraconazole and posaconazole) against *R. oryzae* [141]. It was observed that these drugs exhibited increased activity and lower MICs at higher temperatures in a dose-dependent manner. Higher temperatures were found to favour more elevated levels of ROS accumulation, leading to metacaspase activation and apoptosis. Hyperthermia was proposed as a therapy for mucormycosis, alone or combined with triazoles and tacrolimus. The authors suggest that local thermal delivery is a potential application of this finding. Further in vivo studies are required.

9. Insights from In Silico Studies

A study by Jain et al., 2013 identified six potential targets based on sequence differences in humans. Out of these, three were shortlisted due to the presence of just one copy [142]. These are riboflavin synthase, riboflavin biosynthesis protein RibD domain-containing protein, and 3,4-dihydroxy-2-butanone 4-phosphate synthase. All these genes belong to the riboflavin synthesis pathway, which is essential in microorganisms and absent in humans. Studies are required to determine whether the organism can take up riboflavin from the host. B-glucan synthase is involved in glucan synthesis, contributing to cell wall synthesis. A study by Sharma and Kaur identified 1–8 cineole, a bioactive compound from eucalyptus oil, as an inhibitor of this target using in silico methods [143]. They obtained a high-affinity docking score when the combination docked with the C-terminal end, responsible for catalysis. Further, they obtained good levels of pharmacokinetic and drug-likeness properties using online tools.

10. Conclusions

This review discusses the risk factors and diagnosis associated with mucormycosis. Some possible links between COVID-19 and mucormycosis are also explored. Although only a few treatments are currently recommended to manage mucormycosis, other treatments must be explored due to the development of resistance to mucormycosis. Several therapies have been tested at various levels and have proved successful in treating mucormycosis. These treatments require further evaluation for administration to humans and treatment of CAM.

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Review

Current Effective Therapeutics in Management of COVID-19

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Abstract: The current pandemic due to the SARS-CoV-2 virus has caused irreparable damage globally. High importance is placed on defining current therapeutics for Coronavirus Disease 2019 (COVID-19). In this review, we discuss the evidence from pivotal trials that led to the approval of effective therapeutics in the treatment and prevention of COVID-19. We categorize them as effective outpatient and inpatient management strategies The review also attempts to contextualize the efficacy of therapeutics to the emerging variants. Vaccines, which remain the most effective prevention against hospitalization and deaths is not included in this review.

Keywords: COVID-19; therapeutics; omicron; coronavirus disease 2019; monoclonal antibodies; Casirivimab plus imdevimab; Sotrovimab; bebtelovimab; remdesivir; molnupiravir; paxlovid; evusheld; corticosteroids; baricitinib; tocilizumab; Anakinra; anticoagulation; timeline

1. Introduction

Identifying effective therapeutic strategies for Coronavirus Disease 2019 (COVID-19) in a timely manner has been one of the most significant challenges. As of 15 June 2022, there is an excess of 85 million cases and 1 million deaths in the United States [1], as well as over 534 million confirmed cases and 6.3 million deaths globally [2]. At the time of writing this paper, we have at our disposal several effective therapeutics that may be used based on the timing of patient presentation and disease severity. Additional considerations for the treatment prioritization and management of high-risk patients include old age, high body mass index (BMI) and underlying comorbidities, including but not limited to diabetes, hypertension, obesity and chronic lung and heart diseases [3,4]. While there is a constantly changing landscape with the emergence of new variants, we discuss in this brief review the therapeutics that have shown efficacy in the pivotal trials.

1. Outpatient management: monoclonal antibodies, nirmatrelvir [PF-07321332] and ritonavir (Paxlovid), molnupiravir, remdesivir (Veklury) and bebtelovimab.
2. Inpatient management: remdesivir, corticosteroids, tocilizumab, baricitinib and anakinra.

Vaccines remain the most effective preventive strategy and a discussion is beyond the scope of this brief review. Heavily explored treatment options, such as hydroxychloroquine and Ivermectin, previously presented results indicating effectiveness against COVID-19 [5,6]. However, both were proven to exhibit no significant reduction in mortality [7]. Great financial expenditures and human risk were taken in trials for hydroxychloroquine and ivermectin to present no significant findings, and even increased mortality rates [7,8]. Preventative and cost-effective measures in the US remain in support of masks [9]. Additional effective measures include increased physical distancing [10] and ventilation of closed spaces [11].

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2. Outpatient Management

2.1. Monoclonal Antibodies

Two monoclonal antibody regimens including (a) Casirivimab plus imdevimab (C+I) and (b) Sotrovimab (S) were approved for use in non-hospitalized patients with mild to moderate COVID-19 prior to the current Omicron surge [11]. C+I bind to the spike protein epitope, preventing attachment to the ACE 2 receptor [12]. Sotrovimab is a recombinant human IgG1-kappa mAb that also binds to an epitope on the spike protein receptor binding domain [12]; however, it does not compete with ACE-2 binding and likely inhibits an undefined step of viral replication at a later stage [13]. Both were approved for use in patients with the Delta variant who have risk factors for progression to severe disease (Table 1).

Table 1. Risk factors for progression to severe disease based on FDA and NIH recommendations.

1.	Aged $\geq$ 65 years
2.	Obesity (BMI > 30)
3.	Diabetes mellitus type 2
4.	History of CAD, hypertension, congenital heart disease
5.	History of respiratory disease, such as COPD, moderate or severe persistent asthma, interstitial lung disease, cystic fibrosis, pulmonary hypertension
6.	Sickle cell disease
7.	Immunosuppressive regimen
8.	History of: cancer, chronic liver disease, chronic lung diseases, dementia or other neurological conditions, diabetes, Down syndrome, HIV infection, Immunocompromised, mental health conditions: depression, schizophrenia, sickle cell disease, tuberculosis, substance use disorders, stroke or cerebrovascular disease, organ or blood stem cell transplant
9.	Chronic kidney disease
10.	Are overweight, obese, pregnant, smoke [14].

NOTE: FDA = Food and Drug Administration; NIH = National Institutes of Health; BMI = Body Mass Index; CAD = Coronary Artery Disease; COPD = Chronic Obstructive Pulmonary Disease; HIV = Human Immunodeficiency Virus.

A number of questions remain unanswered and require further research. One of the key questions is the subsequent effect on vaccine-induced immune responses following monoclonal antibody treatment. Additionally, given the heterogeneity in patients who progress to severe disease, it may be possible to have a more precision-medicine-like approach in identifying the patients at the highest risk for progression. With the emergence of new variants, the efficacy of monoclonal antibodies would remain to be studied. Both C+I and S are effective for use against the Delta variant, and are approved for use with Delta. Though Sotrovimab was shown to significantly benefit patients across all Omicron subgroups compared to C+I in a recent study [15], due to changes in the binding site of the Omicron variant, they are not recommended for use in Omicron and BA.2 variants [16–18].

2.2. Bebtelovimab

Bebtelovimab is a new monoclonal antibody (mAb) to be used in patients with mild/moderate COVID-19 disease severity. As with C+I and S, bebtelovimab is a recombinant neutralizing mAb that also binds to the spike protein of SARS-CoV-2, but with increased efficacy for newer COVID-19 variants compared to the previous mAbs [19,20]. The NIH is advising that bebtelovimab be injected at 175 mg as a single IV injection, administered over 30 s in patients who are high-risk but non-hospitalized [11]. According to the BLAZE-4, a randomized phase 2 trial clinical trial that studied viral clearance in patients with mild to moderate COVID-19 at risk for progression, showed that the drug remains effective against the virus, but there are limited clinical efficacy data available. Currently, bebtelovimab is effective in vitro against all Omicron subgroups [11]. The BLAZE-4 trials

began enrollment on 17 June 2020 and concluded the study on 20 October 2021 [21]. The FDA issued its emergency use authorization (EUA) on 11 February 2022 [22]. However, since there are no clinical efficacy data from placebo-controlled trials that evaluated the use of bebtelovimab in patients who are at high risk of progressing to severe COVID-19, the NIH recommends its use only in patients at risk of progression to severe COVID-19 for whom all other options are unavailable [11]. Bebtelovimab is shown to be effective in vitro against the BA.1, BA.1.1 and BA.2 Omicron subvariants [22]. Its use is authorized in adults, aged 12 years or older, and pediatric patients. This EUA excludes bebtelovimab use in patients with severe COVID-19 or who require oxygen therapy [22].

2.3. Remdesivir

Remdesivir is an antiviral treatment used in both hospitalized and non-hospitalized patients for mild/moderate and severe COVID-19 disease. Remdesivir prevents the RNA transcription of SARS-CoV-2 by binding to the viral RNA-dependent RNA polymerase, blocking viral replication [23]. The NIH advises 200 mg IV on Day 1 of symptom onset, along with 100 mg IV once daily on Days 2 and 3 in non-hospitalized patients, within the first 7 days of symptom onset [11]. According to the PINETREE trial, the number needed to treat to prevent hospitalization in non-hospitalized patients was 20 [24], and the hazard ratio (HR) was 0.13 with a 95% CI of 0.03–0.59 [25]. Remdesivir is the only drug that is FDA approved, securing approval on October 22nd, 2020. It is expected to be active in-vitro against the B.1.1.529 Omicron variant (Figure 1) [11,23,26]. However, there are limited in vivo data on remdesivir's effects against Omicron [26].

2.4. Molnupiravir

Molnupiravir is an antiviral treatment for those with mild/moderate COVID-19 disease severity. The active form of molnupiravir is utilized as the substrate for viral RNA- dependent RNA polymerase instead of the coronavirus RNA genome. Therefore, replication of the COVID-19 genome is prevented and a mutated RNA is synthesized in its place [27]. The NIH recommends administering molnupiravir in non-hospitalized patients age 18 or older, 800 mg orally, twice daily for 5 days, only when paxlovid and remdesivir are unavailable [11]. In the MOVe-OUT trial, the number needed to prevent hospitalization for molnupiravir is 33; the treatment difference is −6.8% with a 95% CI= −11.3 to −2.4 [28]. The most interesting finding of the trial was the discrepancy between the interim results (48.2% efficacy) and the final results (29.9% efficacy) [28,29]. This was attributed in part to the emergence of new variants, and it is possible that the drug is much less effective against Delta and subsequent variants. The MOVe-OUT trial began enrollment on 6 May 2021 and completed data collection on 4 November 2021. The FDA issued an emergency use authorization (EUA) on 23 December 2021. The EUA states that molnupiravir is not recommended for pregnant patients; however, it can be considered when these patients are at high risk of progressing to severe COVID-19 without other therapeutic options [11]. Molnupiravir has lower efficacy than the preferred treatment options. It is suspected to be effective against the BA.1 Omicron variant; however, in vitro and in vivo data are limited [26].

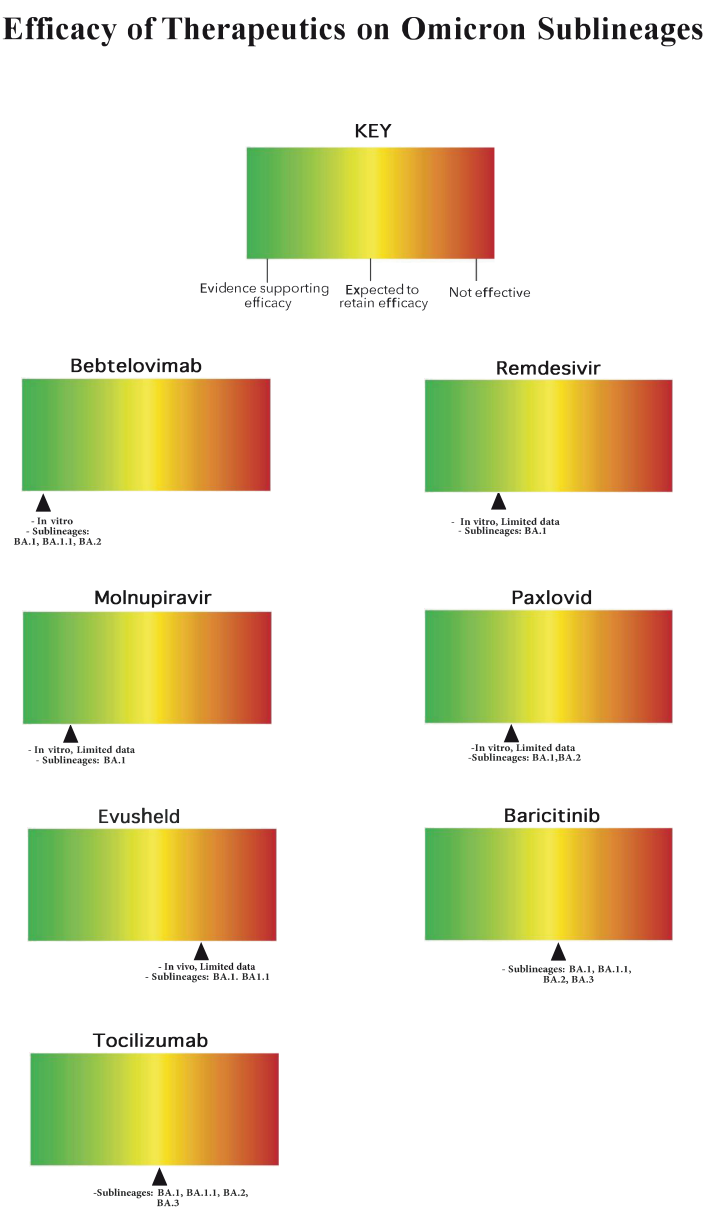

Figure 1. Therapeutic efficacies on Omicron sub-lineages.

2.5. Nirmatrelvir+Ritonavir (Paxlovid)

Protease inhibitors nirmatrelvir [PF-07321332] and ritonavir are included within the oral antiviral paxlovid. Nirmatrelvir [PF-07321332] is a selective protease inhibitor of M^{pro}, also known as 3CL, a major enzyme necessary for SARS-CoV-2 replication [30]. PF-07321332 binds to 3CL through reversible thioimidate bond formation of Cys145 with a nitrile carbon. PF-07321332 is the antiviral portion of paxlovid and prevents replication, while ritonavir is a pharmacokinetic enhancer [31]. Ritonavir primarily inhibits cytochrome P450 enzymes, preventing the metabolism of protease inhibitors such as PF-07321332 [32,33]. Paxlovid

contains nirmatrelvir [PF-07321332] and ritonavir in combination to ensure the highest efficacy of the antiviral effects.

Paxlovid has been approved for emergency use (EUA) on 22 December 2021, in high-risk adults and high-risk pediatric patients aged 12 and older with a minimum weight of 40 kg [34]. Paxlovid should be used to treat mild-to-moderate symptoms after a confirmed positive test result. Paxlovid should not be used in circumstances of pre-exposure or prevention. Refer to Table 1 for the FDA definition of high-risk categories. The dosing recommendations are 300 mg of nirmatrelvir with 100 mg of ritonavir twice daily for 5 days.

Extra precaution should be taken for those with a history of liver or kidney disease [35]. As paxlovid is renally cleared, dosing changes are recommended for those with eGFR $\geq$30 to <60 mL/min, with a decrease to 150 mg of nirmatrelvir with 100 mg of ritonavir, twice daily for 5 days [36]. Its use is not recommended in patients with severe renal impairment of eGFR <30 mL/min or severe hepatic impairment, as the use of paxlovid has not been studied enough in significant renal or hepatic dysfunction [36].

A double-blind clinical trial was conducted on non-hospitalized adults with specific high-risk factors with a confirmed positive COVID-19 test result. No patient had received a COVID-19 vaccine or had a history of infection. The results indicated that paxlovid reduced the risk of hospitalization or death by 89% if taken within 3 days of symptom onset [37].

There are still many concerns regarding the future direction of paxlovid, as well as many other approved outpatient therapeutics. One concern is the consideration from an ethical standpoint. The encouragement of the public to receive vaccinations is contrasted by only testing these therapeutics on unvaccinated people, leaving the potentially harmful effects of these regimens on vaccinated individuals still in question [38]. These concerns are also relevant to those with a history of COVID-19 infection, as paxlovid was also not studied in this population. Furthermore, there have been case reports of patients testing positive with COVID-19 again shortly after being treated with paxlovid; these patients would typically improve following treatment, with a recurrence of mild COVID-19 symptoms several days afterward, or would be asymptomatic with only a positive PCR test [39,40]. No known cases have progressed to severe COVID-19 as of yet, but further research needs to be conducted to better evaluate the frequency of recurrence and the implications for paxlovid therapy [41]. Lastly, while paxlovid is proven effective for the SARS-CoV-2 variants of concern, Delta and Omicron, studies have not yet confirmed paxlovid's effects on subsequent Omicron sub-lineages [42]. Paxlovid is suspected to be effective against the B.1.1.519 and BA.2 Omicron sub-lineages [43] [Figure 1]. However, this remains in question as limited in vivo data and clinical efficacy are presented.

2.6. Evusheld

Evusheld is a combination of two monoclonal antibodies, tixagevimab and cilgavimab [44]. Tixagevimab and cilgavimab effectively work together to block the receptor binding protein of SARS-CoV-2 spike protein from binding the human ACE2 receptor, inhibiting viral attachment [45]. Thus, it is used as a pre-exposure prophylaxis, and is meant for those who are immunocompromised or immunosuppressed, who have not been recently exposed to an infected individual [46]. It is administered by injecting a 300 mg dose of tixagevimab and 300 mg of cilgavimab intramuscularly in non-exposed, immunocompromised individuals [46], as pre-exposure prophylaxis. Patients are tentatively recommended to receive injections at 6-month intervals, as the exact timing between dosing is not yet known [47]. According to the Phase III PROVENT trial, the relative risk reduction was 0.77, with a 95% CI of 0.46 to 0.90 [48]. The FDA issued Evusheld an EUA on 8 December 2021 and recently revised the EUA on 24 February 2022 [47,49]. Evusheld has shown to be efficacious against Omicron subvariants BA.1, BA.1.1 and BA.2 [50]. Only the Omicron BA.2 subvariant remains fully susceptible to Evusheld as BA.1 and BA.1.1 now have decreased susceptibility [51].

3. Inpatient Management

3.1. Remdesivir

As mentioned previously, remdesivir is an intravenous inhibitor of viral RNA-dependent RNA polymerase that is highly conserved across many coronaviruses, which makes remdesivir widely applicable as an antiviral agent [52], particularly in the SARS-CoV-2 virus. In the inpatient setting, remdesivir is recommended as a five-day total course of 200 mg IV on the first day, then 100 mg IV on each subsequent day prior to discharge, for a maximum of four additional days [23]. In the CATCO trial, remdesivir was associated with a small but significant reduction in progression to mechanical ventilation: 8.0% of patients on remdesivir required mechanical ventilation over the hospitalization, compared to 15% of patients randomized to receive standard of care at that time [53]. The SOLIDARITY trial showed a small but statistically significant mitigation of progression of disease and decreasing mortality in patients who were not ventilated; those that required ventilation showed no difference in being treated with either remdesivir or a placebo [54]. Interestingly, an earlier publication of the SOLIDARITY trial showed no difference in outcomes after administering remdesivir; this may be due to the smaller sample size of the earlier trial (2750 patients compared to a final count of 8275) or to a small clinical effect [55]. According to the ACCT trials, the number needed to treat for hospitalized patients was 26; the HR was 0.73 with a 95% CI of 0.52–1.03 [56]. For those with severe COVID-19, remdesivir is frequently used in conjunction with dexamethasone [57].

3.2. Corticosteroids

Of all the therapies studied thus far, corticosteroids have had the most unequivocal impact on mortality. The RECOVERY trial findings [58], released in July 2020, showed a significant reduction in 28-day mortality with dexamethasone compared to standard of care (age-adjusted rate ratio (aRR), 0.83; 95% confidence interval (CI), 0.75 to 0.93). Of note, there was a significant interaction with oxygen dependency. Among patients on mechanical ventilation (MV), the aRR was 0.64 (95% CI, 0.51 to 0.81), while, among those receiving supplemental oxygen without MV, the aRR was 0.82 (95% CI, 0.72 to 0.94). Additionally of importance, in patients not requiring oxygen supplementation, dexamethasone use, while not associated with a benefit, trended towards harm (aRR, 1.19; 95% CI, 0.91 to 1.55) [58]. In a large observational analysis from our New York City center of 1806 patients [59], we found similar results. Among patients with admission C-reactive protein (CRP) levels of $\geq$20 mg/dL, denoting a significant inflammatory burden, corticosteroid treatment was associated with a 75–80% reduction in the composite severe outcome of MV and mortality (adjusted odds ratio (aOR), 0.23; 95% CI, 0.08–0.70), while, among those with CRP $\leq$10 mg/dL, corticosteroid treatment was associated with severe COVID-19 outcomes (aOR, 2.64; 95% CI, 1.39–5.03). Several trial findings published later and analyzed in a WHO meta-analysis [60] have reinforced the findings that corticosteroids have a mortality benefit in the critically ill patients with COVID-19, as defined in Table 2.

Table 2. Summarizing the indications for use of corticosteroids in COVID-19.

Corticosteroids are beneficial	
1.	Moderate to severe ARDS (defined using Berlin Criteria) and need for invasive mechanical ventilation
2.	Moderate to severe ARDS requiring non-invasive mechanical ventilation (high flow nasal cannula)
3.	Mild ARDS (pao2/fio2 < 300) and requiring oxygen support
4.	Pneumonia severity index (PSI) > 130
Corticosteroids may be beneficial	
1.	ARDS and elevated inflammatory markers (CRP > 20 mg/dL)
Corticosteroids may be harmful	
1.	Mild to moderate disease not requiring oxygen support
2.	Mild to moderate disease and low inflammatory markers (CRP < 20 mg/dL)

NOTE: ARDS = Acute Respiratory Distress Syndrome; CRP = C-reactive Protein.

While great progress has been made in utilizing corticosteroids for COVID-19 treatment, several clinically relevant questions warrant further research and are discussed below.

3.3. Heterogeneity of Response across the Clinical Severity Spectrum

In the RECOVERY trial, patients requiring supplemental oxygen but not on MV included those who received both low and high oxygen supplementation [58]. While this subgroup overall benefited from corticosteroids, the differences in response based on a low versus high level of oxygen requirement were not established. Given the risk of harm in patients with milder disease, further stratifying this subgroup for granular assessment of response to corticosteroids among those requiring low oxygen supplementation is clinically relevant.

In addition, inflammatory biomarkers could also play an important role in risk stratification. Patients with a low oxygen requirement but high inflammatory burden may represent a subgroup at risk for progressing to a critical disease state and could be more likely to benefit from corticosteroids than patients with a low oxygen requirement and low inflammatory burden or even no oxygen requirement and high inflammatory burden. Further studies to prognosticate based on clinical variables will be informative.

3.4. Impact on Long-Term Autoreactivity

Recent studies have demonstrated heightened autoreactivity in patients with severe COVID-19 [61–63]. Patients with a higher inflammatory burden, based on elevated CRP, are likely to test positive for antinuclear antigen (ANA) and rheumatoid factor (RF) [64]. In an elegant study, using Rapid Extracellular Antigen Profiling (REAP), Wang et al. [63] have demonstrated a diffuse array of autoantibodies directed against cytokines and chemokines. While the functional effect of these antibodies remains unclear, early data suggest that they may directly neutralize the activity of cytokines/chemokines and alter immune function in COVID-19 patients [63]. Increased autoreactivity seems to correlate with severe disease [63]. Whether this is a direct effect of pathogenic antibodies or an uncontrolled response to the persistence of antigens is unclear. Patients with demonstrable antibodies to interferon-α had a persistently higher viral load compared to antibody-negative controls, suggesting impaired clearance due to an impaired interferon-α-mediated viral clearance pathway [65]. Whether these antibodies cause tissue-specific damage and are associated with persistent symptoms as seen in "long-COVID" patients remain unclear.

Corticosteroids are well-known inhibitors of cytokines and chemokines, and effective in reducing inflammation and autoantibody production. This inhibition has to be balanced against the deleterious effect of inhibiting interferon-α-mediated viral clearance. It may be possible that corticosteroids are most effective in patients who have demonstrable increased autoreactivity, and further research should test this hypothesis.

3.5. Predictors of Early Response

In a recent observational study of 2707 patients, of whom 324 received corticosteroids, a CRP response, defined as a $\geq$ 50% reduction from admission value within 72 h, was associated with a significant reduction in mortality compared to CRP non-response (adjusted OR 0.27; 95% CI 0.14, 0.54) [45,64]. This suggests that CRP may be a biomarker to predict the early response to corticosteroids.

Other clinical variables and biomarkers that could predict early response are of great interest. Candidates include the neutrophil lymphocyte ratio (NLR), neutrophil monocyte ratio (NMR) and d-dimer. COVID-19 is associated with lymphocyte and monocyte recruitment to the lungs, the primary site of injury, facilitated by cytokines such as interleukin (IL-6) and monocyte chemoattractant protein-1 (MCP-1). Corticosteroid-treated patients may show improvements in lymphocyte counts, monocyte counts and perhaps d-dimer.

3.6. Reactivation of Latent Infections

The impact of corticosteroids on infections, both new and reactivated, is an important consideration. Strongyloides hyperinfection and reactivation due to corticosteroid therapy is well established [66,67]. Disseminated Strongyloidiasis, associated with high mortality, can occur with corticosteroids, other immunomodulatory agents and hematologic malignancies [66]. Such cases have been reported even with low-dose and short-duration corticosteroid therapy (3 mg dexamethasone equivalent and duration 5 days) [67]. Empiric prophylactic therapy with ivermectin in patients in endemic areas, or more broadly in countries other than Australia, North America or Western Europe, may be a reasonable strategy. Disseminated Strongyloidiasis should be considered as a differential in COVID-19 patients on corticosteroids with unexplained Gram-negative bacteremia and acute clinical decompensation [68].

Other latent infections of concern include tuberculosis, hepatitis B and herpes. Dexamethasone stimulates the reactivation of HSV-1 ex vivo [69,70] and in animal studies, and it may reactivate the closely related bovine herpesvirus 1 (BHV-1) in latently infected calves [71]. There are little data on the reactivation of hepatitis B and tuberculosis with short-term steroid use.

Corticosteroids are one of the few therapies with an unequivocal benefit in COVID-19, including a mortality benefit in the subgroup of severely ill patients. They are inexpensive and available universally, including in regions with limited resources. However, it is important to take into account the potential for harm due to corticosteroids. Biomarkers such as CRP may help to stratify patients who are more likely to benefit and can also serve as an early therapeutic response biomarker. Patients on corticosteroids should be monitored for the reactivation of Strongyloides and prophylactic ivermectin should be considered in patients from highly endemic areas.

3.7. Baricitinib

Baricitinib belongs to a class of medications called Janus kinase inhibitors, or JAK inhibitors. These medications act by inhibiting signal transducer and activator of transcription proteins, also known as STAT proteins. STAT proteins play integral roles in cellular replication, regulating processes such as growth, replication, signaling and apoptosis [72]. JAK inhibitors are frequently used in oncologic settings, in order to attempt to control rapidly dividing cancer cells. By the same token, JAK inhibitors were trialed to treat COVID-19 with the rationale that they might be able to inhibit the overactivation of the immune system [73]. Interestingly, of the JAK inhibitors, only baricitinib and tofacitinib have been shown to have efficacy in treating COVID-19. In the ACTT-2 trial, baricitinib with remdesivir was shown to increase the recovery rate by a day (7 days compared to 8 days) when compared to remdesivir alone [74]; the study also showed a small improvement in outcomes overall at day 15, though it was not statistically significant. A subsequent study, the COV-BARRIER trial, also established the benefit of baricitinib when used in conjunction with standard of care, most notably corticosteroids. The COV-BARRIER trial showed that although bariciticib did not impact the overall progression of the disease, defined as increasing oxygen requirements including mechanical ventilation, it did improve all-cause mortality at 28 days, with a low number needed to treat of 20 patients [75]. The primary limitation of baricitinib is renal dysfunction, and it is explicitly not recommended to be used in patients with eGFR < 15. The recommended dosing is based on renal clearance (4 mg daily for those with eGFR > 60, 2mg daily for those with eGFR 30–60, 1mg daily for eGFR 15–30), and the treatment duration is up to 14 days or until hospital discharge. Patients most likely to benefit from baricitinib are those with high oxygen requirements, defined as BiPAP or HFNC, with an unclear though possible benefit in those patients requiring mechanical ventilation [75].

3.8. Tocilizumab

Tocilizumab is a monoclonal antibody instructed for use in hospitalized patients with both mild/moderate and severe COVID-19 symptoms. Tocilizumab is effective in treating COVID-19-induced cytokine storms since it is an IL-6 receptor antagonist [76]. The advised use of tocilizumab consists of injecting 8 mg per kg of patient body weight as a single IV dose [56]. It has been shown to be highly effective in hospitalized COVID-19 patients presenting with hypoxia with oxygen saturation of <92% and elevated markers of systemic inflammation, most notably CRP $\geq$ 75 mg/L, when administered in addition to dexamethasone [65]. According to the RECOVERY clinical trial, the number needed to treat was 33, with a risk ratio of 0.85 and 95% CI of 0.76 to 0.94 [25,77]. This trial began enrollment on 23 April 2020 and ended on 24 January 2021. Limitations of this treatment include the use of tocilizumab in combination with baricitinib due to increased risk of infection from potent immunosuppressors. The FDA issued an EUA on 24 June 2021, for tocilizumab use.

3.9. Anakinra

During a COVID-19 infection, many inflammatory markers are increased, including interleukin-1. Anakinra is a recombinant IL-1 receptor antagonist, most commonly used in the treatment of rheumatoid arthritis and cryopyrin-associated periodic syndromes [78]. In the SAVE-MORE trial, treatment with anakinra yielded improved outcomes for patients with hypoxia requiring supplemental oxygen and a suPAR biomarker at a serum concentration of $\geq$6 ng/mL [79]. Specifically, the incidence of severe respiratory failure was decreased from 59.2% in standard of care to 22.3% in those treated with anakinra, with a 10.8% improvement in 30-day mortality as well when compared to standard of care [79]. Despite these promising results, other studies, including REMAP-CAP and CORIMUNO-ANA-1, found no benefit for the use of anakinra in patients with COVID-19 at large [80,81]. Thus, there is an apparent importance of risk stratification with suPAR, which is an assay that is not readily available in many countries, including the United States. As a result, there is no recommendation for the use of anakinra in the United States, either in favor or against. In Europe, anakinra is approved for use in patients with COVID-19 who require supplemental oxygen with a suPAR level of $\geq$6 ng/mL, at a dose of 100 mg as a subcutaneous injection for 10 days [82]. Anakinra is expected to be effective against the Omicron variant, though there are no known active studies investigating this specifically [83].

3.10. Anticoagulation

Heparin is an anticoagulant utilized for treatment in hospitalized patients with mild/moderate and severe COVID-19 symptoms. While the specific mechanism of heparin's action is unknown, there is great evidence for low-molecular-weight heparin exhibiting anti-inflammatory and anti-viral benefits in patients with severe SARS-CoV-2 [84]. It is advised to use heparin in different manners depending on the therapeutic or prophylactic dose usage. The NIH panel recommends administering a prophylactic dose of heparin in non-pregnant, hospitalized patients requiring mechanical ventilation [56]. A therapeutic dose is preferred in patients who have moderate disease, defined as having symptomatic COVID-19 disease but not requiring mechanical ventilation, HFNC, CPAP, BiPAP or pressor support and with no contraindications to anticoagulation, such as platelets <50×10^9/L, hemoglobin <8 g/dL, being on dual antiplatelet therapy or having had major bleeding within the past month [14]. According to the RAPID trial, the number needed to treat was 8, with an indicated relative risk of 0.68 and with a 95% CI of 0.49 to 0.96 [85]. The FDA issued an abbreviated new drug application (ANDA) approval for heparin in relation to COVID-19 treatment on 15 July 2020.

A timeline of FDA approvals for each drug mentioned in this review can be seen in Figure 2.

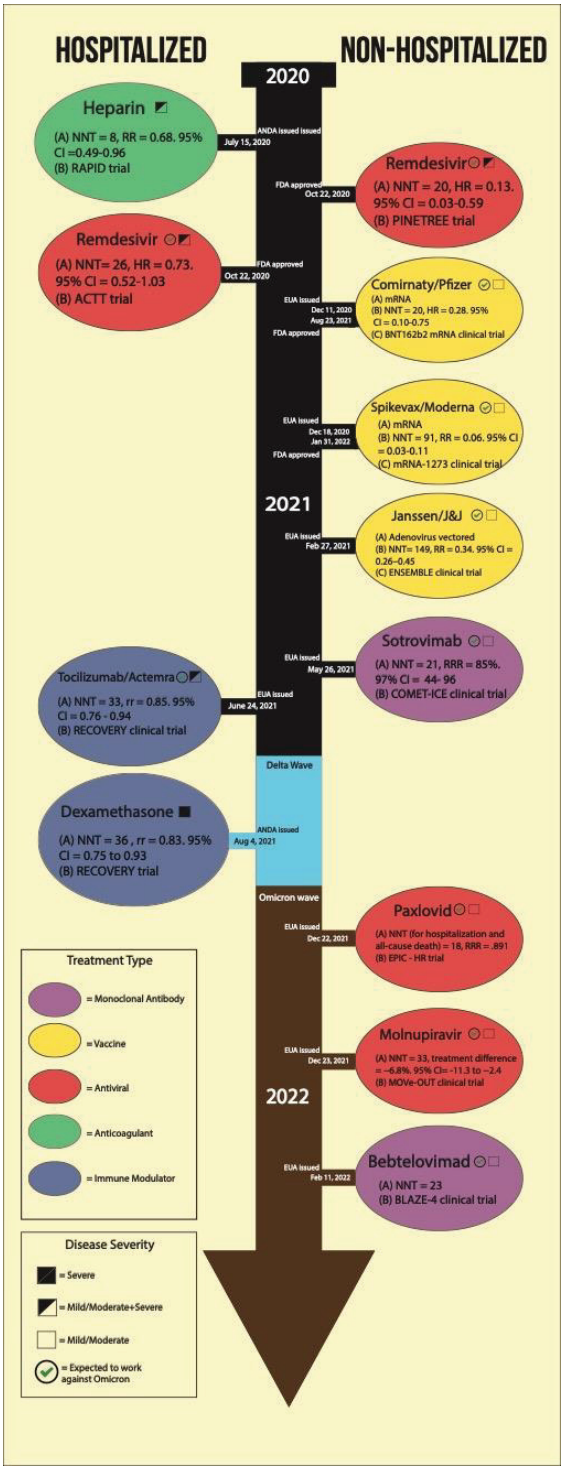

Figure 2. Timeline of COVID-19 therapeutics and authorization use issuance at the FDA.

4. Conclusions

In this rapidly evolving landscape, it is imperative to stay abreast of current therapeutics and their efficacy, particularly against newer and rapidly changing strains of COVID-19. In this brief yet comprehensive review, we discuss the therapeutics available for the treatment of COVID-19 infection that are shown to be effective in well-designed randomized controlled trials. It is worth noting the rapid speed with which many of these therapeutics were identified and developed, which is a testament to the massive undertaking that many international consortia performed, including platform trials such as RECOVERY (Randomised Evaluation of COVID-19 Therapy), REMAP-CAP (A Randomised, Embedded, Multi-factorial, Adaptive Platform Trial for Community-Acquired Pneumonia), ACTIV-IV (Accelerating COVID-19 Therapeutic Interventions and Vaccines) and ATTACC (Antithrombotic Therapy to Ameliorate Complications of COVID-19). It cannot be overstated how much progress has been made in these last two years, and how far we have come from March 2020, when our only interventions were a trial of hydroxychloroquine, a ventilator and a strong dash of hope.

Finally, though beyond the purview of our article, vaccines against the SARS-CoV-2 virus still remain the mainstay of saving lives, and their importance as the most effective preventative measure cannot be emphasized enough. Nevertheless, our aim with this article is to educate providers of the breadth of therapeutics available in both inpatient and outpatient settings, tailored to disease severity. In doing so, we hope to facilitate the selection of the most appropriate agent in each clinical setting and continue to improve outcomes in the treatment of COVID-19.

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Relation of Pulmonary Diffusing Capacity Decline to HRCT and VQ SPECT/CT Findings at Early Follow-Up after COVID-19: A Prospective Cohort Study (The SECURe Study)

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Abstract: A large proportion of patients exhibit persistently reduced pulmonary diffusion capacity after COVID-19. It is unknown whether this is due to a post-COVID restrictive lung disease and/or pulmonary vascular disease. The aim of the current study was to investigate the association between initial COVID-19 severity and haemoglobin-corrected diffusion capacity to carbon monoxide (DLco) reduction at follow-up. Furthermore, to analyse if DLco reduction could be linked to pulmonary fibrosis (PF) and/or thromboembolic disease within the first months after the illness, a total of 67 patients diagnosed with COVID-19 from March to December 2020 were included across three severity groups: 12 not admitted to hospital (Group I), 40 admitted to hospital without intensive care unit (ICU) admission (Group II), and 15 admitted to hospital with ICU admission (Group III). At first follow-up, 5 months post SARS-CoV-2 positive testing/4 months after discharge, lung function testing, including DLco, high-resolution CT chest scan (HRCT) and ventilation-perfusion (VQ) single photon emission computed tomography (SPECT)/CT were conducted. DLco was reduced in 42% of the patients; the prevalence and extent depended on the clinical severity group and was typically observed as part of a restrictive pattern with reduced total lung capacity. Reduced DLco was associated with the extent of ground-glass opacification and signs of PF on HRCT, but not with mismatched perfusion defects on VQ SPECT/CT. The severity-dependent decline in DLco observed early after COVID-19 appears to be caused by restrictive and not pulmonary vascular disease.

Keywords: SARS-CoV-2; COVID-19; long COVID; SPECT; HR-CT scan; lung function test

1. Introduction

After the first wave of the global coronavirus disease 2019 (COVID-19) pandemic, it became increasingly clear that the pulmonary sequelae often persist far beyond the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Apart from the diverse cluster of symptoms collectively coined "long COVID" [1] (breathlessness, chest pain, and fatigue), several studies have documented various degrees of reduced pulmonary diffusing capacity of carbon monoxide (DLco) in previously hospitalised patients up to 12-months post-discharge [2–9]. In many cases, concomitant residual radiological abnormalities are present on high-resolution chest CT, (HRCT) most typically ground-glass opacities (GGO), interlobular septal thickening, and reticulations [4,8].

The mechanisms of post-COVID-19 DLco reduction and the associated symptoms are currently unknown. While previous studies have reported relatively few patients with signs of overt pulmonary fibrosis (PF) on HRCT post-COVID-19 [10], it is still not known if changes on HRCT such as GGO, interlobular septal thickening, and reticulations will remain and for how long. Given that both in situ pulmonary thrombosis and thromboembolism, triggered by aberrations in the coagulation system and pulmonary endothelialitis [11], are considered cardinal in the conspicuous and "silent" hypoxaemia often observed in COVID-19 [12], this may also contribute to late stage changes in lung function. Thus, apart from post-viral PF, persistent pulmonary thromboembolic disease may contribute to persistent DLco reduction and associated symptoms after COVID-19 [6,13].

This paper is the first report from the Danish SECURe (Sequelae of COVID-19, Copenhagen University Hospital, Rigshospitalet) to present a prospective cohort study monitoring the severity and duration of post-COVID complications by the use of extensive clinical, physiological, and radiologic assessments, both in previously hospitalised and non-hospitalised COVID-19 patients.

The aim of the current study was to investigate the association between initial COVID-19 severity and haemoglobin-corrected diffusion capacity to carbon monoxide (DLco) reduction at follow-up. Furthermore, the aim was also to analyse if DLco reduction could be linked to pulmonary fibrosis (PF) and/or thromboembolic disease within the first months after the illness.

2. Materials and Methods

2.1. Study Design and Setting

The SECURe study is an ongoing prospective cohort study of individuals with polymerase chain reaction (PCR) confirmed SARS-CoV-2 infection conducted at Copenhagen University Hospital, Rigshospitalet, a tertiary health care centre, aimed to assess long-term sequalae of COVID-19.

The protocol was developed based on early reports from China [14,15] and on follow-up data from the first SARS outbreak in Hong Kong in 2002–2003 [16]. In Denmark, as elsewhere, the COVID-19 treatment strategies have been modified during the study period along with the availability of scientific data. Thus, steroids were first implemented from June 2020 [17,18]. Likewise, some patients admitted during the early epidemic were included in the remdesivir trial, the usage of which increased from May 2020 and became widely available from August 2020 [17,19].

Inclusion was closed ultimo March 2021 due to the significant decline in SARS-CoV-2 transmission rates in Denmark and closure of our dedicated COVID-19 ward. We enrolled 190 participants.

2.2. Study Participants

All COVID-19 patients admitted to Rigshospitalet, March 2020–March 2021 were invited to participate. Additionally, non-hospitalised SARS-CoV-2 infected patients were offered inclusion with the aim of including 200 patients, $\geq 2/3$ hereof being hospitalised.

Exclusion criteria included dementia, living at an old age facility and being unable to come for follow-up visits.

The initial SECURe study visit was planned to be conducted 3–4 months after SARS-CoV-2 positive testing/post-discharge for non-hospitalised and hospitalised study participants, respectively. Due to a high workload at the participating departments, it was not always possible to adhere fully to this time-plan (see below).

Here, we report on all participants (n = 67) who had completed their first follow-up by 31 December 2020.

2.3. Recruitment

Patients were invited to participate in the study at discharge and/or at a post-discharge telephone consultation. Non-admitted patients were identified through the affiliated testing site and by word of mouth among health care personnel.

2.4. Data Sources

Age, sex, Charlson co-morbidity index [20], date of testing SARS-CoV-2 positive, initial COVID-19 symptoms and duration thereof prior to admission, treatment during hospitalisation including maximal oxygen demand, ICU admission, mechanical ventilation and/or extra-corporal membrane oxygenation (ECMO) and duration thereof, as well as total duration of hospitalisation were extracted from the participant's electronic health record. Even though there is now consensus regarding a more advanced disease severity classification system [21,22], this had not yet been established at the time of this study, and we therefore pragmatically used a trinary system to classify the patients according to the clinical severity of the initial COVID-19 disease, similar to previous studies patients not requiring hospitalization (Group I), patients requiring hospitalization but not ICU admission (Group II), and patients requiring both hospitalisation and ICU admission (Group III) [23–30].

At the follow-up visit, participants were questioned about post-COVID-19 symptoms and respiratory complaints according to the chronic obstructive pulmonary disease assessment test (CAT) [31]. Furthermore, participants completed the health-related quality of life SF-36 questionnaire [32], had an extended assessment of physical performance including Hand Grip strength (HGS) and 30-s Sit-To-Stand Test (STS) muscle strength tests and the Six-Minutes' Walk Test (6MWT) [33–35] (Supplemental File S1), lung function testing [36–38], HRCT with subsequent scoring [39] and ventilation-perfusion (VQ) scintigraphy [40,41] (Supplemental Files S2–S4, and described briefly below).

Participants with signs of post-COVID-19 sequelae were offered re-assessment at 12 months.

2.5. Lung Function Testing

Dynamic spirometry, body plethysmography and single breath measurement of DLco were performed in accordance with the ERS/ATS guidelines [36–38]. Forced expiratory volume in the first second (FEV1), forced expiratory volume (FVC), FEV1/FVC-ratio, total lung capacity (TLC), residual volume (RV), RV/TLC-ratio, Hb corrected DLco and diffusion coefficient for CO (Kco) were measured. A FEV1/FVC-ratio and a TLC below the lower limit of normal was classified as an obstructive and restrictive ventilation defect, respectively [42,43].

2.6. HRCT Chest Scan

HRCT was obtained both after a breath-hold at deep inspiration and deep expiration. The scans were divided into six zones (three on each side), and evaluated for GGO, PF, and honeycombing (HC). PF was indicated by reticulation, traction and bronchiectasi, in combination or separate. For each of these findings, the extent in every zone was scored from 0 to 4 (Supplemental File S3) [39]. All scans were scored by two experienced readers (AK (radiologist) and TKL (pulmonologist)). The readings were carried out as a multidisciplinary reading with consensus. The two readers were blinded to the clinical and functional data.

At the starting point of the SECURe study, there were no validated CT scoring systems in the context of COVID alterations, so we had to choose a system. The scoring system chosen here was based on the system developed in the "Scleroderma Lung Study" [39]. A proportion of scleroderma patients have lung involvement with both GGO of PF and the scoring system was transferable to this population. There is no consensus regarding which scoring system to use, and various methods have historically been used.

2.7. VQ Scintigraphy

VQ scintigraphy was conducted as single photon emission computed tomography (SPECT) with a low dose CT used for attenuation correction. The European Association of Nuclear Medicine interpretation criteria were applied [41]. Perfusion and ventilation defects were visually identified, localised, and classified as mismatched (only defect in perfusion), matched (both perfusion and ventilation defects) or inversely mismatched (only defect in ventilation), and sized as subsegmental or segmental. A matched or inversely mismatched ventilation defect was classified as a ventilatory abnormality, regardless of concomitant HRCT findings, while a mismatched perfusion defect without any concomitant signs of fibrosis in the same area on HRCT, including reticulation with or without GGO, was classified as a vascular abnormality, most likely pulmonary embolism. However, if the HRCT showed signs of fibrosis precisely corresponding to a perfusion defect, it was interpreted as a ventilatory abnormality. Various studies have shown that interstitial lung fibrosis may cause mismatched perfusion defects that may incorrectly be interpreted as pulmonary embolism if not correlated to concomitant CT findings [44–46]. All scans were read independently by two experienced pulmonary nuclear medicine specialists (JM & RB) and discrepancies were resolved in consensus. The readers were blinded to the clinical and functional data.

2.8. Statistical Analyses

All data were entered into REDCap (10.6.18 ©2021 Vanderbilt University, Nashville, TN, USA). Clinical characteristics, lung function, HRCT, VQ scintigraphy, and physical performance were summarised as percentage (n), mean with standard deviation (SD) for normally distributed variables or median [interquartile range, IQR] for non-normally distributed variables. The differences between clinical severity groups were assessed using Fisher's exact test for dichotomous and categorical data, Kruskal-Wallis H test for non-normally distributed data, or one-way ANOVA for normally distributed data. If a difference was found, bivariate comparisons with Bonferroni correction for multiple comparisons were made. Wilcoxon rank-sum test was used to assess the difference in groups for time from discharge to follow-up. Fisher's exact test was used to assess the association between VQ defects and HRCT chest findings of GGO and signs of PF. Univariate linear regression models were used to assess the association between CAT score, VQ defects or HRCT findings with DLco. Multivariable logistic regression models were used to assess the association between VQ defects, HRCT findings or DLco with admission to ICU, age and sex.

Data for physical performance were presented as raw scores and presented as % of age and sex adjusted reference norms.

For all data, a two-sided $p < 0.05$ was considered statistically significant. Statistical analyses were performed using STATA 12 (StataCorp., Stata Statistical Software: College Station, TX, USA: StataCorp LLC).

3. Results

Patients were evaluated a median 5 months after testing SARS-CoV-2 positive and 4 months after hospital discharge for those admitted (Table 1). Patients from a higher clinical severity group were older, predominantly of male sex, and had greater pre-COVID comorbidity compared with patients from a lighter clinical severity group. Most patients (93%) reported persistent complaints and had a reduced physical performance and lower SpO_2 and approximately 25% of the patients had not resumed work (Supplemental File S5).

Table 1. Characteristic of patients with COVID-19 (n = 67) and difference between patients who were not hospitalised, hospitalised without ICU and with ICU treatment.

	All	Group I	Group II	Group III	*p*-Value (between Groups) #
N	67	12	40	15	
Age, years	52.7 ± 14.8	41.8 ± 8.5	54.2 ± 15.6	57.7 ± 12.5	0.012 [A]
Sex, male	39 (58.2)	3 (25.0)	24(60.0)	12 (80.0)	0.016 [B]
CCI *†	2 [1;3]	1 [0;2]	2 [0;>3] *	2 [2;>3]	0.073
CAT score *	5 [2;8]	2 [1.5;5.5]	5 [1;6] *	8 [2;10]	0.084
Co-morbidity	36 (53.7)	1 (8.3)	21 (52.5)	14 (93.3)	<0.001 [C]
Anticoagulation treatment **	29 (46.0)	1 (12.5) **	13 (32.5)	15 (100)	<0.001 [D]
Before diagnosis **	3 (4.8)	0 (0.0) **	3 (7.5)	0 (0.0)	
After diagnosis **	26 (41.3)	1 (12.5) **	10 (25.0)	15 (100)	
Time from positive SARS CoV-2 PCR test to 3 months follow-up, days	154 [132;191]	175.5 [150;222]	154 [120;187.5]	151 [141;170]	0.349
Time from discharge to follow-up, days ***	130 [98;167]	N/A	139.5 [98;174] ***	113 [95;140]	0.203

Data are expressed as mean ± SD, median [interquartile range] or n (%) as appropriate. CAT score: chronic obstructive pulmonary disease assessment test. † Charlson Comorbidity Index (CCI) values > 3, were recorded as 4 for calculation of the median. * Missing data from one patient (n = 66). ** Missing data from four patients (n = 63). *** Missing data from two patients (n = 53). # Fisher's exact test, Wilcoxon rank-sum test, Kruskal-Wallis H test or one-way ANOVA where appropriate and if significant followed by bivariate comparison with Bonferroni correction for multiple comparisons. [A]: Difference between not hospitalised and hospitalised without ICU, and not hospitalised and hospitalised with ICU. [B]: Difference between not hospitalised and hospitalised with ICU. [C]: Difference between all groups. [D]: Difference between not hospitalised and hospitalised with ICU and hospitalised without ICU and hospitalised with ICU.

For two study participants, smoking status was not available. Among the remaining participants, only one reported being a current smoker. Previous smoking was, however, often reported with a gradient across the clinical severity groups, 18, 38 and 60 % in Groups I, II and III, respectively.

3.1. Lung Function

Half of the patients had an abnormal lung function: 25% in Group I, 47% in Group II, and 79% in Group III ($p = 0.02$) (Table 2). FEV1 was normal in (94%) and not significantly different between groups, but FVC, TLC and RV were progressively lower in the clinical severity group. A reduced DLco was the most common abnormality across groups; the frequency and severity depended on the clinical severity group, notably in patients with a concomitantly low TLC (Table 2). In 75% (21/28) of the patients with a low DLco, there were no signs of either a low FEV1/FVC or a low TLC, and this pattern was not associated with clinical severity.

3.2. HRCT

Most patients (63%) had GGO and the frequency depended on the clinical severity group, with GGO being present in all patients in Group III, where the extent of GGO was also rated as higher ($p < 0.001$). Likewise, signs of PF were noted in 44%, also dependent of the clinical severity group ($p < 0.001$) and was observed in all Group III patients. None of the patients in Group III had HC or a history of prior lung disease. PF was associated with the presence of GGO score > 25% ($p < 0.001$) (Supplemental Table S2). One third of patients had bronchiectasis, the proportion of which was higher in Group III than Group II (Table 3). Examples of HRCT findings are depicted in Figure 1.

Table 2. Lung function outcome 4 months after COVID-19 (n = 67) and differences between patients who were not hospitalised, hospitalised without ICU and with ICU treatment.

	All (n = 67)	Group I (n = 12)	Group II (n = 40)	Group III (n = 15)	*p*-Value (between Groups) #
FEV1 %P	109.1 ± 19.0	112.5 ± 14.4	109.7 ± 17.5	104.9 ± 25.7	0.564
FVC %P	112.6 ± 20.0	124.8 ± 17.6	112.0 ± 16.5	104.7 ± 26.7	0.031 [A]
FEV/FVC	79.1 ± 5.7	76.8 ± 5.4	79.3 ± 5.8	80.4 ± 5.3	0.236
TLC %P *	99.9 ± 15.8	113.5 ± 12.2	100.2 ±13.2	87.6 ± 16.4 *	0.001 [B]
RV %P *	88.5 ± 18.7	99.6 ± 14.6	91.6 ± 17.2	70.4 ± 13.5 *	<0.001 [C]
RV/TLC %P *	82.9 ± 11.4	84.4 ± 10.5	85.4 ± 11.1	74.7 ± 9.6 *	0.007 [D]
DLco %P *	79.6 ± 16.7	94.3 ± 16.2	80.3 ± 13.9	64.9 ± 12.6 *	<0.001 [B]
Kco %P *	92.7 ± 16.2	95.5 ± 17.2	94.1 ± 16.8	86.6 ± 12.7 *	0.272
Ventilation					
Restriction *	10 (15.2)	0 (0)	4 (10.0)	6 (42.9) *	0.005 [C]
Obstruction	2 (3.0)	1 (8.3)	1 (2.5)	0 (0)	0.400
Both restriction and obstruction *	0 (0)	0 (0)	0 (0)	0 (0) *	-
Diffusion					
Reduced DLco *	28 (42.4)	2 (16.7)	16 (40.0)	10 (71.4) *	0.014 [A]
DLco > LLN *	38 (57.6)	10 (83.3)	24 (60.0)	4 (28.6) *	
DLco 60%P-LLN *	20 (30.3)	2 (16.7)	13 (32.5)	5 (35.7) *	0.020 [E]
DLco < 60 %P *	8 (12.1)	0 (0)	3 (7.5)	5 (35.7) *	
Both ventilation and diffusion					
Normal *	33 (50.0)	9 (75.0)	21 (52.5)	3 (21.4) *	0.020 [A]
Restriction + low DLco *	6 (9.1)	0 (0)	1 (2.5)	5 (35.7) *	0.004 [C]
Restriction + normal DLco *	4 (6.1)	0 (0)	3 (7.5)	1 (7.1) *	1.000
Obstruction + low DLco *	1 (1.5)	0 (0)	1 (2.5)	0 (0) *	1.000
Obstruction + normal DLco *	1 (1.5)	1 (8.3)	0 (0)	0 (0) *	0.182
Low DLco only *	21 (31.8)	2 (16.7)	14 (35.0)	5 (35.7) *	0.461

Data are expressed as mean ± SD or number (%) when not specified. # Fisher's exact test or one-way ANOVA where appropriate and if significant followed by bivariate comparison with Bonferroni correction for multiple comparisons. * Missing data from one patient (n = 66). [A]: Difference between not hospitalised and hospitalised with ICU. [B]: Difference between all groups. [C]: Difference between not hospitalised and hospitalised with ICU and hospitalised without ICU and hospitalised with ICU. [D]: Difference between hospitalised without ICU and hospitalised with ICU. [E]: Difference between not hospitalised and hospitalised with ICU and between hospitalised without ICU and hospitalised with ICU comparing the normal and moderately-severely reduced DLco.

Table 3. HRCT findings in patients 4 months after COVID-19 (n = 63) and differences between patients who were not hospitalised, hospitalised without ICU and with ICU treatment.

	All (n = 64)	Group I (n = 12)	Group II (n = 38)	Group III (n = 14)	*p*-Value (between Groups) #
Any GGO	40 (62.5)	1 (8.3)	25 (65.8)	14 (100)	<0.001 [A]
Only GGO	12 (18.8)	1 (8.3)	11 (29.0)	0 (0)	0.027 [D]
>25% GGO *	17 (26.6)	0 (0)	7 (18.4)	10 (71.4)	<0.001 [B]
Fibrosis (PF + HC)	28 (43.8)	0 (0)	14 (36.8)	14 (100)	<0.001 [A]
Air trapping	10 (15.6)	2 (16.7)	4 (10.5)	4 (28.6)	0.308
Bronchiectasis	20 (31.3)	3 (25.0)	8 (21.1)	9 (64.3)	0.013 [C]
Tracheobronchomalacia	5 (7.8)	0 (0)	4 (10.5)	1 (7.1)	0.814
Other **	22 (34.4)	6 (50.0)	11 (29.0)	5 (35.7)	0.441

Data are expressed as n (%). GGO: ground-glass opacities, PF: pulmonary fibrosis, HC: honeycombing. # Fisher's exact test and if significant followed by bivariate comparison with Bonferroni correction for multiple comparisons. * In more than one zone; ** Noduli, enlarged truncus pulm, emfysem etc. [A]: Difference between all groups. [B]: Difference between not hospitalised and hospitalised with ICU and hospitalised without ICU and hospitalised with ICU. [C]: Difference between hospitalised without ICU and hospitalised with ICU. [D]: No difference between groups with Bonferroni correction.

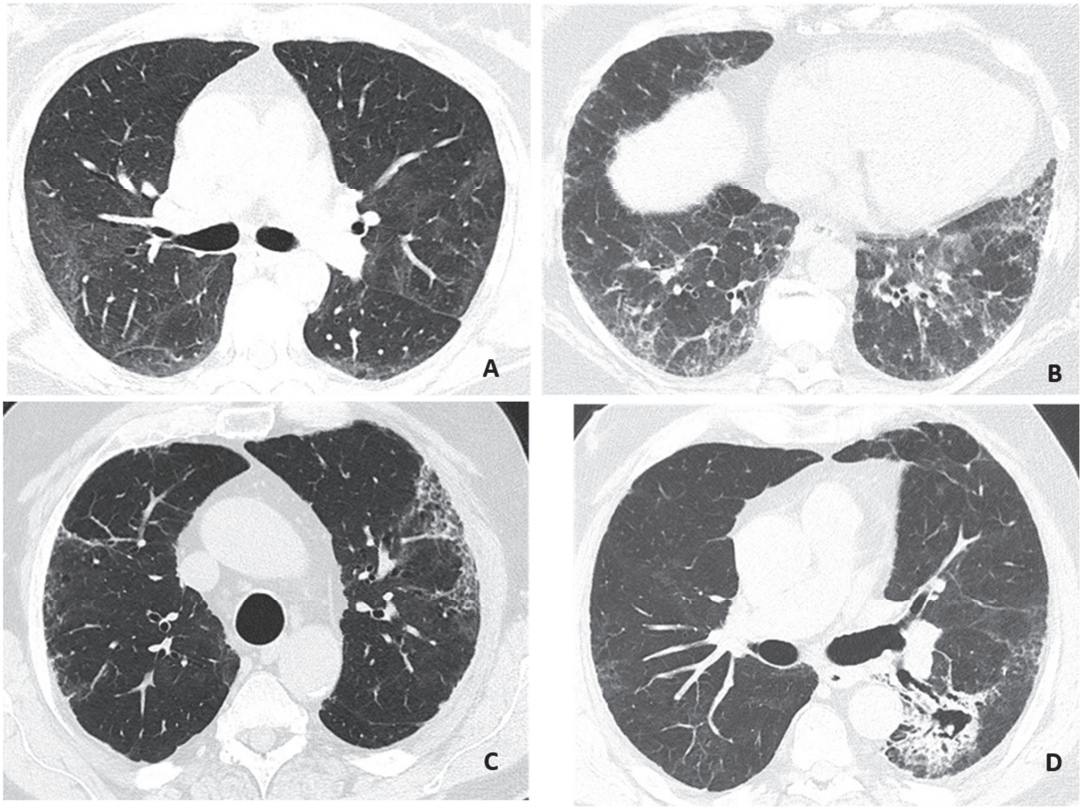

Figure 1. Representative findings on HRCT-scans. (**A**) Ground-glass opacity with discrete interlobular lines. (**B**) Ground-glass opacity with a reticular pattern. (**C**) Discrete ground-glass opacity with reticular pattern and honeycombing. (**D**) Fibrosis with traction bronchiectasis and infarct sequelae with possible fungus ball in the cavity. Images from three patients, (**C**,**D**) is from the same patient.

3.3. VQ SPECT

Most patients (80%) had a some ventilatory abnormality; this was more common in Group III than in Group I. Vascular abnormalities were rare and not related to the clinical severity group. Ninety-five percent of participants had at least one type of VQ defect with a mean of five, with a higher proportion in Group II than Group I; however, there was no distinct relation between clinical severity group and the specific type of VQ defect. Thus, mismatched perfusion defects were identified in almost 2/3 of patients; this was not related to the clinical severity group, neither was it associated with the presence of matched perfusion defects, GGO nor PF on HRCT (Supplemental Table S2). Likewise, the presence of matched VQ defects was neither associated with GGO nor PF on chest HRCT. Only 14% had a normal VQ SPECT, the frequency of which was independent of the clinical severity group (Table 4). Examples of VQ SPECT findings are shown in Figure 2.

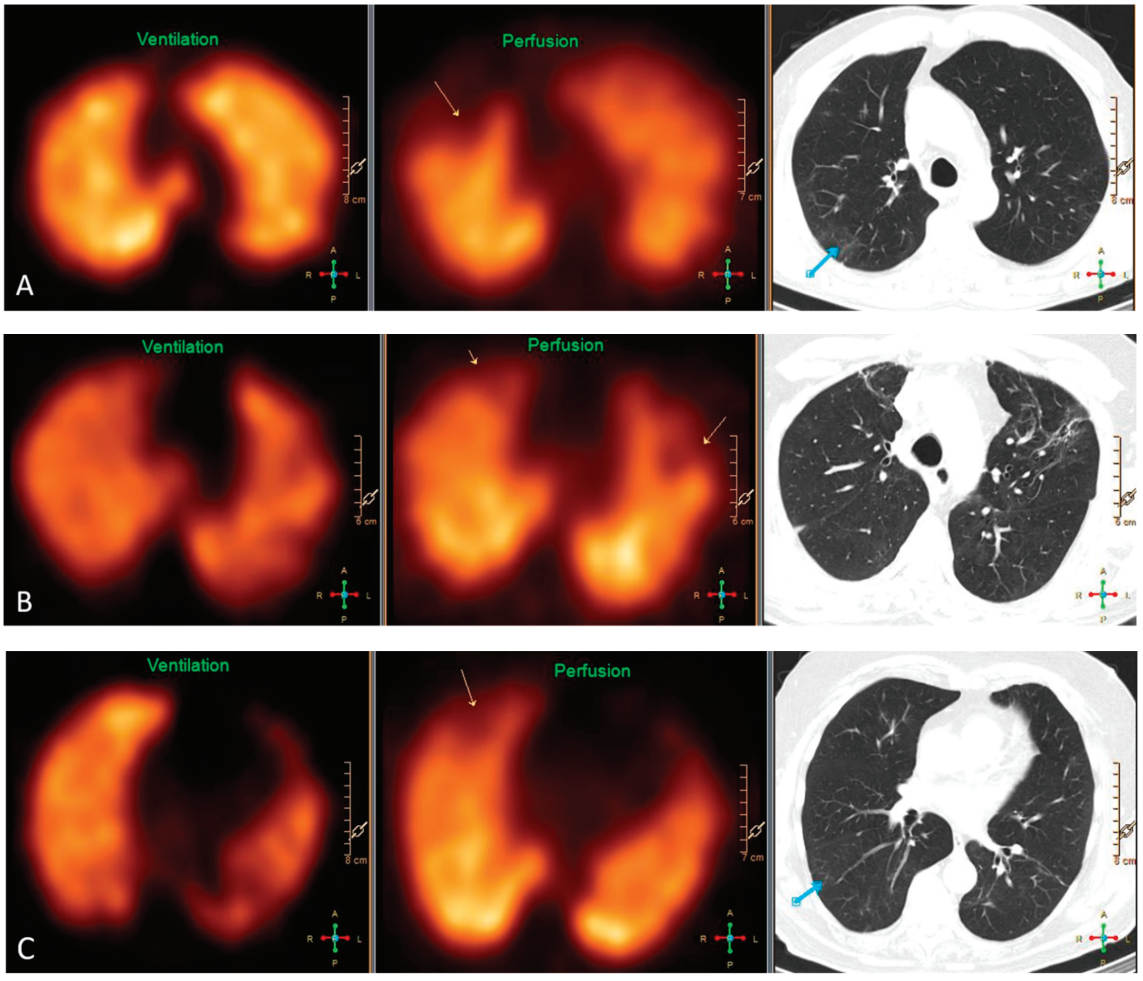

Figure 2. Representative findings on VQ SPECT and HRCT of three patients. (**A**) Pulmonary embolism in the right upper lobe causing a segmental mismatched perfusion defect on SPECT (yellow arrow) without any abnormality in the same area on HRCT. The blue arrow depicts ground-glass opacities dorsally in the right upper lobe without any defect on SPECT. (**B**) HRCT shows signs of fibrosis in the upper lobes causing partially mismatched subsegmental perfusion defects on SPECT (yellow arrows). (**C**) Pulmonary embolism in the right upper lobe causing a subsegmental mismatched perfusion defect on SPECT (yellow arrow) without any abnormality on HRCT in the same area. The blue arrow depicts discrete ground-glass opacities and signs of hypoventilation dorsally in the upper part of the right lower lobe.

Table 4. VQ scintigraphy findings in patients 4 months after COVID-19 (n = 65) and differences between patients who were not hospitalised, hospitalised without ICU and with ICU treatment.

	All (n = 65)	Group I (n = 12)	Group II (n = 38)	Group III (n = 15)	*p*-Value (between Groups) #
Ventilatory abnormality	52 (80)	7 (58.3)	30 (79.0)	15 (100)	0.019 [A]
Vascular abnormality	14 (21.5)	2 (16.7)	11 (29.0)	1 (6.7)	0.215
V/Q defects	62 (95.4)	10 (83.3)	38 (100)	14 (93.3)	0.038 [B]
Subsegmental, total	254	27	153	74	
Subsegmental, ratio	4.1	2.7	4.0	5.3	
Segmental, total	83	8	51	24	
Mismatched Q defects	43 (66.2)	6 (50.0)	26 (68.4)	11 (73.3)	0.424
Subsegmental, total	86	7	53	26	
Subsegmental, ratio	2.0	1.2	2.0	2.4	
Segmental, total	2	0	2	0	
Matched V/Q defects	26 (40.0)	4 (33.3)	15 (39.5)	7 (46.7)	0.831
Subsegmental, total	36	5	18	13	
Subsegmental, ratio	1.4	1.3	1.2	1.9	
Segmental, total	11	2	3	6	
Reverse mismatched V defects	49 (75.4)	7 (58.3)	30 (79.0)	12 (80.0)	0.353
Subsegmental, total	132	15	82	35	
Subsegmental, ratio	2.7	2.1	2.7	2.9	
Segmental, total	70	6	46	18	
Normal V/Q scan	9 (13.9)	3 (25.0)	6 (15.8)	0 (0)	0.126
Follow-up V/Q scan needed	40 (61.5)	4 (33.3)	24 (63.2)	12 (80.0)	0.050 [A]

V = ventilation; Q = perfusion. Mismatched Q defects = perfusion defects, but normal ventilation in the area. Reverse mismatched V defects = ventilation defects, but normal perfusion in the area. Data are expressed as n (%), total sum of defects or ratio between number of subsegmental defects and number patient with subsegmental defects. # Fisher's exact test and if significant followed by bivariate comparison with Bonferroni correction for multiple. [A]: Difference between not hospitalised and hospitalised with ICU. [B]: Difference between not hospitalised and hospitalised without ICU.

3.4. Factors Associated with Reduced DLco

In univariate linear regression analysis, reduced DLco was associated with a higher CAT score, the extent of GGO and PF on HRCT, as well as the number of matched, but not mismatched defects on VQ SPECT (Table 5). In multivariable logistic regression, Group III allocation predicted both GGO > 25% on HRCT, the presence of PF, and reduced DLco, but not the presence of defects on SPECT (Table 6). Age, but not sex, was also predictive for GGO > 25% and PF.

Table 5. Association between CAT score, V/Q scintigraphy defects or HRCT findings with diffusion capacity (DLco %predicted) in patients 4 months after COVID-19 (n = 64) using univariate linear regression.

	B	95% CI	*p*-Value
Clinical findings			
CAT score	−0.89	−1.58;−0.19	0.013
HRCT findings			
GGO extent *	−1.64	−2.19;−1.10	<0.001
PF extent *	−2.67	−3.74;−1.60	<0.001
SPECT findings			
Number of V/Q defects	−1.52	−2.66;−0.39	0.009
Number of mismatched Q defects	−2.09	−5.22;1.03	0.186
Number of matched V/Q defects	−3.69	−7.03;−0.34	0.031
Number of reversed V defects	−0.98	−2.29;0.34	0.143

* Data missing from one patient (n = 63). CAT score: chronic obstructive pulmonary disease assessment test, GGO: ground-glass opacities, PF: pulmonary fibrosis.

Table 6. Association between V/Q scintigraphy defects or HRCT findings or diffusion capacity with admission to ICU, age and sex in patients 4 months after COVID-19 (n = 67) using multivariable logistic regression.

	Odds Ratio	95% CI	*p*-Value
Mismatched Q defects **			
ICU admission	1.77	0.47;6.67	0.400
Age in years	1.00	0.96;1.03	0.876
Female sex	1.52	0.50;4.63	0.460
Matched V/Q defects **			
ICU admission	1.64	0.48;5.58	0.427
Age in years	1.00	0.96;1.03	0.845
Female sex	1.49	0.52;4.32	0.459
GGO > 25% ***			
ICU admission	15.48	2.96;80.89	0.001
Age in years	1.10	1.03;1.17	0.003
Female sex	0.59	0.10;3.48	0.561
PF ***			
ICU admission	†	†	†
Age in years	1.10	1.04;1.18	0.002
Female sex	1.96	0.41;9.45	0.402
Reduced DLco *			
ICU admission	4.14	1.07;16.03	0.040
Age in years	1.03	1.00;1.07	0.088
Female sex	0.98	0.32;3.04	0.976

† Omitted from multivariable logistic regression due to collinearity. ICU admission perfectly predicts pulmonary fibrosis (PF). GGO: ground-glass opacities. * Missing data from one patient (n = 66), ** Missing data from two patients (n = 65), *** Missing data from three patients (n = 64).

4. Discussion

In this Danish cohort of patients with mild to severe COVID-19 the majority had subjective health complaints 5 months after testing SARS CoV-2 positive, irrespective of disease severity. The most common lung function abnormality was reduced DLco. Indeed, both the frequency and severity of reduced DLco differed between clinical severity groups, as did HRCT findings of GGO and fibrosis, and the number of matched defects on VQ SPECT. In contrast, the frequency and extent of mismatched perfusion defects and other signs or pulmonary vascular disease were neither related to reduced DLco nor to clinical severity group.

DLco has been reported at various follow-up times after COVID-19. As in the present study, a reduced DLco is typically noted as part of a restrictive lung disease pattern with a reduced TLC, while signs of obstructive lung disease with a concomitantly low FEV1/FVC is rare [2,4,7,47–50]. We found that the prevalence of reduced DLco was 17% in Group I. Previous studies have likewise found that a reduced DLco is common in this group within the first months after COVID-19 and vary markedly from 6 to 43%. In our study, the prevalence of reduced DLco was 40% and 70% in Group II and III, respectively. This is consistent with previous findings from Germany and USA, where reduced DLco was reported in 1/3 of Group II patients and >90% among Group III patients [24–26]. In contrast, one study, reported lower prevalence of reduced DLco in Group III compared to Group II patients [23], perhaps reflecting selection bias in the former group due to a high mortality rate in patients admitted to the ICU in this population. Thus, in the current and other studies, indices of severity, such as ICU admission, high-flow nasal cannula oxygen therapy, mechanical ventilation and duration thereof have been found to predict the prevalence and extent of DLco reduction [8,23]. Of note, DLco has been reported to gradually increase with time in most Group II patients, but it remains pathologically low at 12-month follow-up in more than half of the patients with a reduced DLco at 3-month follow-up [8]. While the exact prevalence estimates are difficult to compare between countries,

due to the differences in the extend of the COVID-19 epidemic, healthcare capacity, as well as, preventive, diagnostic, and therapeutic strategies including hospital/ICU admission thresholds, it can be inferred that a pathologically reduced DLco is exceedingly common after COVID-19, and the prevalence increases with the acute phase clinical severity.

GGO was the most common finding in HRCT, which agrees well with other studies conducted at various follow-up times within the first year after infection (1–12 months) [4,6,8,9,25]. In accordance with previous studies [23,24], we found a gradient across the severity groups with a GGO prevalence of 8, 66 and 100% in Groups I, II and III, respectively. GGO indicate localised infection, inflammation, or fluid in the interstitial or alveolar space, none of which are mutually exclusive. They occur from the onset of COVID-19, and GGO may reflect residual changes from the acute infection [8,9]. The extent of GGO after COVID-19 has previously been associated with peak HRCT pneumonia scores during hospitalisation, and the GGO scores gradually decrease over the first 12 months. Moreover, in accordance with previous studies [4,6,8,9,25], GGO provide a mechanistic link to reduced DLco. The same pathological changes within the lung parenchyma that cause GGO may thus also adversely affect DLco.

Fibrosis was another key HRCT finding, in most cases in the form of reticulation. This was not observed in Group I, but was present in 37% of Group II patients, and all Group III patients. We identified a broad spectrum from very little to substantial fibrosis, but without HC, which would have indicated end-stage pulmonary fibrosis. At follow-up five months after testing SARS CoV-2 infected (and four months after discharge (for those admitted)), fibrosis was notably seen in Group III patients, while some studies [6,9,23,24,49], but not all [8], have also found fibrosis in Group II patients. Though group III included individuals with asthma and or current/past tobacco usage, none of them were registered in the electronic patient file system with a chronic lung disease diagnosis, nor was this disclosed at the initial encounter due to COVID-19 (data not shown). It is therefore unlikely that the difference in CT-scan findings between the groups was (fully) due to pre-existing signs of fibrosis among the SECURe patients requiring treatment at the ICU unit.

The presence of pulmonary fibrosis was associated with both the presence of GGO and reduced DLco. We speculate that the presence of GGO and pulmonary fibrosis reflect a spectrum of underlying interstitial lung changes that may lead to varying degrees of restrictive lung disease with reduced DLco in a severity-dependent fashion. Accordingly, it is well established that long-standing pulmonary inflammation may facilitate pulmonary fibrosis [51,52], and, recently, several elevated plasma biomarkers of pulmonary fibrosis have been reported in COVID-19 patients across severity groups in a manner that is associated with the concurrent decline in DLco [26]. However, further evaluation of this link is needed.

Though there is an overlap in the CT features found in conjunction with and at follow-up after various viral infections, including influenza- and coronaviruses, differences also exist [53]. Models have been developed to differentiate between COVID-19 vs. Influenza A (H1N1) pneumonia based on clinical and radiologic features [54]. With the availability of effective and easily accessible microbiological tests, the differentiation based on radiological findings, including CT features, is not necessary. However, identification of the various patterns and understanding the reasons behind it might be helpful for evaluating treatment response.

To the best of our knowledge, this is the first study to report on systematic VQ SPECT/CT in the follow-up of COVID-19 patients. We found that 95 % had V/Q defects, which was slightly more prevalent in Group II and III (though also highly prevalent in Group I). Sixty-six percent had mismatched defects, all of which were small subsegmental and 40 % had matched defects, the majority segmental and larger. In addition, reverse ventilatory mismatched defects were very prevalent (75%). The high frequency of ventilatory defects (matched and reverse matched) might have made it difficult to identify possible associations between mismatched defects and DLco (Table 4). It is well-documented that pulmonary vascular disease may complicate COVID-19 in the acute stage and contribute to

hypoxaemia and respiratory failure [55–57], but it is unknown whether this also contributes to the post-COVID decline in DLco observed in many patients. In the present study, more than 20% showed evidence of vascular disease, notably mismatched perfusion defects. Apart from in situ thrombosis and/or pulmonary embolism, this may also reflect the long-term effects of the remarkable COVID-19-associated loss of pulmonary microvasculature recently reported and is also consistent with fibrosis-like inflammatory processes in the lung parenchyma [58]. However, this was neither related to the clinical severity group nor to DLco. Rather, reduced DLco was associated with the number of matched VQ defects, indicating ventilatory disturbance, although the association with clinical severity groups was less clear than for HRCT. This provides a functional correlate of the structural lung parenchymal changes seen on HRCT associated with reduced DLco.

There are several study limitations, which may limit the generalisability. Firstly, although all patients discharged from Rigshospitalet were invited to participate, several patient groups were not included in the current analysis, including patients with dementia and patients living at old age facilities. These patients have a higher risk of developing severe COVID-19 and possibly, consequently hereof, more marked long-term sequelae. Conversely, patients with symptoms believed to be related to their COVID-19 might be more inclined to participate. Furthermore, many patients chose not to participate in the study. Among the patients without the need for hospitalisation, there was an overrepresentation of health care workers.

Due to the epidemic and the ensuing strain on the health care system, the follow-up exams could not always be performed at 3–4 months post infection/discharge; however, the divergence from this timing was limited.

5. Conclusions

In conclusion, the post-COVID-19 lung is prone to exhibit a severity-dependent decline in DLco approximately five months after testing SARS-CoV-2 positive, which is caused by a fibrosis-like restrictive lung disease and not pulmonary vascular disease. While it remains to be determined to which extent these features of the post-COVID-19 lung are reversible, our results underline the need of preventive measures for severe COVID-19 and targeted post-COVID rehabilitation.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm11195687/s1, File S1: Physical performance testing; File S2: Lung function testing; File S3: HRCT chest scan; File S4: VQ scintigraphy; File S5: Subjective complaints, physical performance and employment status; Table S1: Physical performance outcome median 5 months after testing SARS CoV-2 positive (n = 65) and difference between patients who were not hospitalised, hospitalised without ICU and hospitalised with ICU treatment; Table S2: Association between V/Q scintigraphy defects and HRCT findings of ground glass opacities (GGO) and sign of fibrosis (PF) in patients 5 months after testing SARS CoV-2 positive (n = 65). References [33–41,44–46,59] are mentioned in Supplementary Materials.

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Informed Consent Statement: Oral and written informed consent was obtained from all subjects involved in the study.

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Review

Practical Recommendations for Optimal Thromboprophylaxis in Patients with COVID-19: A Consensus Statement Based on Available Clinical Trials

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Abstract: Coronavirus disease 2019 (COVID-19) has been shown to be strongly associated with increased risk for venous thromboembolism events (VTE) mainly in the inpatient but also in the outpatient setting. Pharmacologic thromboprophylaxis has been shown to offer significant benefits in terms of reducing not only VTE events but also mortality, especially in acutely ill patients with COVID-19. Although the main source of evidence is derived from observational studies with several limitations, thromboprophylaxis is currently recommended for all hospitalized patients with acceptable bleeding risk by all national and international guidelines. Recently, high quality data from randomized controlled trials (RCTs) further support the role of thromboprophylaxis and provide insights into the optimal thromboprophylaxis strategy. The aim of this statement is to systematically

review all the available evidence derived from RCTs regarding thromboprophylaxis strategies in patients with COVID-19 in different settings (either inpatient or outpatient) and provide evidence-based guidance to practical questions in everyday clinical practice. Clinical questions accompanied by practical recommendations are provided based on data derived from 20 RCTs that were identified and included in the present study. Overall, the main conclusions are: (i) thromboprophylaxis should be administered in all hospitalized patients with COVID-19, (ii) an optimal dose of inpatient thromboprophylaxis is dependent upon the severity of COVID-19, (iii) thromboprophylaxis should be administered on an individualized basis in post-discharge patients with COVID-19 with high thrombotic risk, and (iv) thromboprophylaxis should not be routinely administered in outpatients. Changes regarding the dominant SARS-CoV-2 variants, the wide immunization status (increasing rates of vaccination and reinfections), and the availability of antiviral therapies and monoclonal antibodies might affect the characteristics of patients with COVID-19; thus, future studies will inform us about the thrombotic risk and the optimal therapeutic strategies for these patients.

Keywords: anticoagulation; COVID-19; COVID-19 therapeutics; dosage; mortality; thromboprophylaxis; treatment

1. Introduction

The relationship between the Coronavirus disease 2019 (COVID-19) and venous thromboembolism (VTE) was first reported as a case report in March 2020, close to the onset of the pandemic [1]. Since then, an enormous amount of evidence has emerged and nearly ten thousand articles on COVID-19 and VTE have been published within the last two years [2]. COVID-19 is associated with an increased VTE risk [3] that can be attributed to factors related to (i) the virus and the induced thromboinflammation observed in severe infection per se; (ii) the hospitalization conditions (immobilization); and (iii) the individual patient risk factors for VTE, most of which are also risk factors for severe COVID-19 [4].

While the pathophysiological mechanisms are not clearly defined, hospitalized patients with severe COVID-19 exhibit an increased inflammatory status both at the systemic (cytokine storm) and local (endothelial injury with thromboinflammation) level [5–7]. COVID-19 associated coagulopathy mainly manifests with a prothrombotic tendency, as platelet count is preserved, coagulation function tests are normal or minimally prolonged, and bleeding events are uncommon [8]. These features can be distinguished from a diagnosis of disseminated intravascular coagulation (DIC), which can occur in patients with critical infectious illness [8]. Interestingly, COVID-19 associated coagulopathy and the related microthrombi formation mainly affects the lung vessels, as confirmed by autopsy studies [5,9].

The prevalence of pulmonary embolism (PE) and deep vein thrombosis (DVT) in hospitalized patients with COVID-19 varies widely and is likely due to across study differences in patient characteristics and VTE diagnostic and screening protocols [4]. In a meta-analysis of 47 studies (n = 6459 patients), where all patients were subjected to imaging diagnostic evaluation for PE/DVT, the prevalence of PE and DVT in hospitalized patients with COVID-19 was about 32% and 27%, respectively [10]. Importantly, a two-fold increased risk for death was demonstrated in patients with VTE compared to those without VTE [10].

Considering the increased VTE risk of COVID-19 and the association between VTE and mortality, it is not surprising that pharmacologic thromboprophylaxis has been shown to offer significant benefits in terms of reducing not only VTE events but also mortality, especially in cases of severe COVID-19 [11–14]. Thus, thromboprophylaxis is currently recommended by multiple national and international clinical practice guidelines for hospitalized patients with an acceptable bleeding risk [15–21]. Yet, the main source of evidence has been derived from observational studies with important methodological limitations.

Recently, randomized trials have investigated the role of thromboprophylaxis and provide insights into the optimal thromboprophylaxis strategy.

The aim of this statement is to systematically review all the available evidence derived from randomized clinical trials (RCTs) regarding the role of thromboprophylaxis in adult patients with COVID-19 (both in the inpatient and outpatient setting), to address specific key questions, and to transform this evidence into practical lessons to be implemented in daily clinical practice.

2. Materials and Methods

A systematic PubMed search was conducted in line with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) recommendations independently by two investigators (KGK and IGK) [22]. The literature search was conducted using the algorithm ("coronavirus 2019" OR "2019-nCoV" OR "SARS-CoV-2" OR "COVID-19" OR COVID OR COVID19) AND (thrombotic OR thrombosis OR "deep vein" OR "pulmonary embolism" OR thromboemboli* OR heparin) AND randomi* until August 10, 2022. Articles were also identified from references of relevant articles using the snowball procedure. Disagreements were resolved by consensus with senior authors. Eligible studies were RCTs regarding different thromboprophylaxis strategies in patients with COVID-19 in different settings (either inpatient or outpatient). Data concerning the population characteristics, the interventions/comparators, and the main conclusions of each RCT were extracted and tabulated.

3. Results—Key Questions and Practical Recommendations

Among the 352 articles initially retrieved, 20 fulfilled the inclusion criteria and were included in the systematic review (Table 1). Clinical questions accompanied by practical recommendations were formed according to available data derived from the included RCTs.

3.1. Hospitalized Patients

3.1.1. Does Thromboprophylaxis Offer a Benefit to All Hospitalized Patients with COVID-19?

Medically ill patients with infectious diseases requiring hospitalization usually receive thromboprophylaxis based upon their VTE and bleeding risk [23]. Given the increased risk for VTE in hospitalized patients with COVID-19, thromboprophylaxis seems a reasonable approach; yet no RCT comparing thromboprophylaxis versus placebo was identified. Despite this lack, the evidence from large-scale observational studies is consistent and in favor of thromboprophylaxis in hospitalized patients, and this has been translated into recommended practice [16,19].

In fact, the earliest evidence is derived from an observational study that reported decreased mortality in patients with COVID-19, who received thromboprophylaxis with low-molecular-weight heparin (LMWH), compared with those who did not [14]. Additional studies reporting beneficial effects of anticoagulant prophylaxis in patients with COVID-19 have subsequently been published [11–13]. A cohort study of 4297 hospitalized patients with COVID-19 showed that the early (within 24 h of hospitalization) initiation of thromboprophylaxis versus no anticoagulation resulted in a 27% decreased risk for 30-day mortality for those receiving anticoagulation [12]. In this study, 70% of patients received LMWH [12]. In another study, no anticoagulation was associated with increased risk for the composite outcome of death, VTE, intensive care unit (ICU) admission compared with LMWH use, irrespective of the dose intensity (prophylactic, intermediate, or therapeutic dosages) [11]. In the latter study, thromboprophylaxis use was additionally associated with a significant decrease in acute phase inflammatory indices such as ferritin, interferon gamma, or interleukin-6 [11].

Conclusion—Recommendation: *Thromboprophylaxis is associated with survival benefit (low dose compared to no thromboprophylaxis) and is recommended for all hospitalized patients with COVID-19 with an acceptable bleeding risk profile.*

3.1.2. Which Is the Drug of Choice for Inpatient Thromboprophylaxis?

Among all the anticoagulants, LMWH is the most studied drug that has been used for thromboprophylaxis in hospitalized patients with COVID-19 and is currently recommended as the first option by most guidance reports [16]. Unfractionated heparin (UFH) and fondaparinux are considered when LMWH is contraindicated (e.g., UFH in severe renal failure or fondaparinux in patients with history of heparin-induced thrombocytopenia, respectively) [16]. The majority of RCTs examining thromboprophylaxis strategies (16 out of 20) reported in Table 1 included interventions mainly with LMWH, especially enoxaparin. Head-to-head comparison of LMWH with direct oral anticoagulants (DOACs) has not been done but indirect data can be extracted from the AntiCoagulaTIon cOroNa virus (ACTION) trial [24]. In this randomized study, therapeutic versus prophylactic dosage of thromboprophylaxis was compared among 615 hospitalized patients with COVID-19 [24]. A total of 90% of the therapeutic arm received rivaroxaban, while 84% of the prophylactic arm received LMWH. No statistically significant difference was observed in the primary efficacy outcome (any VTE, myocardial infarction, stroke, systemic embolism, and major adverse limb events) but bleeding events were more frequent in the therapeutic rivaroxaban arm [24]. Conclusions regarding the comparison of LMWH and DOACs cannot be drawn since different dosages were implemented and different durations of treatment were planned (i.e., inpatient administration of prophylactic dose LMWH but up to 30-days post-discharge for therapeutic dose rivaroxaban) [24].

LMWH is the established drug class of choice in hospitalized patients with COVID-19 because of its anticoagulant effects coupled with putative pleiotropic anti-viral and anti-inflammatory properties [25]. LMWH has an important role in suspending the entry of the virus into the host cells and in modulating the inflammatory state and cytokine storm [11,25]. Moreover, it seems to present the least interactions with anti-viral or other drugs used in the treatment of COVID-19 infection [26–28] compared to other anticoagulants. Importantly, for hospitalized patients that are already treated with oral anticoagulants (vitamin K antagonists [VKA] or DOACs), a switch to LMWH can be considered (and is preferred in critical disease) because of the fewer potential drug–drug and drug–food interactions [26–28]. A recent meta-analysis showed that the prevalence of new-onset atrial fibrillation in hospitalized patients with COVID-19 was 7.4% [29]. LMWH can be suggested as the preferred anticoagulation regimen for hospitalized patients with COVID-19 and new-onset atrial fibrillation with a high CHA_2DS_2-VASc score, especially those with critical disease, mainly due to the abovementioned fewer interactions, whereas DOACs would be preferred for long-term anticoagulation afterwards [30]. A recent Good Practice Guidance Statement by the International Society on Thrombosis and Haemostasis (ISTH) also recommends LMWH as the anticoagulant of choice for hospitalized patients with COVID-19 [31].

Conclusion—Recommendation: *LMWH has the largest body of evidence regarding the beneficial role of thromboprophylaxis in hospitalized patients with COVID-19 and should be currently regarded as the drug of choice.*

3.1.3. What Is the Optimal Dosage for Inpatient Thromboprophylaxis? What Is the Role of Timing of Thromboprophylaxis Initiation?

Anticoagulation options include prophylactic dose, intermediate dose (doses higher than the prophylactic ones but lower than the therapeutic ones), and therapeutic dose anticoagulant regimens. Initial guidance recommendations relating to COVID-19 favored prophylactic dose regimens with higher doses being considered for selected patients, such as those with severe disease [16].

Several RCTs have addressed the issue of the optimal anticoagulant dosage for hospitalized patients with COVID-19 (Table 2). The Intermediate vs. Standard-Dose Prophylactic Anticoagulation in Critically ill Patients With COVID-19: An Open Label Randomized Controlled Trial (INSPIRATION) was the first RCT that addressed this issue comparing intermediate versus prophylactic dosages in patients with COVID-19 admitted to the ICU [32]. The findings of this trial did not show any benefit for the intermediate over

standard prophylactic dosage either in the primary analysis [32] or in the 90-day follow-up sub-analysis [33]. In their conclusion, the authors recommended against the routine empirical use of intermediate dosage anticoagulation in patients with COVID-19 admitted to the ICU. However, it is important to mention that this was an open-label trial and patients were randomized 12 days (median) after the onset of symptoms with details regarding their previous anticoagulation regimens missing. This fine point is of potential importance since recent data support the idea that timing of initiation of anticoagulation may be equally important as optimal dosage and therefore the results should be interpreted with caution [34].

In line with the above assumption, the multiplatform RCT combining Randomized, Embedded, Multifactorial Adaptive Platform Trial for Community-Acquired Pneumonia (REMAP-CAP), A Multicenter, Adaptive, Randomized Controlled Platform Trial of the Safety and Efficacy of Antithrombotic Strategies in Hospitalized Adults with COVID-19 (ACTIV-4a) and Antithrombotic Therapy to Ameliorate Complications of COVID-19 (ATTACC) (REMAP-CAP, AC-TIV-4a, and ATTACC), showed a benefit of the therapeutic versus prophylactic dosage only when the former was administered to non-critically ill patients [35]. The same study group failed to prove a similar benefit when the comparison was made in the setting of critically ill patients [36]. The importance of the prompt initiation of the increased dosages in high-risk patients has been implied, to gain benefit from this intervention [35,36]. The HEP-COVID trial demonstrated a reduction in the composite endpoint of major thromboembolic events and mortality in selected non-ICU patients with highly elevated (>4 $\times$ ULN) D-dimer levels or a sepsis-induced coagulopathy (SIC) score $\geq$4 receiving therapeutic versus lower dosages [37]. Once more, the beneficial effect of the therapeutic dosage was not demonstrated in ICU patients. The Therapeutic Anticoagulation versus Standard Care as a Rapid Response to the COVID-19 Pandemic (RAPID) trial showed similar results in reduction of the secondary outcome of all-cause mortality at 28 days in moderately ill patients with increased D-dimer levels [38]. Moreover, the small HESACOVID trial revealed a decreased need for mechanical ventilation and improved gas exchange in patients with severe COVID-19 receiving therapeutic enoxaparin compared to standard prophylactic anticoagulation [39]. In the same context, Oliynuk et al. conducted a small, randomized trial comparing prophylactic enoxaparin versus therapeutic enoxaparin or UFH [40]. Hospitalized ICU patients that were not intubated prior to study enrollment were included. The authors concluded that there was an increased risk for intubation or death in the prophylactic enoxaparin treatment arm compared to the therapeutic dosage treatment groups. On the other hand, the results of the AntiCoagulaTIon cOroNavirus (ACTION) trial do not support the use of therapeutic doses due to no improvement in clinical outcomes and increased bleeding events with therapeutic over prophylactic dosages [24]. It is noteworthy that in the ACTION trial, randomization was done up to 14 days after the onset of symptoms with previous anticoagulation status remaining unclear. Notably, the majority of patients in the therapeutic arm (90%) received rivaroxaban while patients in the prophylactic arm received enoxaparin (85%) or UFH (15%). It is also noteworthy that the therapeutic arm was treated for 30 days after hospital discharge while prophylactic anticoagulation was administered only during the hospital stay. Additionally, COVID-HEP included 159 patients with COVID-19 (28% in ICU setting) and compared therapeutic versus prophylactic dose for acutely ill and intermediate versus prophylactic dose for critically ill patients [41]. Both higher anticoagulation dosages failed to offer clinical benefits; however, the study was prematurely discontinued due to low recruitment rate [41]. The BEMICOP study compared therapeutic versus prophylactic dosage of bemiparin in 65 moderately ill patients with increased D-dimer and failed to demonstrate a protective role of therapeutic dosage [42]. Perepu et al. did not demonstrate a significant benefit of the intermediate dose over prophylactic dose heparin in both ICU and non-ICU patients; however, in 61% of the study sample, obesity and weight-adjusted doses were used (obese patients in the standard dose arm received either 30 mg or 40 mg of enoxaparin twice daily whereas in the intermediate dose arm, all obese patients received 0.5 mg/kg twice

daily) [43]. Finally, the small X-COVID trial showed a potential benefit of the intermediate over prophylactic dose heparin but was underpowered and prematurely discontinued [44] (Table 2).

Apart from data derived from RCTs, some observational studies demonstrated a benefit to patients receiving higher than prophylactic dose regimens [11,13,45,46]. Interestingly, a recent meta-analysis reported a trend for fewer VTE events with increasing dosages of anticoagulation [47]. However, observational studies are inevitably subjected to several forms of bias—including indication bias and selection bias from lack of randomization. Thus, patients with more severe disease usually tend to receive more intense therapeutic interventions, the beneficial impact of which may be hard to determine. This indication bias has been shown in a recent meta-analysis, where a trend for survival benefit was observed for the therapeutic over prophylactic dose only in the adjusted (for several confounders) analyses, while the opposite trend was revealed for the unadjusted analysis [46]. Interestingly, in the same meta-analysis a survival benefit was shown for intermediate over prophylactic dose heparin regimens [46].

Most guidelines initially recommended prophylactic dose anticoagulation for hospitalized patients and the consideration of a higher dose regimen in those at increased VTE risk [16]. The most recent formal guidelines using accepted methodology from the ISTH [17] and guidance from American College of Chest Physicians (ACCP/CHEST) [21] provide an updated approach based on recent findings from RCTs and meta-analyses [48]. The CHEST clinical guidance suggests that the severity of COVID-19 should be assessed before a decision for thromboprophylaxis [21]. The ISTH Guidelines propose that for hospitalized non-critically ill patients at increased risk for VTE [e.g., elevated D-dimer levels (>2 $\times$ ULN) or with need for oxygen requirements or low baseline oxygenation] with low bleeding risk, therapeutic dosage thromboprophylaxis is recommended. If a therapeutic dosage cannot be administered, a prophylactic (and not intermediate) dosage should be considered [17,21]. On the other hand, in critically ill patients (ICU) or those in a step-down or ward setting receiving high-flow nasal cannula oxygenation, prophylactic over intermediate or therapeutic dose heparin is recommended [20,21]. The National Institute for Health (NIH) [20] and the American Society of Hematology (ASH) guidance documents [15] are aligned with these recommendations. The NIH guidance and ISTH guidelines documents further recommend decreasing the anticoagulation intensity in the case of clinical deterioration when a patient changes from acutely to critically ill [17,20].

The CHEST guidance document discourages the use of intermediate dose anticoagulation based on lack of supportive RCT evidence and the potential for dose regimen confusion in clinical practice. It should be noted that intermediate dosages have been traditionally used in both observational [47] and randomized studies (Table 2). Three RCTs exclusively used intermediate dosages compared with prophylactic anticoagulation dosages [32,43,44]. Two of them were conducted mainly in an ICU setting and did not demonstrate any clinical benefit [32,43]; however, one of these trials was conducted in general wards and showed a marginal benefit in favor of the intermediate dosage [44]. Other studies have used mixed dosage strategies and consequently possible positive effects of intermediate dosages may have been blunted [35] (Table 2). In a recent meta-analysis including both data from RCTs and observational studies, but with the latter providing adjusted analyses for confounders, a beneficial effect of the intermediate over prophylactic dosage was observed, especially in the non-ICU setting [46]. It should be highlighted that the intermediate dosage is understudied in RCTs including acutely ill non-ICU or ward patients. Thus, at present, current data from RCTs support the use of a therapeutic dosage in acutely ill non-ICU or ward patients, discourage an escalation strategy with worsening status, and suggest a prophylactic dosage in critically ill patients, especially in the ICU.

Conclusion-Recommendation:

- *All hospitalized patients with COVID-19 should at least receive timely prophylactic anticoagulation. In the case of high risk for bleeding/active bleeding, mechanical prophylaxis should be used.*
- *In high thrombotic risk, non-critically ill (non-ICU) patients, a therapeutic dose of heparin (LMWH/UFH) is recommended, taking into consideration the individual patient's bleeding risk. The role of the intermediate dose heparin in such patients has not been adequately studied in RCTs.*
- *For critically ill (ICU) patients, higher dosages do not offer a benefit and increase the bleeding risk; therefore, a prophylactic dosage should be administered, preferably with LMWH/UFH.*

3.1.4. What Is the Role of the Antiplatelet Therapy in Hospitalized Patients with COVID-19 in the Context of Thromboprophylaxis? What about Patients Already on Antiplatelet Treatment?

Antiplatelet drugs are not recommended for thromboprophylaxis in general. Four RCTs evaluated the role of antiplatelet drugs in hospitalized COVID-19 patients [49–51] and outpatients [52] without demonstrating any significant benefit (Table 1). It should be noted that in these trials, hospitalized patients were already receiving anticoagulation for thromboprophylaxis in various dosages.

Regarding patients already receiving antiplatelet drugs, the following should be taken into consideration: (i) the indication of the antiplatelet treatment (secondary cardiovascular prevention—strong evidence; primary cardiovascular prevention—weak evidence [53]); (ii) the thrombotic and bleeding risk; and (iii) the benefit/safety of the co-administration of complex antiplatelet regimens and anticoagulants (e.g., dual antiplatelet treatment after a recent acute coronary event or percutaneous coronary intervention—in this case additional prophylactic thromboprophylaxis should be considered in addition to antiplatelet regimen, on an individualized basis and with periodic assessment of the bleeding risk). According to the recent Good Practice Guidance Statement by the ISTH, add-on antiplatelet therapy should not be routinely initiated in hospitalized patients with COVID-19 [31]. The exception could be in critically ill patients with COVID-19, with a low risk for bleeding, and treated with prophylactic dose LMWH and gastric protection with a proton pump inhibitor. In this subset, the addition of antiplatelet therapy (aspirin 81 mg or clopidogrel 75 mg daily) might reduce mortality at 90 days after discharge, as shown in the REMAP-CAP trial [50].

Conclusion—Recommendation: *Antiplatelet drugs should not be routinely initiated for thromboprophylaxis and concomitant administration with anticoagulants should be considered on an individualized basis, taking into consideration the indication for antiplatelet treatment and the thrombotic/bleeding risk of each patient.*

3.1.5. What Is the Bleeding Risk Associated with Thromboprophylaxis?

Thromboprophylaxis is widely regarded in most patients as having a net therapeutic benefit when balancing efficacy (to prevent thrombosis) and safety (bleeding risk), whereas mechanical methods of thromboprophylaxis are recommended only in a minority of patients with high bleeding risk [16]. Risk factors for bleeding are patient-specific and include age, underlying disease severity (e.g., COVID-19- or sepsis-associated coagulopathy), comorbidities (e.g., impaired renal or hepatic function), as well as the type and intensity of anticoagulant used.

An important part of the RCTs' objectives was not only to address the efficacy of thromboprophylaxis interventions, but also to verify the safety of these strategies in terms of clinically significant and important major bleeding events. The majority of the RCTs demonstrated the low bleeding risk of the thromboprophylaxis strategies (Table 1). Two trials in non-ICU patients demonstrated increased major bleeding events with therapeutic dosages [24,35]. Another two trials using antiplatelet drugs in addition to thromboprophylaxis anticoagulation found that this intervention was associated with increased incidence of bleeding events [49,50].

Conclusion-Recommendation: *Thromboprophylaxis should be regarded as a clinically beneficial and low bleeding risk intervention for most hospitalized patients with COVID-19. Detailed individualized bleeding risk assessment should be conducted, especially in cases where increased dosages are considered.*

3.2. Outpatients and Post-Discharge Patients—Practical Considerations for Outpatients and Post-Discharge Patients

The question of whether outpatients and post-discharge patients with COVID-19 should receive thromboprophylaxis was raised early. COVID-19 associated coagulopathy was more thoroughly investigated and a proportion of COVID-19 mortality was largely attributed to thrombotic events. Moreover, the main impetus for post-discharge prophylaxis was the premise that the at-risk period persists after hospitalization. Additionally, using anticoagulants in ambulatory patients with COVID-19 could possibly attenuate the pneumonitis and ventilation/perfusion (V/Q) mismatch related to inflammation and microthrombi. Nevertheless, inconclusive data were primarily available and only one third of the available guidance reports referred to outpatients and post-discharge patients, mainly recommending non-pharmacological thromboprophylaxis measures (e.g., increased mobilization and hydration) [16]. During the pandemic, it was demonstrated that thrombotic events tend to occur early in the clinical course of COVID-19 [54]. Moreover, in the outpatient setting, the incidence of VTE is higher among outpatients with certain characteristics (older age, male sex, obesity, inherited thrombophilia, no or partial vaccination) [55]. In this context, early initiation of thromboprophylaxis in outpatients with adverse prognostic factors for severe disease (candidates for hospitalization) and increased VTE risk could be regarded as a reasonable approach. With the increased use of oral antivirals such as Paxlovid (nirmatrelvir/ritonavir) for outpatients at high risk for COVID-19 progression, the co-administration of anticoagulants can be problematic because many DOACs share the same (CYP-450) metabolic pathway as ritonavir (which, in fact, is used to increase the bioavailability of the active anti-coronavirus agent nirmatrelvir), with the potential for DOAC bioaccumulation and an increased bleeding risk. Management options in anticoagulated patients who require Paxlovid include reducing the dose of the DOAC, using a DOAC with less drug-drug interaction potential (e.g., edoxaban), or switching to a LMWH [28]. Five RCTs have addressed the question of outpatient thromboprophylaxis [52,56–59].

The first randomized trial that assessed the efficacy and safety of an antithrombotic agent in the outpatient setting was the study by Gonzalez-Ochoa et al. [57]. The investigators randomized 243 outpatients at high risk for severe clinical progression within 3 days of COVID-19 clinical onset to receive sulodexide 1000 lipase releasing units/day or placebo for 21 days. Sulodexide is a natural glycosaminoglycan composed of 80% fast moving heparin plus 20% dermatan sulfate [60]. Its in vitro antihemostatic effects have been shown to be at least comparable with those of enoxaparin [61]. The authors concluded that patients treated with sulodexide had a significantly lower risk for hospitalization and supplemental oxygen need along with improved laboratory parameters without significantly increased major bleeding risk. The ACTIV-4B COVID-19 Outpatient Thrombosis Prevention Trial studied symptomatic but clinically stable outpatients receiving aspirin or therapeutic or prophylactic dose of apixaban or no anticoagulation [52]. The trial was terminated early due to low event rates and failed to conclude if there are improvements in clinical outcomes in the aspirin or apixaban groups over no anticoagulation in outpatients. The OVID study randomized 472 outpatients to receive prophylactic enoxaparin dosage versus standard of care (no thromboprophylaxis) and showed a similar risk of hospitalization and death between the two treatment arms. Similar to the ACTIV-4B study, the OVID study was terminated early due to low event rates and failed to conclusively assess the futility of thromboprophylaxis under the initial study design assumptions. The same results and conclusions were reached from the investigators of the ETHIC study that randomized 219 outpatients to a prophylactic dose of enoxaparin versus standard of care (no thromboprophylaxis) [58]. The ETHIC study was also terminated early due to low event rates.

The Medically Ill Hospitalized Patients for COVID-19 Thrombosis Extended Prophylaxis With Rivaroxaban Therapy (MICHELLE) trial randomized post-discharge patients at increased risk for VTE (International Medical Prevention Registry on Venous Thromboembolism [IMPROVE] VTE score of $\geq$4 or 2–3 with a D-dimer >500 ng/mL) to rivaroxaban 10 mg/day or no anticoagulation for 35 days [56]. Results demonstrated a reduction in the composite endpoint of major thromboembolic events and cardiovascular mortality in the prophylactic group and overall no major bleeding risk in either group. The authors concluded in favor of the use of prophylactic dosages of rivaroxaban in high-risk post-discharge patients.

Conclusion-Recommendation:

- ***Outpatients:***
  - *Available data indicate against routine pharmacologic thromboprophylaxis in outpatients with COVID-19 in general.*
  - *It is reasonable to suggest individualized thromboprophylaxis in outpatients at high risk for disease worsening (with adverse prognostic factors for severe disease, potential candidates for hospitalization or "hospital-at home programs") and/or increased VTE risk after careful assessment of the bleeding risk.*
  - *Regular assessment and reevaluation for disease worsening and bleeding risk is strongly recommended.*
- ***Post-discharge:*** *Post-hospital discharge prophylactic anticoagulation with rivaroxaban 10 mg once daily for approximately 1 month is recommended in high VTE risk patients if no drug-drug interactions are expected.*

Table 1. Randomized Controlled Trials regarding thromboprophylaxis strategies in patients with COVID-19.

Hospitalized						
Study	**N**	**Setting**	**Comparator**	**Intervention**	**Findings**	
					Efficacy	**Safety**
X-COVID-19 [44]	183	General Wards	Prophylactic Enoxaparin	Intermediate Enoxaparin	Intermediate: ↓ PE No DVT in both groups (underpowered study/premature discontinuation)	↔ Major bleedings
HEP-COVID [37]	253	D-dimer > 4 ULN or SIC score ≥ 4 ICU 33%	Prophylactic or Intermediate LMWH/UFH	Therapeutic Enoxaparin	Therapeutic: ↓ VTE/ATE/Death	↔ Major bleedings
RAPID [38]	465	General Wards (moderately ill + increased D-dimer)	Prophylactic LMWH/UFH	Therapeutic LMWH/UFH	Therapeutic: ↓ Death	↔ Major bleedings
Perepu et al. [43]	176	ICU and/or coagulopathy † ICU 62%	Prophylactic Enoxaparin	Intermediate Enoxaparin	↔ VTE/ATE/Death	↔ Major bleedings
ACTION [24]	615	Hospitalized + increased D-dimer ICU 6%	Prophylactic Enoxaparin/UFH (mainly Enoxaparin)	Extended Therapeutic Rivaroxaban/ Enoxaparin/UFH (mainly Rivaroxaban)	↔ Duration of hospitalization or oxygen supply/VTE/ATE/Death	Therapeutic: ↑ Bleeding events
INSPIRATION [32]	562	ICU 100%	Prophylactic Enoxaparin	Intermediate Enoxaparin	↔ VTE/ATE/ECMO/Death	↔ Major bleeding
HESACOVID [39]	20	IMV ICU 100%	Prophylactic Enoxaparin/UFH	Therapeutic Enoxaparin	Therapeutic: ↑ PaO_2/FiO_2 ↓ need for IMV	↔ Major bleeding
Oliynyk et al. [40]	126	Severely ill ICU 100%	Prophylactic Enoxaparin	Therapeutic Enoxaparin/UFH	Therapeutic enoxaparin/UFH: ↓ intubation/death	↔ Major bleeding

Table 1. *Cont.*

Hospitalized						
Study	**N**	**Setting**	**Comparator**	**Intervention**	**Findings**	
					Efficacy	**Safety**
REMAP-CAP, ACTIV-4a and ATTACC Critically ill [36]	1098	Critically ill ICU 100%	Prophylactic or Intermediate LMWH/UFH	Therapeutic LMWH/UFH	↔ VTE/ATE/Organ support-free days/Death (premature discontinuation—futility)	↔ Major bleedings
REMAP-CAP, ACTIV-4a and ATTACC Non-critically ill [35]	2219	Non-critically ill ICU 0%	Prophylactic or Intermediate LMWH/UFH	Therapeutic LMWH/UFH	Therapeutic: ↑ Organ support–free days ↓ Death (premature discontinuation—superiority)	↔ Major bleeding
COVID-HEP [41]	159	Acutely ill + increased D-dimer or critically ill ICU 28%	Prophylactic (acutely) or Intermediate (critically) enoxaparin/UFH	Therapeutic enoxaparin/UFH	↔ VTE/ATE/DIC/Death (premature discontinuation—low recruitment rate)	↔ Major bleeding
BEMICOP [42]	65	General Wards (moderately ill + increased D-dimer)	Prophylactic Bemiparin	Therapeutic Bemiparin	↔ VTE/ATE/development of ARDS/Need for mechanical ventilation support/ICU admission/Death	↔ Major bleeding
RECOVERY [49]	14892	Hospitalized * ICU 5%	Standard of Care	Standard of care + Aspirin 150 mg	↔ Progressing to IMV or Death	Aspirin: ↑ Major bleeding
REMAP-CAP [50]	1557	Critically ill § ICU 100%	No antiplatelet therapy	Aspirin or P2Y12 inhibitor	↔ Organ support-free days (premature discontinuation—futility)	Antiplatelets: ↑ Major bleeding

Table 1. *Cont.*

Hospitalized						
Study	**N**	**Setting**	**Comparator**	**Intervention**	**Findings**	
					Efficacy	**Safety**
ACTIV-4a [51]	562	Non critically ill ICU 0%	Therapeutic Heparin	Therapeutic Heparin + P2Y12 inhibitor	↔ VTE/ATE//Organ support-free days/Death	↔ Major bleeding
Non-Hospitalized						
Study	**N**	**Setting**	**Comparator**	**Intervention**	**Findings**	
					Efficacy	**Safety**
Gonzalez-Ochoa et al. [57]	243	Outpatients at high risk for severe clinical progression within 3 days of COVID-19 clinical onset	Placebo	Sulodexide (oral 1000 LRU/d) for 21 days	Sulodexide: ↓ Hospitalization/supplementary oxygen need/d-dimer/CRP	↔ Major bleeding
ACTIV-4B [52]	657	Symptomatic but clinically stable outpatients	Placebo	1. Prophylactic Apixaban (2.5 mg twice daily) 2. Therapeutic Apixaban (5 mg twice daily) 3. Aspirin (81 mg once daily)	↔ VTE/ATE/ Hospitalization/Death (premature discontinuation—low event rate)	↔ Major bleeding
ETHIC [58]	219	Outpatients $\geq$ 30 years with symptomatic COVID-19 + one risk factor for severe disease	Standard of Care (No thromboprophylaxis)	Prophylactic Enoxaparin	↔ Hospitalization/Death (premature discontinuation—low event rate)	↔ Major bleeding

Table 1. *Cont.*

Non-Hospitalized						
Study	**N**	**Setting**	**Comparator**	**Intervention**	**Findings**	
					Efficacy	**Safety**
OVID [59]	472	Outpatients ≥ 50 years with respiratory symptoms and body temperature > 37.5 °C	Standard of Care (No thromboprophylaxis)	Prophylactic Enoxaparin	↔ Hospitalization/Death (premature discontinuation—low event rate)	↔ Major bleeding
MICHELLE [56]	320	Post-discharge with increased VTE risk ¶	Prophylactic Rivaroxaban (10 mg) for 35 days	No anticoagulation	Rivaroxaban: ↓ VTE/ATE/Death	↔ Major bleeding

ARDS, acute respiratory distress syndrome; ATE, arterial thromboembolism; CRP, C-reactive protein; DIC, disseminated intravascular coagulopathy, DVT; Deep vein thrombosis; ECMO, extracorporeal membrane oxygenation; ICU, intensive care unit; IMV, invasive mechanical ventilation; P2Y12 inhibitor, clopidogrel, ticagrelor or prasugrel; LMWH; Low molecular weight heparin; LRU, lipase releasing units; PE; Pulmonary embolism; SIC, sepsis-induced coagulopathy; UFH, Unfractionated heparin; ULN, upper limit of normal; VTE, venous thromboembolism; * Patients were already receiving anticoagulation thromboprophylaxis (33% high LMWH dosage, 60% prophylactic LMWH, 7% no anticoagulation); † Coagulopathy defined as modified (International Society on Thrombosis and Haemostasis) ISTH Overt disseminated intravascular coagulation (DIC) score ≥ 3; § Patients were already receiving anticoagulation thromboprophylaxis (therapeutic 11%, intermediate 58%, prophylactic 18%, unknown 13%); ¶ All patients received prophylactic LMWH/UFH/fondaparinux during hospitalization. ↑, Intervention increased the endpoint versus comparator; ↓, Intervention decreased the endpoint versus comparator; ↔, No difference in the endpoint between intervention and comparator.

Table 2. Randomized Clinical Trials evaluating the optimal dosage of thromboprophylaxis in hospitalized patients with COVID-19.

Study	N	ICU (%)	Comparator	Intervention	Result-Conclusion
X-COVID-19 [44]	183	0	Prophylactic Enoxaparin	Intermediate Enoxaparin	Underpowered Fewer pulmonary embolism events with Intermediate
HEP-COVID [37]	253	33	Prophylactic or Intermediate LMWH/UFH	Therapeutic Enoxaparin	Improved clinical outcomes with Therapeutic only in non-ICU patients
RAPID [38]	465	0	Prophylactic LMWH/UFH	Therapeutic LMWH/UFH	Fewer deaths with Therapeutic
Perepu et al. [43]	176	62	Prophylactic Enoxaparin	Intermediate Enoxaparin	No difference
ACTION [24]	615	6	Prophylactic Enoxaparin/UFH (mainly Enoxaparin)	Extended Therapeutic Rivaroxaban /Enoxaparin/UFH (mainly Rivaroxaban)	No difference
INSPIRATION [32]	562	100	Prophylactic Enoxaparin	Intermediate Enoxaparin	No difference
HESACOVID [39]	20	100	Prophylactic Enoxaparin/UFH	Therapeutic Enoxaparin	Improved oxygenation parameters with Therapeutic
Oliynyk et al. [40]	126	100	Prophylactic Enoxaparin	Therapeutic Enoxaparin/UFH	Improved clinical outcomes with Therapeutic
REMAP-CAP, ACTIV-4a and ATTACC Critically ill [36]	1098	100	Prophylactic or Intermediate LMWH/UFH	Therapeutic LMWH/UFH	No difference
REMAP-CAP, ACTIV-4a and ATTACC Non-critically ill [35]	2219	0	Prophylactic or Intermediate LMWH/UFH	Therapeutic LMWH/UFH	Improved clinical outcomes with Therapeutic
COVID-HEP [41]	159	28	Prophylactic (acutely) or Intermediate (critically) enoxaparin/UFH	Therapeutic enoxaparin/UFH	No difference
BEMICOP [42]	65	0	Prophylactic Bemiparin	Therapeutic Bemiparin	No difference

ICU, intensive care unit; LMWH; Low molecular weight heparin; UFH, Unfractionated heparin.

4. Conclusions

Thromboprophylaxis has been regarded as one of the most important therapeutic interventions for patients with COVID-19 since the onset of the pandemic. Most guidance recommendations have been primarily based on data derived from observational studies. Recently, high quality RCTs have been published shedding light on the optimal strategies that should be followed. Careful interpretation and implementation of their findings should be the cornerstone of the physicians' practices in addressing everyday clinical problems and providing the best health services to these patients. LMWH represents the most well-studied type of thromboprophylaxis in hospitalized patients. At present, current randomized data support the use of therapeutic dosage in acutely ill non-ICU or ward patients with high thrombotic risk, discourage escalation strategy with worsening status, and suggest prophylactic dosage in critically ill patients, especially in the ICU. Yet, the role of the intermediate dosage in high thrombotic risk hospitalized patients without critical disease (non-ICU) has not been extensively studied in the context of RCTs. Thromboprophylaxis should not be routinely administered in outpatients; however thromboprophylaxis should be administered on an individualized basis in post-discharge patients with COVID-19 with high thrombotic risk. Moreover, the change in the dominant SARS-CoV-2 variants, the

wide immunization status (increasing rates of vaccination and natural immunity), and the availability of antiviral therapies and monoclonal antibodies in the outpatient setting might affect the characteristics of the patients with COVID-19; thus, further studies are needed for the optimal management of their thrombotic risk.

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Prediction Value of KREBS Von Den Lungen-6 (KL-6) Biomarker in COVID-19 Patients: A Systematic Review and Meta-Analysis

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Abstract: The SARS-CoV-2 (COVID-19) pandemic is a major issue that necessitates the use of cutting-edge disease prediction models. The aim of the study was to assess the existing evidence regarding association between Krebs von den Lungen-6 levels and COVID-19 severity. A literature search was performed on Web of Science, PubMed, Scopus and Cochrane Central Register of Controlled Trials databases from 1 January 2020 up to 2 August 2022. The electronic database search was supplemented by searching Google Scholar. In addition, reference lists of relative articles were also reviewed. KL-6 levels among COVID-19 positive vs. negative patients varied and amounted to 443.37 ± 249.33 vs. 205.73 ± 86.8 U/mL (MD = 275.33; 95%CI: 144.57 to 406.09; $p < 0.001$). The KL-6 level was 402.82 ± 261.16 U/mL in the severe group and was statistically significantly higher than in the non-severe group (297.38 ± 90.46 U/mL; MD = 192.45; 95%CI: 118.19 to 266.72; $p < 0.001$). The KL-6 level in the mild group was 272.28 ± 95.42 U/mL, compared to 268.04 ± 55.04 U/mL in the moderate COVID-19 group (MD = −12.58; 95%CI: −21.59 to −3.57; $p = 0.006$). Our meta-analysis indicates a significant association between increased KL-6 levels and SARS-CoV-2 infection. Moreover, KL-6 levels are significantly higher in patients with a more severe course of COVID-19, indicating that KL-6 may be a useful predictor to identify patients at risk for severe COVID-19.

Keywords: biomarker; COVID-19; Krebs von den Lungen-6; SARS-CoV-2; severity

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has contributed to millions of deaths worldwide since its outbreak [1]. Despite vaccines, new antiviral drugs, greater treatment experience, and survival-favorable virus mutations, the pandemic is not completely controlled as of yet, and continues to threaten humans' lives [2,3]. Moreover, there is still a risk of overloading health care systems due to the increased infectivity of new variants of the virus [3]. This, in turn, may contribute to increased mortality from causes other than COVID-19. Symptoms of disease caused by SARS-CoV-2 vary significantly from only

fever and cough, to acute respiratory distress syndrome (ARDS), and often change dynamically within the progression of the disease. Hence, it is necessary to identify biomarkers that can stratify patients into cohorts of those who may develop severe diseases, versus populations who can be released from the hospital while still in the early stage of their disease. This is also important because of the possibility of offering intensified therapy with new drugs to patients at higher risk of severe COVID-19. Although biomarkers may enhance prognosis and outcomes, their high interpatient variability may have an impact on the investigations' results. There are several biomarkers that may be used to evaluate the degree of COVID-19 infection. These markers may have a number of advantages, including the ability to recognize at-risk patients, stratify COVID-19 severity, help with the establishment of admission or intensive care criteria, provide treatment guidance through response assessment, evaluate prognosis, and frame ICU or regular ward discharge criteria.

The most commonly tested inflammatory biomarkers, including C-reactive protein (CRP), IL-6, and Procalcitonin (PCT), have proven insufficient in prospectively identifying patients who will suffer the severe course of COVID-19 [4]. However, combining several additional factors can increase their predictive value [5]. Additional prospective disease severity predictor candidates have been developed with other molecules, e.g., ferritin, lactate dehydrogenase, serum amyloid A or soluble interleukin-2-receptor (sIL2-R), but these have also proven mostly insufficient and not specific enough [6–9]. Finally, soluble urokinase-type plasminogen activator receptor (suPAR) is a promising predictor factor, but more data are needed to establish its potential clinical utility [10].

Krebs von den Lungen-6 (KL-6) is a high-molecular-weight glycoprotein that is released by type II alveolar pneumocytes and bronchial epithelial cells and has been shown to be a useful biomarker of alveolar epithelial proliferation and damage. KL-6 level has been reported to be increased in diseases such as acute respiratory distress syndrome (ARDS), pulmonary sarcoidosis, idiopathic interstitial pneumonia, hypersensitivity pneumonia, and collagen vascular disease-associated interstitial pneumonia. Moreover, KL-6 is associated with clinical outcomes and has been suggested for use evaluating disease activity [10–12]. Due to these properties, KL-6 has gained attention in COVID-19 evaluation as a molecule that may predict a more severe disease course.

In assessing the severity of COVID-19, computed tomography (CT) lung evaluation is of great value. The extent of lung involvement provides a more accurate assessment of disease severity than relying on somatic symptoms alone. Of note, the CT score correlates with serum KL-6 levels ($p = 0.035$) and was significantly higher in those with high KL-6 levels (>400 U/mL; 12.00, IQR 5.00–18.00, p-value 0.027). In addition, the KL-6 level was also significantly higher in COVID-19 positive subjects, compared to the negative group [10]. Interestingly, abnormal CT scans after 12 weeks from the onset of COVID-19 significantly correlated with elevated KL-6 levels upon admission [13].

Moreover, KL-6 has been positively correlated with CRP and IL-6 levels in patients with a severe course of COVID-19. The combination of these three prognostic factors differentiated severe from the mild-to-moderate disease [5]. An elevated KL-6 level on admission was an independent risk factor for prolonged hospitalization [14]. The above information indicates the value of baseline serum KL-6 level as a predictor of a more severe course of SARS-CoV-2 infection.

Conversely, some have reported no significant relationship between KL-6 levels and COVID-19 disease severity. KL-6 has also been demonstrated to be unrelated to persistent symptoms such as a feeling of shortness of breath 12 weeks after COVID-19 [13]. Given the inconsistencies in the data, our purpose was to perform a meta-analysis to summarize the information available in the literature on KL-6 and its utility to evaluate COVID-19 progression.

2. Materials and Methods

This systematic review and meta-analysis were prepared in accordance with the preferred reporting items for systematic reviews and meta-analyses (PRISMA) statement [15]

and was registered with PROSPERO prior to completion of the initial search (registration No: CRD42022349526).

2.1. Search Strategy

Two reviewers (M.P. and A.N.) independently searched four major electronic databases (Web of Science, PubMed, Scopus, and Cochrane Central Register of Controlled Trials) from 1st, January 2020 up to 2nd, August 2022, to identify studies examining the prognostic value of Krebs von den Lungen-6 in COVID-19-hospitalized patients. The electronic database search was supplemented by searching Google Scholar. A specific and appropriate search strategy was used for each database. We used the following searching terms: "Krebs von den Lungen-6" OR "KL-6" AND "SARS-CoV-2" OR "COVID-19". Search results were managed using EndNote software (version X7; Thomson Reuters). Additionally, reference lists of relative articles were also reviewed.

2.2. Study Selection

We included original studies that report the Krebs von den Lungen-6 levels among patients with COVID-19 on at least one or more of the following outcomes such as COVID-19 severity. Original articles available in English were included. The exclusion criteria for the meta-analysis were as follows: (1) studies involving data from pediatric patients; (2) case reports, editorial, conference papers, reviews; (3) studies published in other than English language; (4) studies lacking research indicators required for meta-analysis.

Two reviewers (L.S. and M.P.), independently and in duplicate, screened the titles and abstracts of the studies retrieved by the databases against the search criteria. Afterwards, the full texts of all potentially relevant articles were retrieved and independently assessed by the same reviewers. If any disagreement arose regarding the selection of literature papers disagreement was resolved through discussion with another reviewer (A.D.).

2.3. Data Extraction

Two investigators (L.S. and M.P.) performed study selection independently to select studies that met the above inclusion criteria. If potential disagreement arose, data extraction was resolved through discussion with another reviewer (A.D.). Data were collected using a predefined form. Data extracted included details regarding the publication data (i.e., first author name, year of publication, study design), population data (i.e., number of participants, age, male sex), KL-6 levels in predefined groups (COVID-19 positive and negative patients; mild and moderate COVID-19 severity groups; severe and non-severe COVID-19).

2.4. Quality and Risk of Bias Assessment

Two reviewers (M.P. and A.D.) independently assessed the individual studies for risk of bias. Any disagreements were also resolved by discussion with the third reviewer (L.S.). We used the Newcastle–Ottawa scale (NOS) to assess the methodological quality of observational studies with its design [16]. NOS score was categorized into three levels: low, moderate, and high quality, with the NOS scores of 0–5, 6–7, and 8–9. We performed funnel plot tests for asymmetry to investigate potential publication bias if there were more than 10 trials in a single meta-analysis.

2.5. Statistical Analysis

The meta-analysis was conducted in accordance with the Cochrane handbook. We analyze data using the STATA 14 software (StataCorp LP, College Station, TX, USA) and the RevMan 5.4 software (The Nordic Cochrane Center, The Cochrane Collaboration, 2014, Copenhagen, Denmark). For assess the KL-6 levels, we used mean differences (MDs) as the effect measure with 95% confidence intervals (CIs). In the case when KL-6 levels were reported as median with interquartile range, estimated means and standard deviations with the formula described by Hozo were used [17]. Heterogeneity was quantitatively assessed

using Cochran's Q statistics and Higgins's index (I^2), with 25%, 50%, and 75% considered moderate, substantial, and considerable heterogeneity, respectively [18]. The random-effects model was used for $I^2 > 50\%$; otherwise, the fixed effects model was employed. The Egger's test was used to provide quantitative evidence. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Study Characteristics

Our electronic literature search yielded 109 potentially relevant articles and one article was identified by hand searching. After elimination of duplicates, 88 records remained. Subsequent screening of titles and abstracts of the remaining records led to exclusion of 65 irrelevant records, leaving 23 articles. These articles were re-evaluated based on full-text contents, resulting in exclusion of 8 articles. Finally, 15 studies met the inclusion criteria and were included in our meta-analysis (Figure 1) [5,8,12,14,19–29]. All selected studies were published between 2020 and 2022. Detailed characteristics of the patients included in the meta-analysis is presented in Table 1.

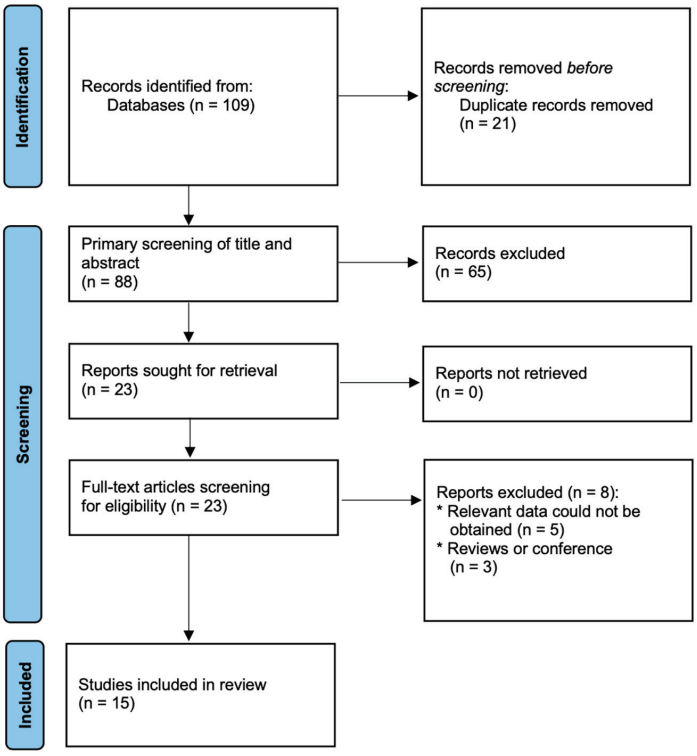

Figure 1. Flow chart of literature search and selection.

Six studies reported KL-6 levels among COVID-19 positive vs. negative patients, eleven studies among severe vs. non-severe COVID-19 patients and three studies compared KL-6 levels between mild vs. moderate COVID-19 severity. Of the fifteen trials, six were performed in China, four in Japan, three in Italy, and one in each of the following countries: Belgium and Indonesia. The NOS scores of the eight included studies were $\geq$8 (Table 1).

Table 1. A summary of study characteristics.

Study	Country	Study Group	No. of Participants	Age (Years)	Sex, Male (*n*, %)	NOS Score
Anastasi et al., 2022 [12]	Italy	COVID-19 (+)	37	66.5 ± 6.08	18 (48.6%)	8
		COVID-19 (−)	26	71.81 ± 7.27	10 (38.5%)	
Awano et al., 2020 [8]	Japan	Severe	21	65.5 ± 6.36	15 (71.4%)	9
		Non-severe	33	40.75 ± 4.91	23 (69.7%)	
Bergantini et al., 2021 [5]	Italy	Severe	10	65.2 ± 8	8 (80.0%)	8
		Non-severe	14	62.2 ± 15.6	11 (78.6%)	
		COVID-19 (−)	30	59 ± 9.8	18 (60.0%)	
Chen et al., 2021 [14]	China	Mild	37	NS	NS	8
		Moderate	298	NS	NS	
		Severe	29	NS	NS	
d'Alessandro et al., 2020 [19]	Italy	Severe	12	63 ± 2.34	9 (75.0%)	8
		Non-severe	10	60.75 ± 3.71	6 (60.0%)	
Deng et al., 2021 [20]	China	Severe	17	57.75 ± 4.35	9 (52.9%)	9
		Non-severe	149	48.13 ± 7.94	65 (43.6%)	
Frix et al., 2020 [21]	Belgium	COVID-19 (+)	83	71 ± 4	52 (62.6%)	8
		COVID-19 (−)	70	58 ± 3	35 (50.0%)	
He et al., 2021 [22]	China	COVID-19 (+)	28	64.56 ± 1.55	14 (50.0%)	8
		COVID-19 (−)	25	64.93 ± 1.63	16 (64.0%)	
Peng et al. 2021 [23]	China	Mild	49	44.5 ± 14	25 (51.0%)	9
		Moderate	28	51 ± 13.86	12 (42.9%)	
		Severe	36	56.5 ± 16.74	24 (66.7%)	
		COVID-19 (−)	65	47.75 ± 13.56	28 (43.1%)	
Saito et al., 2020 [24]	Japan	COVID-19 (+)	12	65.1 ± 10.7	7 (58.3%)	9
		COVID-19 (−)	34	49.6 ± 15.7	14 (41.2%)	
Suryananda et al., 2021 [25]	Indonesia	Severe	57	50.5 ± 13.85	38 (66.7%)	9
		Non-severe	18	49.75 ± 15.59	8 (44.4%)	
Wang et al., 2021 [26]	China	Severe	12	NS	NS	8
		Non-severe	52	NS	NS	
Xue et al., 2021 [27]	China	Severe	63	61.38 ± 4.19	31 (49.2%)	8
		Non-severe	226	54.75 ± 4.17	99 (43.8%)	
Yamada et al., 2022 [28]	Japan	Severe	27	64.25 ± 6.05	21 (77.8%)	8
		Non-severe	108	47 ± 12.85	48 (44.4%)	
Yamaya et al., 2021 [29]	Japan	Severe	60	NS	NS	8
		Non-severe	296	NS	NS	

Legend: NS: not specified.

3.2. KL-6 Meta-Analysis

Pooled analysis of KL-6 levels among COVID-19 positive vs. negative patients varied and amounted to 443.37 ± 249.33 vs. 205.73 ± 86.8 U/mL (MD = 275.33; 95%CI: 144.57 to 406.09; $p < 0.001$; Figure 2).

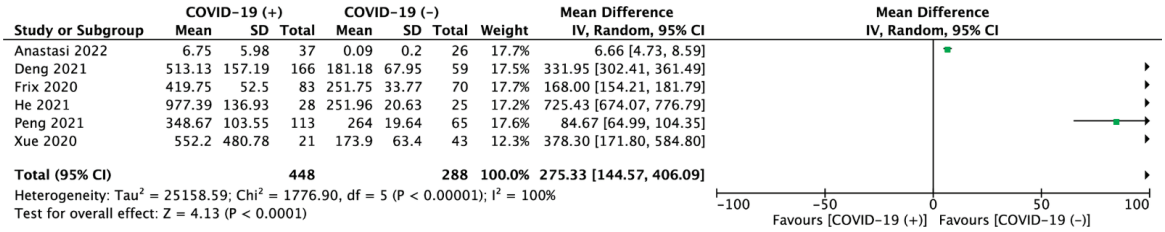

Figure 2. Forest plot of Krebs von den Lungen-6 levels (U/mL) among COVID-19 positive vs. negative patients. The center of each square represents the mean differences for individual trials, and the corresponding horizontal line stands for a 95% confidence interval. The diamonds represent pooled results [12,20–23,27].

12 studies reported KL-6 levels in severe and non-severe COVID-19 patients. Pooled analysis showed that KL-6 level was 402.82 ± 261.16 U/mL in severe group and was statistically significantly higher than in non-severe group (297.38 ± 90.46 U/mL; MD = 192.45; 95%CI: 118.19 to 266.72; $p < 0.001$; Figure 3).

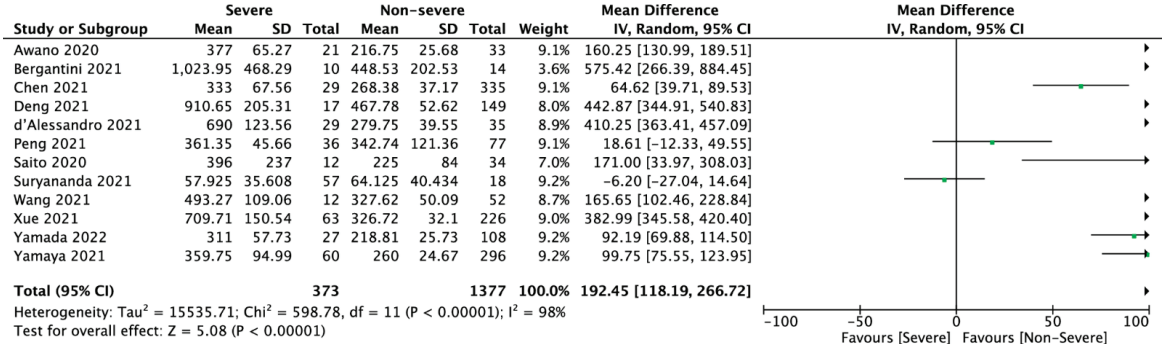

Figure 3. Forest plot of Krebs von den Lungen-6 levels (U/mL) among severe and non-severe COVID-19 patients. The center of each square represents the mean differences for individual trials, and the corresponding horizontal line stands for a 95% confidence interval. The diamonds represent pooled results [5,8,14,19,20,23–29].

Three studies compere KL-6 marker among mild and moderate COVID-19 patient groups. KL-6 in mild group was 272.28 ± 95.42 U/mL, compared to 268.04 ± 55.04 U/mL in moderate COVID-19 group (MD = −12.58; 95%CI: −21.59 to −3.57; $p = 0.006$; Figure 4).

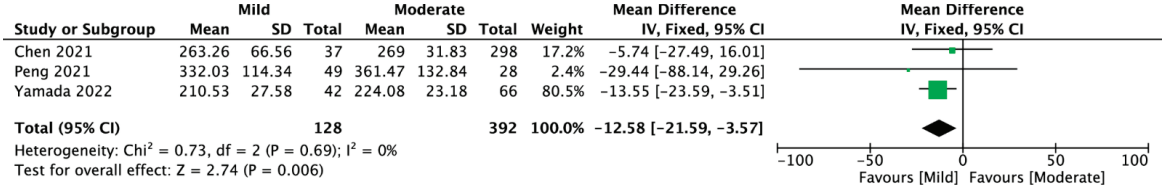

Figure 4. Forest plot of Krebs von den Lungen-6 levels (U/mL) among mild and moderate COVID-19 patient groups. The center of each square represents the mean differences for individual trials, and the corresponding horizontal line stands for a 95% confidence interval. The diamonds represent pooled results [14,23,28].

Sensitivity analysis based on the leave-one-out analysis showed that the pooled results were not influenced by a single trial. The above dependence applied to all comparisons included in the meta-analysis.

4. Discussion

We found that high levels of KL-6 are highly correlated with severe courses of COVID-19, and thus this marker may have the potential to be an excellent tool for the early identification of patients most likely to benefit from early antiviral therapy. Conversely, low KL-6 levels were associated with a mild disease course, and if validated, may be a useful tool in predicting a population who may be successfully managed in an outpatient environment.

The primary target of SARS-CoV-2 is definitely the lungs. Post-mortem studies have shown that the virus causes diffuse alveolar damage. In addition, COVID-19 has a higher incidence of thrombosis in the pulmonary vasculature compared to ARDS from other causes [30]. This is believed to be a function of the fact that SARS-CoV-2 uses the ACE2 receptor, and subsequently the Toll-like receptor, to invade pneumocytes and replicate its genome. Because KL-6 is a glycoprotein released by the type II alveolar pneumocytes and bronchial epithelial cells in various pulmonary diseases [30], the injury of pneumocytes and alveoli in COVID-19 may be pathophysiologically associated with elevated levels of KL-6 in the blood [31]. A disulfide link near the surface of the type II AECs' epithelial cell membrane may be disrupted as a result of the inflammatory storm, and KL-6 can subsequently diffuse into the fluid and blood flow of the pulmonary epithelial lining [32]. It should be noted that because KL-6 is secreted by lung cells, as opposed to other inflammatory markers such as CRP, which are associated with broad inflammation, it has a substantial advantage when compared to other proinflammatory cytokines [33]. Therefore, KL-6 with the predictive value would predict who will be more likely to experience the fibrosing gradually, which can also be very helpful in assessing the COVID-19 patient's condition, organizing the treatment for pulmonary fibrosis, or determining fibrosis following COVID-19 after the patient has been discharged from the hospital. This is crucial since 32–44.9% of individuals will develop lung fibrosis following COVID-19 [34,35]. Moreover, previous research has shown that the length of the illness plays a significant role in predicting the development of lung fibrosis following ARDS. About 4% of patients with diseases lasting less than a week, 24% of patients with diseases lasting between one and three weeks, and 61% of patients with diseases lasting longer than three weeks developed fibrosis [36].

Six studies in our meta-analysis compared KL-6 concentrations in COVID-19 cases and healthy subjects [12,20–23,27]. Results showed that KL-6 is significantly higher in COVID-19 than in healthy subjects. A previous meta-analysis evaluating KL-6 in COVID-19 positive and negative subjects also indicated significantly higher KL-6 levels in positive than in healthy subjects (standardized mean difference (SMD) = 1.34; 95%CI: 0.60 to 2.08) with high heterogeneity of data ($p < 0.001$, $I^2 = 93\%$) [22]. This indicates that SARS-CoV-2 infection causes an increase in KL-6, regardless of the symptoms caused, but the high heterogeneity of the data limits the usefulness of this information. Further studies are needed to obtain more homogeneous data.

Greater clinical value could be found in using KL-6 at admission to predict the subsequent course of COVID-19. Twelve studies involved in our meta-analysis assessed the KL-6 level according to the disease severity [6,8,14,19,20,23–29]. Our analysis demonstrated that there was a significantly higher level of KL-6 in patients suffering from severe COVID-19 than mild-to-moderate. Unfortunately, the heterogeneity of this data was high ($I^2 = 98\%$, $p < 0.00001$) decreasing the value of these findings. Nevertheless, our results are similar to those obtained in the previous meta-analyses. Ke et al., showed that serum KL-6 in patients with mild-to-moderate COVID-19 were significantly lower (SMD = −0.93; 95%CI: −1.22 to −0.65) than those in severe COVID-19 patients [37], although there was high heterogeneity of data. However, COVID-19 survivors had a significantly lower level of circulating KL-6 than non-survivors (SMD = −1.09; 95%CI: −1.63 to −0.55), and this analysis had low

data heterogeneity ($p = 0.52$, $I^2 = 0\%$) [38]. Likewise, a low heterogeneity meta-analysis, performed by Naderi et al. showed that KL-6 was significantly higher in patients with severe than non-severe COVID-19 (SMD = 1.25; 95%CI: 0.99 to 1.5; $p < 0.001$) [39]. Another meta-analysis conducted by Witarto et al. presented similar results: that patients with severe COVID-19 had a higher level of KL-6 than those with the non-severe disease (SMD = 1.16; 95%CI = 0.69 to 1.63) [40]. In this study, heterogeneity was considered low ($I^2 < 25\%$). Taking the above results into account, it can be concluded that higher levels of KL-6 are associated with a more severe course of COVID-19, and the data in the literature are rather consistent. The problem of the studies that reduce the value of the obtained results is the high heterogeneity of the data. It may be due to the difficulty in defining the severe and non-severe course of the disease.

Our analysis also involved a comparison of KL-6 levels in mild and moderate COVID-19. Three articles contained the necessary data and were included in this analysis [14,23,29]. The results indicate, with low heterogeneity ($I^2 = 0\%$, $p = 0.69$), that mild COVID-19 is characterized by significantly lower KL-6 levels than moderate COVID-19. It illustrates that KL-6 levels increase with the severity of the disease, and further studies are needed that would determine the cutoff points for each degree of disease severity.

The main limitation of our study is the observational type of studies included in the meta-analysis. This results in a significant level of bias risk. Moreover, the analysis showed significant heterogeneity in the data, making it necessary to treat the obtained results with caution. Finally, there is always a risk of publication bias caused by a greater tendency to publish substantial results [41].

KL-6 has a rising clinical role in the research, with over 250 studies employing its clinical potential in the clinical trials registry alone [42]. The role of KL-6 use has already been confirmed in other lung diseases, such as pulmonary fibrosis, interstitial lung disease, idiopathic pulmonary fibrosis, diffuse parenchymal lung disease, and many others [43,44]. However, in the case of COVID-19, current research are not studies of KL-6 itself but already using it in the clinic to estimate patient's lung function when testing new drugs or in patient care and clinical status. Detailed characteristics of studies using KL-6 for COVID-19 infection is presented in Table 2.

Table 2. Currently ongoing research on KL-6 in the context of COVID-19 disease.

ClinicalTrial identifer	Study name	Status	Purpose of using KL-6	Time Frame
NCT04816760 [45]	Immune Cells Phenotypes During COVID-19 (IMMUNO-COVID)	Recruiting	Serum alveolar epithelial and endothelial cells biomarkers during SARS-CoV-2 infection incl. measurement of KL-6 using ELISA.	Day 0, Day 7, Day 14, Day 28
NCT05074875 [46]	COVID-19 Respiratory Outcomes Registry	Active, not recruiting	Examine the effects of COVID-19 on the presence of molecular biomarkers associated with Interstitial Lung Disease. Biomarkers prognostic for progression in PF patients incl. Krebs von den Lungen-6 (KL-6). Biomarkers elevated in PF (vs age-matched controls) incl. Krebs von den Lungen-6 (KL-6).	72 weeks
NCT04392531 [47]	Clinical Trial to Assess Efficacy of cyclosporine Plus Standard of Care in Hospitalized Patients with COVID-19	Completed-No Results Posted	Change in KL-6 change from baseline in KL-6 levels	Days 1, 8, 15, and end of study visit (14 days after discharge or 14 days after end of study treatment)
NCT04390061 [48]	TOFAcitinib Plus Hydroxycloroquine vs Hydroxycloroquine in Patients With COVID-19 Interstitial Pneumonia (TOFACoV-2)	Unknown	Identification of predictors of outcome. Role of some clinical and laboratory factors in predicting outcome incl. KL-6.	14 days
NCT04541680 [49]	Nintedanib for the Treatment of SARS-CoV-2 Induced Pulmonary Fibrosis (NINTECOR)	Recruiting	Compare change in lung injury, pulmonary hypertension, and inflammation biomarkers. Biomarker assay (KL-6, NT-proBNP, CRP, D-dimers)	At inclusion and 12 months

5. Conclusions

Our meta-analysis indicates a significant association between increased KL-6 levels and SARS-CoV-2 infection. Moreover, KL-6 levels are significantly higher in patients with a more severe course of COVID-19, indicating that KL-6 may be a useful predictor to identify patients at risk for severe COVID-19. However, the high heterogeneity of the data warrants cautions in interpreting these results.

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Characteristics of Hospitalized Pediatric Patients in the First Five Waves of the COVID-19 Pandemic in a Single Center in Poland—1407 Cases

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Abstract: This is a single-center, prospective study that compared the clinical presentation and laboratory findings of hospitalized children during the first five waves of the COVID-19 pandemic. Data were collected, according to a standardized questionnaire, from 1407 children from 23 March 2020 to 30 April 2022. Significant differences in clinical courses were found among the five waves probably due to different SARS-CoV-2 variants. The median age was 95.8 months in the first wave versus 14.6–23 months in the others. The number of patients with upper respiratory infection was the highest in the fifth wave (74.4% versus 43.8–56.9% in the others) and for lower respiratory infection in the first wave (50.0% versus 16.4–32.5%). Gastroenterocolitis was more common in the fifth wave (24.4% versus 8.9–16.5%); neurological diagnoses appeared more frequently in the fourth wave (16.6% versus 0.6–9.9%), while anosmia and ageusia were higher in the fifth wave (13% versus 1.5–4%). Life-threatening courses were relatively rare. However, children with pneumonia, dehydration from high fever, gastrointestinal symptoms, loss of smell and taste, and neurological symptoms required hospitalization.

Keywords: COVID-19; SARS-CoV-2; children; pandemic; waves; hospitalization; clinical presentation

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1. Introduction

In February 2020, the World Health Organization (WHO) designated a new strain of betacoronavirus as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative agent of coronavirus disease 2019 (COVID-19) [1]. Fever, cough, and dyspnea were initially indicated as the presenting symptoms of SARS-CoV-2 infection. Severe pneumonia with respiratory failure reported on 31 December 2019 in the region of Wuhan, China, was the reason for hospitalization and life-threatening situations [2].

In the first reports of COVID-19, the frequency of disease in children appeared much lower than in adults. Both in China and in Italy, only 1% of cases were pediatric [2,3]. In the following months, the number of pediatric patients gradually increased. For instance, in the U.S., at the beginning of the pandemic, 2.2–4.2% of the reported cases were pediatric; then, according to reports from the American Academy of Pediatrics (AAP), the rate increased to 14.3% and in 10 states, children accounted for over 18% of cases [4,5]. Data from the European Centre for Disease Prevention and Control (ECDC) showed that up to 17.6% of cases were pediatric [6]. A lot of studies published have confirmed the clinical impression that COVID-19 in children typically presents as a mild (37%) or moderate (45%) upper respiratory tract infection and is rarely severe or critical [7]. Other signs and symptoms described in children include gastrointestinal, anosmia, ageusia, neurological, and dermatologic manifestations [2,3,7–17].

Numerous SARS-CoV-2 variants have circulated globally since the beginning of the pandemic, and differences in their courses have been reported [1,4,10–12,16–29]. The increasing number of pediatric cases and changing clinical disease presentations might require changes in COVID-19 management for children, risk group identification, testing criteria, and indications for hospitalization.

The aim of this paper was to describe the COVID-19 characteristics in hospitalized Polish children during the first five waves and to assess whether there were any differences among the different waves. In particular, the trends in the demographic data, clinical presentation, laboratory findings, and COVID-19 outcomes over two years of the pandemic were analyzed. Our observations may be useful for ongoing guidance for the evaluation, management, and prevention of COVID-19 in children.

2. Materials and Methods

Because of the WHO's announcement of a pandemic and the increasing cases of COVID-19 in Poland, the Department of Infectious Diseases and Pediatrics was instituted to be the central unit for treating pediatric COVID-19 cases in the southern region. The first two children were admitted to hospital on 23 March 2020, which is when this study commenced. Every patient from 0 to 18 years of age with confirmed COVID-19 hospitalized between March 2020 and April 2022 was included.

Following the recommendations from the WHO and the National Institute of Public Health [30,31], COVID-19 was diagnosed using a positive reverse transcription and real-time polymerase chain reaction (RT-PCR) test. Since 30 October 2020, second-generation antigen tests from a nasopharyngeal swab were performed in certified laboratories. Several kits were used: (1) GeneFinder™ COVID-19 Plus RealAmp, Elitech, Biomedica (Oxford, UK); (2) Liferiver, Novel Coronavirus (2019-nCoV) Real Time Multiplex; (3) VIASURE CerTest, Biotec (Zaragoza, Spain); (4) Maccura SARS-CoV-2 Fluorescent PCR, Maccura Biotechnology (Sichuan, China); (5) Homemade DIAGtest SARS-CoV-2 real time RT-PCR; (6) Labsystems Diagnostics (Vantaa, Finland). COVID-19 Real Time Multiplex RT-PCR and the second-generation Abbott Panbio-COVID-19 Ag Rapid Test Device (WHO laboratory 2020, AOTM).

Criteria for hospital admission were similar to other pediatric infection diseases, such as dehydration from fever, vomiting, and diarrhea. According to the Polish Ministry of Health [32], hospitalization was compulsory for every patient with diagnosed SARS-CoV-2 infection up to September 2020. According to Polish expert group recommendations, hospital referrals were also required for children with congenital heart defects, neurologic diseases, genetic disorders, chronic renal diseases, mucoviscidosis, broncho-pulmonary dysplasia, immunodeficiency after organ transplantation, and diabetes mellitus. Included also were newborns, infants, and children with obesity, especially with a body mass index (BMI) >30 kg/m^2 [33].

Discharge criteria were two negative PCR tests taken within 24 h. After 2 September 2020, the only criterion was the condition of the patient.

The disease severity assessment in this analysis was based on the need for oxygen, intravenous rehydration or steroids, and the length of stay. Antiviral therapy was also assessed. Systemic steroid and antiviral therapy were used according to the recommendations from the beginning of the pandemic [33–39] with the following changes: Dexamethasone was used according to the European Medicines Agency's (EMA) recommendations in hospitalized patients, especially in those treated with remdesivir at a dose of 0.1 mg/kg for a maximum of 4 mg/24 h [34]. Dexamethasone was also used in some patients with laryngitis according to references from previous studies [40,41].

Remdesivir was used in our department according to the Food and Drug Administration's (FDA) and EMA's recommendations [35,42]. According to the product characteristics, remdesivir was used in patients 12 years of age and older weighing at least 40 kg. It was also used in pediatric patients weighing at least 3.5 kg with positive results for direct SARS-CoV-2 testing with pneumonia and requiring oxygen supplementation. Baricitinib

was used in patients aged 2–18 years who required non-invasive or invasive mechanical ventilation with recommended dosages under the Emergency Use Authorization (EUA): for patients aged nine years or older, 4 mg once daily, and for those aged two to less than nine years, 2 mg once daily [43]. Data were collected and reported by the physicians working in the department according to a standardized case history questionnaire and a physical examination for every patient. Symptoms were recorded at the time of hospitalization. Standard laboratory tests were conducted for every child diagnosed with COVID-19.

All patients included in the study were symptomatic. The questionnaire included:

1. Demographic data: age, sex, ethnicity, recent contact with patients with COVID-19, and comorbidities (e.g., heart, chronic lung, neurological, or genetic diseases; asthma; developmental delay; diabetes; immunodeficiency, or malignancy).
2. Signs and symptoms: fever, cough, rhinitis, dyspnea, sore throat, weakness, diarrhea, abdominal pain, vomiting, headache, conjunctivitis, nausea, myalgia, rash, ageusia, anosmia, chest pain, or irritability.
3. Disease outcome data: length of hospitalization, complications, oxygen treatment, casual treatment, pediatric intensive care unit (PICU) admission, or death.
4. Laboratory data: complete blood count (CBC) parameters, C-reactive protein (CRP), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatinine kinase (CK), ferritin, vitamin D3 level, prothrombin time, D-dimers, nasal swabs for other viral pathogens (co-infection), and imaging (i.e., lung ultrasound (LU), chest X-ray, and high-resolution computed tomography (HRCT)).
5. Final diagnoses: Upper or lower respiratory tract infection, gastroenterocolitis, or neurological diagnoses.

Lower respiratory infections were diagnosed based on clinical presentation and LU, chest X-ray, and HRCT. The examination taken most often, especially in the youngest children, was LU. The presence of focal, multifocal, and confluent B lines and pleural irregularities were the most common LU findings for diagnosing pneumonia from COVID-19. In chest X-ray examinations, bilateral and multifocal lesions were found most frequently, especially in the lower lobes. The pure ground-glass appearance was also typical for COVID-19 lower respiratory-related findings [44–46]. Regarding gastrointestinal infection, diagnosis was based on clinical presentation (i.e., vomiting or diarrhea) and the exclusion of any other etiology such as rotavirus, adenovirus, and norovirus.

Statistical analysis was performed using SPSS ver. 27 software (Armonk, NY, USA). The results are presented based on the parameters of descriptive statistics, including either the mean value and standard deviation (SD) for the quantitative variables with normal distribution or the median value with the interquartile range in the opposite case. Categorical variables are presented as numbers with percentages. Qualitative values were compared using the chi-squared test. For the analysis of continuous variables, a Kruskal–Wallis test was used. In all cases of statistical significance, a pairwise comparison between groups was performed using a post hoc test. In all of the analyses, $p < 0.05$ was considered statistically significant.

This study was performed in accordance with the ethical standards of the Declaration of Helsinki and its later amendments. It was approved by the Ethics Committee of the Regional Medical Chamber in Krakow, No. OIL/KBL/18/2020, on 10 March 2020.

3. Results

We compared the data characteristics of those children and adolescents admitted with acute COVID-19 during the first five waves of the pandemic.

3.1. Study Groups

This study comprised 1407 patients: 112 (8%) from the first wave (1 March to 30 September 2020); 175 (12.4%) from the second (1 October 2020 to 31 January 2021); 195 (13.8%) from the third (1 February to 31 May 2021); 511 (36.3%) from the fourth (1 October

2021 to 15 January 2022); 414 (29.5%) from the fifth (16 January to 30 April 2022) (Figure 1). All but one of the children were white European; the other was of Asian background.

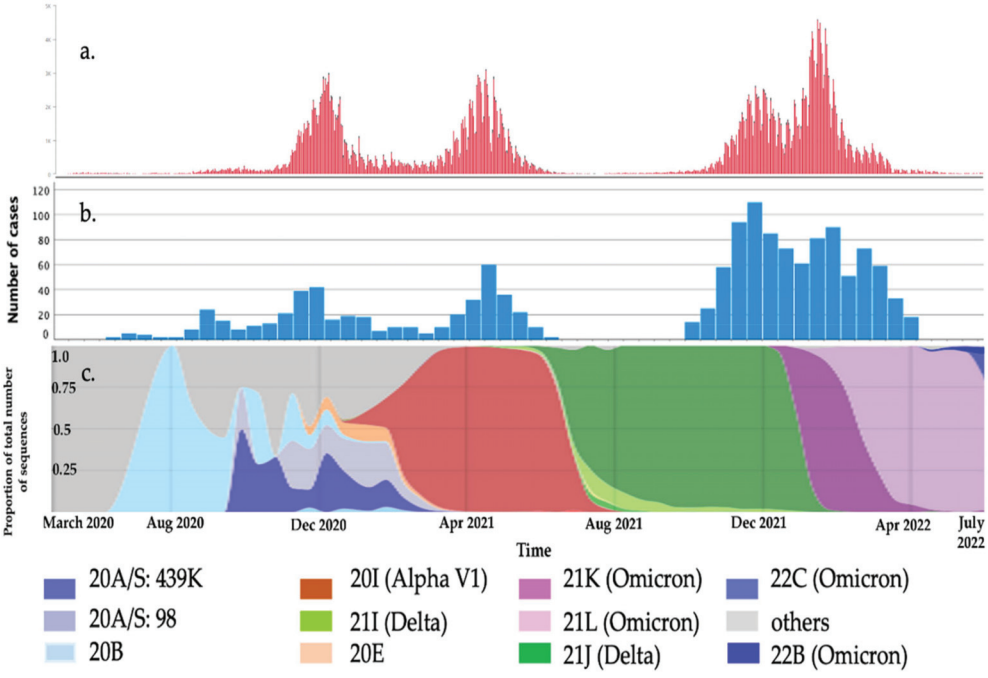

Figure 1. COVID-19 cases in our region: (**a**). Daily COVID-19 cases in the Malopolska region based on [47]. (**b**). Number of hospitalized children with COVID-19. (**c**). The proportion of the total number of SARS-CoV-2 variants over time in Poland based on [48].

Table 1 shows the demographic characteristics of the hospitalized patients.

Table 1. Demographic characteristics of those COVID-19 pediatric patients hospitalized during the first five waves of the pandemic.

	First Wave *n* = 112	Second Wave *n* = 175	Third Wave *n* = 195	Fourth Wave *n* = 511	Fifth Wave *n* = 414	*p*-Value	Post Hoc Analysis
Male sex, *n* (%)	57 (50.9)	99 (56.6)	100 (51.3)	258 (50.7)	228 (55.1)	0.569 *	
Age, months Median (25th–75th percentile)	95.8 (17–130)	23.0 (5.7–85)	20.1 (5.4–55)	17.6 (5.3–68)	14.6 (5.4–43)	<0.001 #	1 vs. 2: <0.001 1 vs. 3: <0.001 1 vs. 4: <0.001 1 vs. 5: <0.001
Patients with chronic diseases, *n* (%)	39 (34.8)	37 (21.1)	63 (32.3)	111 (21.7)	107 (26.8)	0.06 *	
Immunocompromised patients, *n* (%)	2 (1.9)	6 (3.5)	5 (2.6)	4 (0.8)	4 (1.0)	0.064 *	
BCG vaccinated patients, *n* (%)	97 (96)	159 (98)	178 (98)	483 (96)	399 (97)	0.59 *	

* Chi-squared test; # Kruskal–Wallis ANOVA.

In all waves, more boys than girls were hospitalized (from a low of 50.5% in the fourth wave to a high of 56.6% in the second), with no statistical significance between waves. The median age was the highest in the first wave (95.8 months) and significantly lower in others, decreasing in the following waves: 23 months in the second, 20.1 months

in the third, 17.6 months in the fourth, and 14.6 months in the fifth ($p < 0.001$). Chronic comorbidities, which related to high risk of severe COVID-19 were present in 21.1% of patients in the second wave to 34.8% in the first, and there were no statistically significant differences in the comorbidity frequency between waves ($p = 0.06$).

3.2. Clinical Presentation

The clinical presentation of pediatric COVID-19 during the first five waves of the pandemic is shown in Table 2.

Table 2. Clinical characteristics of those COVID-19 pediatric patients hospitalized during the first five waves of the pandemic.

	First Wave *n* = 112	Second Wave *n* = 175	Third Wave *n* = 195	Fourth Wave *n* = 511	Fifth Wave *n* = 414	*p*-Value *
Fever, *n* (%)	76 (69)	117 (67)	146 (75)	347 (68)	296 (71)	0.327
Rhinitis, *n* (%)	24 (21)	48 (28)	78 (40)	266 (52)	193 (47)	<0.001
Cough, *n* (%)	42 (38)	62 (36)	119 (61)	326 (64)	221 (53)	<0.001
Dyspnea, *n* (%)	11 (10)	13 (7.4)	24 (12)	73 (14)	46 (11)	0.143
Vomiting, *n* (%)	7 (6.3)	37 (21)	27 (14)	91 (18)	126 (30)	<0.001
Diarrhea, *n* (%)	22 (20)	54 (31)	39 (20)	91 (18)	105 (25)	0.002
Anosmia, *n* (%)	3 (2.7)	7 (4)	3 (1.5)	8 (1.6)	54 (13)	<0.001
Ageusia, *n* (%)	2 (1.8)	5 (2.9)	3 (1.5)	10 (2)	54 (13)	<0.001
Neurologic symptoms, *n* (%)	15 (14)	19 (11)	9 (4.6)	52 (10)	38 (9.2)	0.077

* Chi-squared test.

The most frequent symptom in all waves was fever. (68% in the fourth wave to 75% in the third), with no statistically significant differences between waves. The fever was defined as a temperature above 37.5 °C (99.5 °F) in axillary, ear, and forehead temperature measurements. In the case of respiratory symptoms, rhinitis was most frequently reported in the fourth wave (52% of patients), and significantly the least in the first (21%), whereas cough was most common in the third (61%) and fourth waves (64%) ($p < 0.001$). Dyspnea was a relatively rare symptom, although the study included only hospitalized patients—7.4% in the second wave to 14% in the fourth, with no statistically significant differences between waves.

In the case of gastrointestinal symptoms, vomiting was the rarest in the first wave (6.3%) and the most common in the fifth (30%) ($p < 0.001$), and diarrhea was the most common in the second wave (31% versus 18–25% in the others) ($p = 0.002$).

Anosmia and ageusia, the most specific COVID-19 symptoms, were rare in the children in the first four waves (1.5–4% of children), but the frequency of these symptoms was much higher in the fifth wave (13%) ($p < 0.001$).

Neurological manifestations (seizures and impaired coordination and balance) appeared in 4.6% of the patients in the third wave to 14% of the patients in the first wave, and the differences between waves were statistically insignificant. Table 2 shows the symptoms by wave.

3.3. Laboratory Findings

The laboratory findings from the children during the first five waves of the pandemic are shown in Table 3. There were no statistical differences between the groups at the CRP level or in the number of neutrophils, but there were differences between the waves in seven parameters. The number of leukocytes was the lowest in the first wave (median of $6.4 \times 10^3/\mu L$) ($p < 0.001$), similar in the others ($7.75–8.9 \times 10^3/\mu L$). The number of lymphocytes was also the lowest in the first (median of $2.3 \times 10^3/\mu L$ vs. 3.49, 3.96, 3.61, and $3.39 \times 10^3/\mu L$ ($p < 0.001$) in waves 2–5, respectively). The first-wave patients also had the lowest platelet count (median of 247 vs. 309, 303, 279, and $281 \times 10^3/\mu L$ in waves 2–5, respectively ($p < 0.001$). There were also differences in alanine transaminase and creatinine

kinase ($p < 0.001$), but the post hoc analysis revealed them to be significantly higher in the fifth wave.

Table 3. Laboratory findings in those COVID-19 pediatric patients hospitalized during the first five waves of the pandemic according to the Kruskal–Wallis and chi-squared tests.

	First Wave $n = 112$	Second Wave $n = 175$	Third Wave $n = 195$	Fourth Wave $n = 511$	Fifth Wave $n = 414$	p-Value	Post Hoc Analysis
CRP (mg/dL) (normal value = 0–5 mg/dL)	2.05 (1–9)	2.1 (1–12.6)	2.05 (1–14)	2.9 (1–10)	3.7 (1–11)	0.11 #	
CRP > 5 mg/dL	37 (38)	57 (38)	67 (36)	187 (38)	160 (41)	0.799 *	
Leukocytes ($10^3/\mu L$) (normal value = 6–10 × $10^3/\mu L$)	6.4 (5–8.1)	8.2 (6–11.6)	8.9 (6.1–12.5)	8.0 (5.8–11)	7.75 (5.9–11.2)	<0.001 #	1 vs. 2: <0.001 1 vs. 3: <0.001 1 vs. 4: <0.001 1 vs. 5: <0.001
Leukocytes ($10^3/\mu L$)							
<4.5	18 (18)	19 (13)	19 (10)	59 (12)	35 (9)		
4.5–13.5	79 (78)	106(69)	131(69)	368 (75%)	301(37)		
>13.5	4 (4)	27 (18)	39 (21)	63 (13)	54 (14)		
Neutrophils ($10^3/\mu L$) (normal value = 1.5–7 × $10^3/\mu L$)	2.89 (1.8–4.2)	2.62 (1.2–4.7)	2.62 (1.4–4.9)	2.59 (1.6–4.7)	2.7 (1.6–4.8)	0.925 #	
Neutrophils ($10^3/\mu L$)							
<1.0	7 (8)	26 (18)	29 (16)	65 (13)	45 (12)	0.016 *	
1.0–6.5	79 (87.6)	95 (64)	119 (68)	348 (74)	284 (73)		
>6.5	4 (4.4)	26 (18)	28 (16)	64 (13)	57 (15)		
Lymphocytes ($10^3/\mu L$) (normal value = 2.5–8.5 × $10^3/\mu L$)	2.3 (1.5–3.2)	3.46 (2.1–5.3)	3.96 (2.4–6.4)	3.61 (2.1–5.7)	3.39 (1.7–5.7)	<0.001 #	1 vs. 2: <0.001 1 vs. 3: <0.001 1 vs. 4: <0.001 1 vs. 5: <0.001
Lymphocytes ($10^3/\mu L$)							
<1.0	10 (11)	12 (8)	6 (3.4)	25 (5.3)	43 (11)	<0.001 *	
1.0–7.0	78 (26)	117 (80)	139 (77)	381 (80.4)	297 (75)		
>7.0	3 (3)	17 (12)	33 (19)	68 (14.3)	48 (24)		
Blood platelets ($10^3/\mu L$) (normal value = 210–560 × $10^3/\mu L$)	247 (192–298)	309 (247–411)	303 (243–360)	279 (215–363)	281 (222–351)	<0.001 #	1 vs. 2: <0.001 1 vs. 3: <0.001 1 vs. 4: 0.005 1 vs. 5: 0.01
Blood platelets < 100 × $10^3/\mu L$	4 (4)	3 (2)	3 (1.6)	10 (2)	3 (0.8)	0.265 *	
Alanine transaminase (U/L) (normal value = 0–55 U/L)	16 (12–24)	18 (12–28)	19 (13–29)	20 (13–29)	22 (15–32)	<0.001 #	5 vs. 1: <0.001 5 vs. 2: 0.015 5 vs. 3: 0.03 5 vs. 4: 0.017
Alanine transaminase >55 (U/L)	3 (3.2)	9 (6.3)	8 (4.5)	21 (4.5)	33 (8)	0.065 *	
Creatinine kinase (U/L) (normal value = 30–170 U/L)	80 (55–114)	84 (63–125)]	108 (71–157)	94 (65–146)	115 (83–168)	<0.001 #	5 vs. 1: <0.001 5 vs. 2: <0.001 5 vs. 4: <0.001
Creatinine kinase							
170 (U/L)	5 (9.4)	12 (12.5)	31 (19)	66 (15.3)	82 (24)	0.005 *	
Lactate dehydrogenase (IU/L) (normal value = 125–220 (U/L)	238.5 (192–293)	268.5 (210–311)	286.0 (236–323)	272.0 (221–313)	293.0 (252–333)	<0.001 #	1 vs. 3: <0.001 1 vs. 4: 0.023 1 vs. 5: <0.001 5 vs. 2: 0.001 5 vs. 4: <0.001
Lactate dehydrogenase >220 IU/L	51 (57)	101 (70)	148 (88)	316 (85)	280 (86)	<0.001 *	
D-dimers (ng/mL) (normal value = 0–500 ng/mL)	407 (256–694)	582 (377–1142)	471 (294–908)	559 (331–1082)	611 (397–1065)	<0.001 #	1 vs. 2: 0.004 1 vs. 4: 0.004 1 vs. 5: <0.001
D-dimers > 500 ng/mL	33 (38)	80 (60)	72 (47)	205 (54)	184 (61)	<0.001 *	

* Chi-squared test, data are presented as n (%); # Kruskal–Wallis ANOVA, data are presented as the median (25th–75th percentile).

The Kruskal–Wallis test showed significant differences between the groups in LDH level ($p < 0.001$); however, in the post hoc analysis, the first and fifth wave groups differed from the others. D-dimers were significantly lower in the first wave versus the second, fourth, and fifth waves ($p < 0.001$). In the chi-squared test, significant differences were found in the leukocyte ($p = 0.003$) and lymphocyte levels ($p < 0.001$), creatinine kinase ($p = 0.005$), and lactate dehydrogenase and D-dimers ($p < 0.001$).

3.4. COVID-19 Severity

The COVID-19 severity data are included in Table 4. Oxygen therapy was required in 0% (first wave) to 4% (fourth wave) of the patients and there were no statistically significant differences between the five waves ($p = 0.071$). Differences were found in the need for intravenous rehydration—most common in the fifth wave (59%) and least in the third wave (9%) ($p < 0.001$). Systemic steroid therapy was used the least in the second wave (1.1%) and the most in the fourth wave (11.2%) ($p < 0.001$). The length of stay was significantly shorter in the fifth wave (median of three days). The post hoc analysis revealed differences between the fifth and all other waves ($p < 0.001$). Only one (0.5%) patient in the third wave and two (0.3%) in the fourth were referred to a PICU, but no one died. One patient in our department needed high-flow nasal oxygen therapy (HFNOT). Nine (1.7%) patients in the fourth wave were treated with remdesivir (0.64% during the whole pandemic) and 1 (0.19%) with baricitinib according to FDA and EMA recommendations [25,30,31]. Two (0.4%) patients in the fifth wave were treated with baricitinib (0.21% during the whole pandemic).

Table 4. COVID-19 outcomes in those hospitalized pediatric patients during the first five waves of the pandemic.

	First Wave *n* = 112	Second Wave *n* = 175	Third Wave *n* = 195	Fourth Wave *n* = 511	Fifth Wave *n* = 414	*p*-Value	Post Hoc Analysis
Oxygen therapy, *n* (%)	0	2 (1.1)	8 (4)	19 (4)	9 (2.2)	0.071 *	
Intravenous fluids, *n* (%)	24 (21)	62 (35)	18 (9)	243 (48)	243 (59)	<0.001 *	
Steroid therapy, *n* (%)	7 (6)	2 (1.1)	18 (9)	57 (11.2)	20 (4.8)	<0.001 *	
Antiviral therapy, *n* (%)	0	0	0	10 (1.9) (remdesivir—9, baricitinib—1)	2 (0.4) (baricitinib—2)	0.67 *	
Length of stay, *n* (days) Median (25th–75th percentile)	4 (2–6)	4 (3–5)	3 (2–6)	4 (3–5)	3 (2–4)	<0.001 #	5 vs. 1: <0.001 5 vs. 2: <0.001 5 vs. 3: <0.001 5 vs. 4: <0.001

* Chi-squared test; # Kruskal–Wallis ANOVA.

3.5. Final Diagnoses

Because of the overlap, there were 1862 diagnoses in the 1407 patients, of whom 235 (16.7%) had more than one final diagnosis: urinary tract infection (UTI) combined with gastroenterocolitis, pneumonia and gastroenterocolitis, and upper respiratory tract infection and seizures or suicide attempts. In the first and second waves, there were 1.2 diagnoses per patient, but that increased in the following waves to 1.26 in the third, 1.35 in the fourth, and 1.39 in the fifth. The average for the whole period was 1.32. This means that through subsequent waves the symptomatology of COVID-19 in children was becoming richer.

The most common final diagnoses were upper respiratory, lower respiratory, and gastrointestinal infections (Table 5). Upper respiratory infections were the most common in the fifth wave (74.3%) and the least in the first wave (43.8%) ($p < 0.001$). Rhinitis and laryngitis were reported the most frequently. Lower respiratory infections were diagnosed based on clinical presentation and LU, chest X-ray, and HRCT. Lung imaging data from the children during the first five waves of the pandemic is shown in Table 6. It was the most common in the first wave (50%) and least common in the fifth wave (16.4%) ($p < 0.001$), whereas gastroenterocolitis was the most frequent in the fifth wave (24.4%) and the least in the first wave (8.9%). Significant differences were observed between the five waves in

the frequency of neurological diagnoses, especially between the second (0.6%) and fourth (16.6%) waves ($p < 0.001$).

Table 5. Final diagnoses during the first five waves of the pandemic.

	First Wave *n* = 134	Second Wave *n* = 212	Third Wave *n* = 247	Fourth Wave *n* = 694	Fifth Wave *n* = 57	*p*-Value
Upper respiratory tract infection	49 43.8%	99 56.9%	95 49.0%	277 54.6%	304 73.4%	<0.001
Lower respiratory tract infection	56 50.0%	53 30.5%	63 32.5%	162 32.0%	68 16.4%	<0.001
Gastroenterocolitis	10 8.9%	26 15.1%	32 16.5%	68 13.4%	101 24.4%	<0.001
Neurological diagnoses	5 4.5%	1 0.6%	16 8.2%	84 16.6%	41 9.9%	<0.001
Other	14 12.5%	33 19.1%	41 21.1%	103 20.3%	61 14.7%	0.07

n, number of diagnoses. Data are presented as *n* (%); *p*-value for chi-squared test.

Table 6. Lung imaging in COVID-19 pediatric patients hospitalized during the first five waves of the pandemic.

	First Wave *n* = 112	Second Wave *n* = 175	Third Wave *n* = 195	Fourth Wave *n* = 511	Fifth Wave *n* = 414
Chest X-ray, *n*	52	33	33	265	71
Positive X-ray, *n (%)*	24 (46)	25 (76)	33 (100)	149 (56)	51 (72)
Lung ultrasound, *n*	7	37	42	256	212
Positive lung ultrasound, *n (%)*	1 (14)	28 (76)	37 (88)	134 (52)	54 (18)
HRCT, *n*	0	0	2	11	0
Positive HRCT, *n* (%)	0	0	2 (100)	11 (100)	0

4. Discussion

To the best of our knowledge, this is the largest single-center study of children hospitalized due to COVID-19 and the first one comparing clinical presentations in children during the first five waves of the pandemic. Although children are considered to be less affected [12,18,19,49–51], 1407 were hospitalized between 23 March 2020 and 30 April 2022. This might have been due to the higher prevalence of SARS-CoV-2 in our local community and the central organization of hospital care in our region. The first wave of the pandemic was very mild in Poland because of the strict lockdown in the spring of 2020, which means that the relatively high number of hospitalized children in the first wave was the result of mandatory hospitalization for every infected SARS-CoV-2 patient [32] (Figure 1).

4.1. Demographic Characteristics

The demographic characteristics of the patients were similar in all five waves. There were no significant differences in sex, but there was a slight male predominance, as in other studies [15,16,20,50,52–54].

The ages of our patients were of particular interest. The median age in the first wave (95.8 months) was higher compared to the others (14.6–23 months). Similarly, infants aged zero to six months represented 26–29% of patients from the second to fifth waves. Other authors have reported the prevalence of both younger [2,15–17,54] and older children [8]. For example, Turan et al. [16] revealed the prevalence of younger children in the second wave compared to the first. It should be noted that, to the best of our knowledge, there has not been such a large study of the prevalence of children with COVID-19 at such a young

age. This can be explained by outbreaks of COVID-19 in large neonatal departments and the referral to our department of children at risk of a severe course of COVID-19. The Polish expert group recommendations also indicate the necessity of hospitalizing the youngest children [33]. It is noteworthy that our study included only hospitalized children.

4.2. Clinical Presentation

Though SARS-CoV-2 infection was common in children, the course of the disease was usually milder than for adults [12,18,19,49–51]. In our department, severe courses of the disease were rare, and there were no significant differences in severity over the five waves, although we did observe increased hospitalizations in the fourth and fifth waves. Similar observations regarding increasing numbers of hospitalization for the delta and omicron variants were reported by Marks et al. and Shi et al. [21–23]. However, we found significant differences in their clinical presentations. Similar observations have been reported by other authors [9,13,14,24–28,33,50].

The basic differences in the clinical presentation were the frequency of respiratory symptoms (rhinitis, cough, dyspnea, auscultatory changes, and lower respiratory infection), which increased from the second to the fourth waves. In contrast, gastrointestinal symptoms (vomiting and diarrhea) were the most common in the second wave. Other authors have reported fever and cough as the most frequent early symptoms [9–12]. During the predominance of the delta and omicron variants, upper respiratory tract symptoms (rhinitis and sore throat) were more common [29].

Anosmia and ageusia, the most significant symptoms of COVID-19, were very rare in the children: Fewer than 4% of the patients in our study, which differed significantly from previous reports. Most authors have emphasized that anosmia and ageusia caused by the omicron variant appeared much less often in the fifth wave [29,55–57]. This might have been caused by the specific nature of our cohort—only hospitalized children, who showed a significant decrease in age from wave to wave (Table 1). In the fifth wave, the median age was 14.6 months. This was a special group of patients who might require hospitalization for dehydration resulting from the refusal to take fluids due to smell and taste disorders. In such cases, medical help was sought, as feeding the youngest children proved difficult. In older children and adults, smell and taste disorders did not usually require hospitalization. It is noteworthy that the results were also affected by the team's increasing experience in COVID-19 diagnosis in the youngest group of patients, who were unable to verbalize their ailments. It is also worth emphasizing that some authors have reported the frequency of smell disorders in the fifth wave of the pandemic as 12% and taste as 23%, which was more frequent than in our cohort (13% in both cases) [58].

Regarding the final diagnoses of the hospitalized COVID-19 pediatric patients, the number of children with upper respiratory or gastroenterological symptoms was the highest in the fifth wave, while that of lower respiratory infection was most common in the first wave. Interestingly, Pokorska-Śpiewak et al. [12] reported in their study that pneumonia was more common in the second than in the first wave, but this can be explained by lower testing for SARS-CoV-2 infection of asymptomatic or mildly symptomatic children in our region during the first wave. We observed more upper than lower respiratory infections and shorter lengths of stay in hospital in the fifth wave. A lot of publications support our study's finding of a milder course for the omicron-dominated fifth wave in both adults and children [59–62]. Marks and Shi reported that the proportions of hospitalized children requiring PICU or intensive mechanical ventilation were similar in the first four waves but lower in the fifth [21–23]. Nevertheless, although most of the patients who contracted the SARS-CoV-2 omicron variant exhibited milder clinical features, severe clinical features, including mortality, were encountered among individuals who were not vaccinated [63].

In our cohort, more neurological symptoms occurred in the fourth wave. Similarly, in London, Molteni compared the disease course during the alpha and delta variant predomi-

nance and found more neurological symptoms (headaches, dizziness, chills, anosmia, and ageusia) during the delta variant period [19].

Antoon et al., who analyzed only serious neurological complications and those of clear significance (seizures, strokes, and encephalopathy), also reported that the most common neurological diagnoses occurred in the delta variant period (37.8%), while during the alpha and omicron periods, they were 5.6% and 5.1%, respectively. They also reported 42.7% of cases from the wild-type variant, otherwise than in our cohort [64]. The majority of our patients (69%) had no history of neurological diseases, and required special attention only when neurological or psychiatric disorders were a symptom of COVID-19. Such a possibility was pointed out by the CoroNerve Study Group in the U.K. [65], but this needs further investigation.

The differences in the course of COVID-19 between the five waves indicate the probable influence of different variants of SARS-CoV-2 on disease presentation. Until the second wave (October 2020 to January 2021), variants were not reported in Poland and SARS-CoV-2 sequencing was only performed occasionally. In the third wave (February to May 2021), the alpha (B.1.1.7) variant predominated and was reported to be associated with increased transmissibility (i.e., more efficient and rapid transmission). In January 2021, U.K. scientists reported evidence that suggested that the B.1.1.7 variant may be associated with an increased risk of death, but early reports found no evidence to suggest any effect on the severity of the disease [66]. In other countries, after the alpha variant announcement in December 2020, there were reports of increased admissions to hospital and more serious illnesses in children, indicating that the B.1.1.7. variant was more pathogenically infectious within this group [24]. Nevertheless, we found no evidence of more severe disease in children during the third wave, and we found that the B.1.1.7 variant did not result in an appreciably different clinical course than the original strain. The fourth wave was dominated by the B.1.617.2 delta variant, which was reported to have increased transmissibility. Many more patients were hospitalized and we observed more severe cases of COVID-19, but these were statistically insignificant. In the fifth wave, omicron (B.1.1.529, BA.1, BA.1.1, BA.2, BA.3, BA.4, and BA.5 lineages) dominated. The CDC announced that it caused a milder disease, although some people experienced a severe course, required hospitalization, and could have died from infection [67]. In this wave, we hospitalized 414 children and observed the shortest hospital stay.

In this study, the Bacillus Calmette–Guérin (BCG) vaccination was also considered to be a factor that influenced COVID-19 severity, because it was hypothesized that countries without widespread tuberculosis prevention policies had a higher percentage of severe cases (Italy, France, and Spain) than countries that adopted long-term widespread prevention (Japan, Denmark, and Korea). In Poland, antituberculosis BCG vaccination was obligatory, so in our pediatric study groups, over 95% of patients had been vaccinated. The lack of BCG vaccination was found in 2–4% of hospitalized children in different waves. We did not observe statistically significant differences in the number of hospitalized BCG-vaccinated and unvaccinated patients. However, various publications have described the results of the first association between BCG vaccination and COVID-19 cases, but these have concerned only adults [68,69].

Our study confirmed that the children had a much milder course of the virus and richer symptoms of COVID-19 compared to adults in all waves. The same has been reported in other studies [12,18,19,49–51].

4.3. Laboratory Findings

Only a few authors have compared the COVID-19 course in children between different waves of the pandemic. Most of them did not consider laboratory findings, while Murugan et al. did not find any significant differences in laboratory results (hemoglobin, total platelet count, creatinine, Alt, prothrombin time, partial thromboplastin time, D-dimer, and C-reactive protein) [9,10,13–15,24–26,70,71]. In our study, we found statistically significant

differences in the first five waves of the pandemic in terms of CRP, blood platelets, and lactate dehydrogenase.

Our study has several limitations. During the first and second waves, primary care for COVID-19 patients was limited, so they were often referred to hospital. The Polish Ministry of Health's recommendations about the rules for COVID-19 isolation and hospitalization changed in the subsequent waves, and this could have influenced the admission criteria and the length of hospitalization. Our experience with pediatric COVID-19 also expanded over the subsequent waves, which could also have influenced hospital admissions and the length of stay.

To the best of our knowledge, this is the first such large single-center study comparing the differences between the clinical course of pediatric COVID-19 in the first five waves of the pandemic.

5. Conclusions

Our findings confirmed that a life-threatening course of COVID-19 in children was relatively rare. However, children with pneumonia, dehydration from fever, gastrointestinal symptoms, and loss of smell and taste, as well as those with neurological symptoms, represented most of the patients requiring hospitalization.

The absolute number of hospitalizations was significantly higher in the fourth and fifth waves than in the first three waves. The clinical course of the disease changed between March 2020 and April 2022 due to the predominance of different SARS-CoV-2 variants.

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Factors Associated with Mortality in Coronavirus-Associated Mucormycosis: Results from Mycotic Infections in COVID-19 (MUNCO) Online Registry

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Abstract: Background: COVID-19-associated mucormycosis (CAM) is associated with high morbidity and mortality. MUNCO is an international database used to collect clinical data on cases of CAM in real time. Preliminary data from the Mycotic Infections in COVID-19 (MUNCO) online registry yielded 728 cases from May to September 2021 in four South Asian countries and the United States. A majority of the cases (694; 97.6%) consisted of a mucormycosis infection. The dataset allowed for the analysis of the risk factors for adverse outcomes from CAM and this analysis is presented in this paper. Methods: The submission of cases was aided by a direct solicitation and social media online. The primary endpoints were full recovery or death measured on day 42 of the diagnosis. All patients had histopathologically confirmed CAM. The groups were compared to determine the contribution of each patient characteristic to the outcome. Multivariable logistic regression models were used to model the probability of death after a CAM diagnosis. Results: The registry captured 694 cases of CAM. Within this, 341 could be analyzed as the study excluded patients with an unknown CAM recovery status due to either an interruption or a lack of follow up. The 341 viable cases consisted of 258 patients who survived after the completion of treatment and 83 patients who died during

the period of observation. In a multivariable logistic regression model, the factors associated with an increased risk of mortality include old age (OR = 1.04, 95% CI 1.02–1.07, $p = 0.001$), history of diabetes mellitus (OR 3.5, 95% CI 1.01–11.9, $p = 0.02$) and a lower BMI (OR 0.9, 95% CI 0.82–0.98, $p = 0.03$). Mucor localized to sinus disease was associated with 77% reduced odds of death (OR = 0.23, 95% CI 0.09–0.57, $p = 0.001$), while cerebral mucor was associated with an increased odds of death (OR = 10.96, 95% CI 4.93–24.36, $p = \leq 0.0001$). Conclusion: In patients with CAM, older age, a history of diabetes and a lower body mass index is associated with increased mortality. Disease limited to the sinuses without a cerebral extension is associated with a lower risk of mortality. Interestingly, the use of zinc and azithromycin were not associated with increased mortality in our study.

Keywords: coronavirus; mucormycosis; steroids

1. Introduction

As of 12 October 2022, over 619 million confirmed cases of COVID-19 and 6.5 million deaths have been globally reported [1]. The SARS-CoV-2 virus emerged in 2019, leading to the beginning of the COVID-19 pandemic. A co-infection of COVID-19 can cause a serious illness and impairment, especially in those who are immunocompromised. A significant increase in cases of mucormycosis, a relatively rare fungal infection, has been seen in patients with COVID-19, termed COVID-19-associated mucormycosis (CAM). The pathophysiology is thought to be related to COVID-19-associated pulmonary aspergillosis (CAPA) and influenza-associated pulmonary aspergillosis (IAPA). CAM emerged as a significant healthcare challenge, with more than 41,000 cases reported as of September 2021 in India alone [2]. It is a life-threatening infection and carries high morbidity and mortality. Several risk factors are associated with an increased likelihood of acquiring the disease and they are seen more commonly in patients with underlying immunosuppression corticosteroid therapy and uncontrolled diabetes, with or without diabetic ketoacidosis [3]. There are other suggested hypotheses, including the role of a zinc supplementation and iron overload states in CAM that remain untested. Zinc is a micronutrient for fungal growth and has been shown to augment growth in vitro [3,4].

Mortality, even with standard care, is high at around 45–50% for rhino-orbital disease, >90% for disseminated disease and it is higher for patients with malignancies (66%) or diabetes (45%) than for immunocompetent hosts (35%) [4]. Although India emerged as the epicenter for CAM, sporadic cases comprised of various mycotic infections aside from mucormycosis were reported from several countries across the world. We established an online registry (Mycotic Infections in COVID-19; MUNCO) to collect clinic–epidemiologic data on CAM and other fungal infections using an online reporting system in real time. Our findings from April to June 2021 were previously reported from the first 65 cases of CAM in the registry [5]. Due to the severity of CAM, urgency was placed on the data collection and analysis [5]. At the time, the number of reported cases available provided a limited ability to fully evaluate the risk factors associated with adverse outcomes in CAM. In this report, we sought to further evaluate the association of the clinic–epidemiologic factors associated with mortality in CAM.

2. Methods

2.1. Data Collection

Cases were collected from July 2021 to June 2022 via an online questionnaire submitted by international physicians to report the case and treatment of CAM in a known patient. This was collected through a REDCap database [6] at http://covidmucor.com, as previously described [5]. The cases were entered at the discretion of the reporting physician, but the case definition included a histopathologically confirmed infection. The outcomes were defined as a full recovery or death at the 6-month time point. Online solicitation for the cases was performed through social media and networking. The authors confirm that the

ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received.

2.2. Statistical Analysis

Descriptive statistics such as the mean with standard deviation (SD), median with an inter-quartile range (IQR) and frequencies (n, %) were generated to summarize the patient characteristics both overall and stratified by the outcome (full recovery, death or a composite of death or vision loss). For each patient characteristic, the groups were formally compared via chi-square or Fisher's exact test for the association (categorical), two-sample t-test or Wilcoxon test (continuous). A multivariable logistic regression model was estimated to model the probability of death after a CAM diagnosis during treatment using Firth's penalized maximum likelihood estimates. Our primary variables of interest included a history of diabetes mellitus, obesity, the location of the disease, zinc and corticosteroid treatments which were examined along with a set of pre-selected covariates (age, location of and days to mucor infection and any known previous ICU stay) assumed to be potential confounders. Odds ratios, 95% confidence intervals and p-values corresponding to Wald chi-square tests for association were generated to summarize the effects. Two-sided p-values less than 0.05 were considered to be statistically significant. All analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA, copyright 2016).

3. Results

A total of 694 patients diagnosed with CAM were recorded in the database. Of these, 353 patients were still alive but had an unknown CAM recovery status due to an interrupted or failure to follow-up, and were thus excluded. Thus, a total of 341 patients were retained in the analysis data set, consisting of 258 patients who successfully completed treatment and survived versus the 83 patients who died.

Table 1a compares the baseline characteristics between survivors and non-survivors as well as the overall dataset. The patient ages ranged from 38 to 64 and the mean age of all the CAM patients was 51.7 years (S.D. 13 years). In total, 79% of the patients were female. The mean body mass index was 24.7 (S.D. 4). Of the overall population, 31% were overweight, 8% were obese and 4.5% were classified as underweight based on their BMI. Among subjects with CAM, 84% (286 patients) were diabetic and 21% (72 patients) had hypertension. The median hemoglobin A1C was 8.8 (IQR 7.4–10.9). A total of 85% of the overall population was unvaccinated. Among the CAM patients with diabetes, 11 of them (3.2%) had a presentation with ketoacidosis at the time of their COVID-19 diagnosis. Other potential risk factors were observed infrequently, such as a history of cancer (0.3%), an organ transplant (2.1%) or an HIV infection (0.3%). The median time from a COVID-19 diagnosis to the diagnosis of CAM was 20 days (IQR 14–30). Patients with CAM had higher C-reactive protein [54.3 mg/L (IQR 22.6–98.5)] and ferritin levels [509 ng/mL (306–931)].

In total, 85.6% (292/341) of all patients were treated with corticosteroids for COVID-19. The median steroid dose was 50 mg prednisone equivalent, with 52% of the overall population receiving >10 days of steroid therapy. The missing data in our data set included vaccination status (11.7%), gender (0.6%), BMI (2.1%), days to mucormycosis infection (10.6%), CRP (49.6%), Ferritin (29.3%), A1c (31.4%), steroid dose (52.5%), steroid duration (30.2%) and steroid type (31.1%).

Overall, 83 patients died due to CAM (24.3%). The mean age of the non-survivors was 6.8 years higher than the survivors (56.9 vs. 50.1 years). The non-survivors had a lower BMI (23.87) than the survivors (25.01) and out of the 15 underweight patients, 10 were non-survivors (66.7%). The non-survivors also had a higher median A1C of 9.6 (IQR 8.3–11.8) compared to 8 (IQR 6.9–10.0) among the survivors. The median time from the COVID-19 diagnosis to the diagnosis of CAM was shorter (17 days, IQR 11–27) in non-survivors as compared to survivors (21 days, IQR 15–30, $p = 0.007$). The non-survivors also had higher levels of CRP and ferritin as compared to the survivors (median ferritin 85.1 mg/L, IQR 47–119 vs. 40.2 mg/L, IQR 18–70; median ferritin 763 ng/mL, IQR 373–1174 vs. 359.5 ng/mL,

IQR 234–578, $p < 0.001$). Among those treated with corticosteroids, the non-survivors were treated with a higher median daily dose of 53 mg prednisone equivalent, (IQR 50–100) as compared to the survivors (median dose 50 mg prednisone equivalent, IQR 40–53.3), though a longer duration did not result in a higher mortality risk when looking at steroid courses ≥10 days or <10 days.

Table 1. Baseline Characteristics.

(**a**) Baseline variables between group with recovery and deceased				
Baseline Characteristic	**Overall N = 341**	**Recovery N = 258**	**Death N = 83**	***p*-Value #**
Age in years	51.72 (13.02)	50.07 (12.70)	56.88 (12.72)	<0.001
Vaccinated	46 (15.3%)	34 (73.9%)	12 (26.1%)	0.71
Female	269 (79.4%)	204 (75.8%)	65 (24.2%)	0.79
Male	70 (20.6%)	52 (74.3%)	18 (25.7%)	
BMI kg/m^2	24.76 (4.11)	25.04 (4.19)	23.87 (3.73)	0.03
BMI Category:				
Underweight (<18.8)	15 (4.5%)	5 (33.3%)	10 (66.7%)	0.001
Normal (18.5 ≤ BMI < 25)	187 (56%)	144 (77%)	43 (23%)	
Overweight (25 ≤ BMI < 30)	105 (31.4%)	83 (79%)	22 (21%)	
Obese (≥30)	27 (8.1%)	22 (81.5%)	5 (18.5%)	
Comorbidities:				
Hypertension	72 (21.1%)	50 (69.4%)	22 (30.6%)	0.17
DM	286 (83.9%)	208 (72.7%)	78 (27.3%)	0.004
DM with ketoacidosis	11 (3.2%)	3 (27.3%)	8 (72.7%)	0.001
Cancer	1 (0.3%)	0 (0%)	1 (100%)	0.08
Organ Transplant	7 (2.1%)	5 (71.4%)	2 (28.6%)	0.79
IDU	4 (1.2%)	1 (25%)	3 (75%)	0.05
HIV+	1 (0.3%)	1 (100%)	0 (0%)	0.57
Asthma	3 (0.9%)	2 (66.7%)	1 (33.3%)	0.72
Laboratory values:				
CRP mg/L	54.3 (22.6–98.5)	40.2 (18.0–69.6)	85.1 (47.0–118.7)	<0.001
Ferritin ug/L	509 (306–931)	359.5 (234–578)	763 (372.9–1174)	<0.001
A1c%	8.8 (7.4–10.9)	8.0 (6.9–10.0)	9.6 (8.3–11.8)	<0.001
Days from COVID-19 diagnosis to mucor	20 (14–30)	21 (15–30)	17 (11–27)	0.01
Corticosteroid Treatment	292 (85.6%)	219 (75%)	73 (25%)	0.49
Dose, prednisone equivalent	50 (40–53.3)	50 (40–53.3)	53.3 (50–100)	<0.001
Type: Dexamethasone	132 (56.2%)	101 (76.5%)	31 (23.5%)	0.43
Methylrednisone	81 (34.5%)	56 (69.1%)	25 (30.9%)	
Prednisone	22 (9.4%)	15 (68.2%)	7 (31.8%)	
Treatment duration 10+ days	124 (52.1%)	98 (79%)	26 (21%)	0.03
(**b**) Mucor location/severity between CAM recovered and deceased				
Location(s) of Mucor Infection	**Overall N = 341 (100%)**	**Recovery N = 258**	**Death N = 83**	***p*-Value #**
Sinus	307 (90%)	239 (77.9%)	68 (22.1%)	0.005
Pulmonary	12 (3.5%)	3 (25%)	9 (75%)	0.001
Cutaneous	4 (1.2%)	2 (50%)	2 (50%)	0.229
Gastric	3 (0.9%)	3 (100%)	0 (0%)	0.324
Ophthalmic	183 (53.7%)	133 (72.7%)	50 (27.3%)	0.167
Cerebral	52 (15.2%)	17 (32.7%)	35 (67.3%)	0.001

Table 1. *Cont.*

(**c**) Medications administered to patients during course of treatment for CAM				
Mucor Treatment(s)	**Overall N = 341 (100%)**	**Recovery N = 258**	**Death N = 83**	***p*-Value #**
Amphotericin B	286 (83.9%)	217 (75.9%)	69 (24.1%)	0.833
Posaconazole	202 (59.2%)	164 (81.2%)	38 (18.8%)	0.004
Isavuconazole	22 (6.5%)	18 (81.8%)	4 (18.2%)	0.486
Surgery	258 (75.7%)	209 (81%)	49 (19%)	0.001
Voriconazole	6 (1.8%)	5 (83.3%)	1 (16.7%)	0.659
Amphotericin B regimen(s)				
Amphotericin B deoxycholate	47 (16.4%)	37 (78.7%)	10 (21.3%)	0.617
Liposomal amphotericin B	269 (94.1%)	209 (77.7%)	60 (22.3%)	0.004
Amphotericin B lipid complex, ABLC	39 (13.6%)	27 (69.2%)	12 (30.8%)	0.297
Amphotericin B cholesteryl sulfate complex	0 (0%)	0 (0%)	0 (0%)	0.297
(**d**) Medications administered to patients during course of treatment for COVID-19				
COVID-19 Treatment(s)	**Overall N = 341 (100%)**	**Recovery N = 258**	**Death N = 83**	***p*-Value #**
Favipiravir	93 (27.3%)	76 (81.7%)	17 (18.3%)	0.110
Remdesivir	161 (47.2%)	121 (75.2%)	40 (24.8%)	0.837
Doxycycline	138 (40.5%)	105 (76.1%)	33 (23.9%)	0.880
Azithromycin	97 (28.4%)	74 (76.3%)	23 (23.7%)	0.865
Ivermectin	146 (42.8%)	118 (80.8%)	28 (19.2%)	0.055
Tocilizumab	14 (4.1%)	8 (57.1%)	6 (42.9%)	0.099
Itolizumab	1 (0.3%)	1 (100%)	0 (0%)	0.570
Zinc	216 (63.3%)	166 (76.9%)	50 (23.1%)	0.500
Other	35 (10.3%)	20 (57.1%)	15 (42.9%)	0.007

Data are presented as N (%), mean (SD) or median (25th–75th percentile). # Corresponds to a chi-square or Fisher's exact test for association (categorical), two-sample *t*-test (mean (SD) presented) or Wilcoxon test (if median (IQR) presented. BMI: body mass index; DM: diabetes mellitus; IDU: injecting drug user; HIV: human immunodeficiency virus; CRP: C-reactive protein; IQR: interquartile range.

Table 1b includes the anatomical location of CAM. The most common site was sinus disease (307 patients, 90%) overall, in both the survivors and non-survivors. Out of the 307 patients with sinus disease, 239 (77.9%) were survivors while 68 (22.1%) were non-survivors. The non-survivors had a higher percentage of cerebral disease (67.3%) and pulmonary disease (75%). An orbital progression occurred in 183 patients (54%), while a cerebral extension occurred in 52 patients (15%). Rarer manifestations included pulmonary mucormycosis (12 patients, 3.5%) and cutaneous mucormycosis (4 patients, 1.2%), while 3 patients (0.9%) had gastric mucormycosis.

Table 1c shows the medications used during the course of treatment for CAM. These include amphotericin B (286 patients, 83.9%) with or without Posaconazole (202 patients, 59.2%). Isavuconazole (22 patients, 6.5%), voriconazole (6 patients, 1.8%) and surgery (258 patients, 75.7%) were also utilized as CAM treatment options. Liposomal amphotericin B was the most commonly used preparation of amphotericin (94.1%). We also noted the use of alternate formulations in combination with, or excluded, liposomal amphotericin B, including amphotericin B lipid complex (ABLC) and conventional amphotericin B deoxycholate (16.4%). Ultimately, 258 (75.7%) patients received a surgical intervention, with this number consisting of 49 non-survivors (19%).

Table 1d shows the treatments given to patients for the management of COVID-19 prior to a diagnosis of CAM. Among COVID-19 therapies, there was no association with a higher risk of CAM mortality with any individual antiviral, antibiotic use or immunomodulator therapy use. Antiviral therapies included remdesivir (161 patients, 47.2%), favipiravir (93 patients, 27.3%) and ivermectin (146 patients, 42.8%). The antibiotics prescribed included azithromycin (97 patients, 28.4%) and doxycycline (138 patients, 40.5%).

In a multivariable logistic regression model (Table 2), a lower BMI (OR 0.9, $p = 0.03$) and a history of diabetes mellitus was associated with increased mortality (OR 3.5, 95% CI 1.01–11.93, $p = 0.02$). Mucor localized to sinus disease was associated with a 77% reduced odds of death (OR = 0.23, $p = 0.001$). Neither azithromycin nor a zinc treatment were associated with the probability of death after adjustment.

Table 2. Logistic regression model for the probability of death.

	Estimated Odds Ratio	*p*-Value
Patient age, years	1.04 (1.02, 1.07)	0.001
Azithromycin treatment	0.99 (0.49, 2.03)	0.76
Zinc treatment	0.76 (0.37, 1.57)	0.46
History of DM	3.47 (1.01, 11.93)	0.02
BMI, kg/m^2	0.90 (0.82, 0.98)	0.03
Steroid treatment *Ref: no steroid treatment*	1.67 (0.68, 4.12)	0.22
Known ICU stay *Ref: no known ICU stay*	1.50 (0.70, 3.25)	0.16
Days to mucor *(continuous)*	0.98 (0.96, 1.00)	0.15
Location of mucor: Sinus *Ref: not sinus*	0.23 (0.09, 0.57)	0.001
Ophthalmic *Ref: not ophthalmic*	0.87 (0.45, 1.69)	0.61
Cerebral *Ref: not cerebral*	10.96 (4.93, 24.36)	<0.0001

DM: diabetes mellitus; BMI: body mass index; ICU: intensive care unit.

4. Discussion

We present the risk factors for mortality from CAM from an online database of reported cases from centers predominantly located in South East Asia. Indeed, >70% of the reported cases in the literature have been from India [7]. Previous observational studies from urban centers in India have reported a 2.1-fold increase in the number of cases of mucormycosis as compared to previous years [8]. The overall mortality of cases in our registry, where data were ascertainable, was 24%. This is consistent with the other published reports [7–9]. The reported mortality has varied from 14% to 64% and may reflect different levels of COVID severity [10], CAM severity and the aggressiveness of a surgical intervention [11] and the time of the ascertainment of the data. Mortality was lowest (14–17%) in studies which reported a high level of surgical intervention and a low COVID-19 disease acuity [11,12]. At the 6-month follow-up, the mortality from CAM was 34% with a combined surgical and medical treatment [13].

While most studies have reported a high male preponderance in cases of CAM [7,8,11], the high female preponderance in this registry is unexplained and may reflect a reporting bias. Consistent with previous studies, our data shows a high proportion of patients with diabetes, and this has a significant association with a reduced survival. The increased risk of mucormycosis in diabetes is well described in earlier studies [8,9] in patients with or without COVID-19 [14,15]. While the exact mechanism is unknown, it is suggested that due to changes in the iron metabolism, pH and the diminished phagocytic response to fungi due to hyperglycemia, as well as increased endothelial receptor expression for fungal ligands, may facilitate angioinvasion [16]. There is a bidirectional relationship between COVID-19 and diabetes where SARS-CoV2 infection may facilitate a dysglycemic state and diabetes may increase the risk of the COVID-19 infection's severity [7,16]. India has the second-largest number of adults aged 20–79 years with diabetes [17].

Interestingly, we did find that a lower BMI was associated with increased mortality. This is not entirely surprising since ketosis and the presence of beta-hydroxybutyrate likely worsen during COVID-19 associated malnutrition states and are known to increase the expression of the host and fungal receptors that contribute to an increased tissue invasiveness [18].

Higher levels of CRP and ferritin were found in non-survivors as compared to survivors. An elevated CRP may reflect elevated IL-6 levels, and both of these can perpetuate the inflammatory diabetogenic process of COVID-19 as well [19]. Since the time to a CAM diagnosis was shorter in non-survivors as compared to survivors, they could plausibly have still been in the heightened inflammatory phase, which may predispose to an invasive and severe disease.

Corticosteroids are known risk factors for mucormycosis. COVID-19 was treated with corticosteroids in 76–87% of the published data on CAM [8,11] and in the study by Patel et al., a corticosteroid dose was appropriate in only a third of treated COVID-19 patients [8]. In our analysis, neither a corticosteroid treatment dose nor its duration was associated with a decreased survival. Prior studies hypothesized that a zinc supplementation would increase the risk of mucormycosis as it may act as a micronutrient for fungal growth [20,21]. Interestingly, in our analysis, neither a zinc treatment nor azithromycin were associated with increased mortality.

Similar to the published data in non-CAM settings [14,22,23], a disease limited to the sinus and surgical debridement are associated with lower mortality, while a CNS extension was associated with a higher mortality risk. This was consistent with our analysis that cerebral disease had much higher odds for death. A surgical intervention has been shown to reduce mortality in CAM patients without CNS involvement, but also reduced the progression of disease in those with CNS involvement, though these data were limited in terms of the duration of the follow-up [11]. It should be noted that the CNS extension of a disease has been noted in 21–50% of case series of CAM [11,13,24,25], while in non-CAM, the CNS extension has been described in 21–25% of the published cases in reports where this is clearly delineated [14,26].

The strength of our study is the large multinational sample size providing real world data about the risks of mortality of CAM. The simple online interface allowed for a rapid deployment and implementation. Limitations include a potential selection bias due to diverse locations with variable access to specialty care in the setting of pandemic shortages, a lack of comparable registry data for an external validation, a lack of a separate verification for the integrity of data at the entry point and a lack of complete follow-up information. Nevertheless, with a lack of widely available data about the clinical course and risks of the adverse outcomes, we feel this is important to leverage such a registry acquisition to disseminate the knowledge of an emerging disease.

5. Conclusions

CAM emerged as a serious life-threatening infection in the middle of the pandemic and this registry was deployed in order to generate data sets that can answer some of the key clinic–epidemiologic questions in our understanding of this disease. In this paper, we discuss the factors associated with mortality in CAM and identify the sites of the disease, old age, a low BMI and a history of diabetes as factors associated with increased mortality. We also identify that the use of zinc is not associated with increased mortality in CAM.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Albert Einstein College of Medicine with approval number 2021-13086 for studies involving humans.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to redcap policies.

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Assessing the Efficacy of Early Therapies against SARS-CoV-2 in Hematological Patients: A Real-Life Study from a COVID-19 Referral Centre in Northern Italy

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Abstract: Early therapies to prevent severe COVID-19 have an unclear impact on patients with hematological malignancies. The aim of this study was to assess their efficacy in this group of high-risk patients with COVID-19 in preventing hospitalizations and reducing the SARS-CoV-2 shedding. This was a single-center, retrospective, observational study conducted in the Fondazione IRCSS Policlinico San Matteo of Pavia, Northern Italy. We extracted the data of patients with hematologic malignancies and COVID-19 who received and did not receive early COVID-19 treatment between 23 December 2021, and May 2022. We used a Cox proportional hazard model to assess whether receiving any early treatment was associated with lower rates of hospitalization and reduced viral shedding. Data from 88 patients with hematologic malignancies were extracted. Among the patients, 55 (62%) received any early treatment, whereas 33 (38%) did not. Receiving any early therapy did not significantly reduce the hospitalization rate in patients with hematologic malignancies (HR 0.51; SE 0.63; *p*-value = 0.28), except in the vaccinated non-responders subgroup of patients with negative anti SARS-CoV-2 antibodies at the time of infection, who benefited from early therapies against SARS-CoV-2 (HR 0.07; SE 1.04; *p*-value = 0.001). Moreover, no difference on viral load decay was observed. In our cohort of patients with hematologic malignancies infected with SARS-CoV-2, early treatment were not effective in reducing the hospitalization rate due to COVID-19, neither in reducing its viral shedding.

Keywords: COVID-19; early remdesivir; molnupiravir; ritonavir-boosted nirmatrelvir; sotrovimab; hematological patients; hospitalizations rate; prolonged viral shedding

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1. Introduction

Patients with hematological malignancies or who underwent hematopoietic stem-cell transplantation (HSCT) are considered at high risk of developing severe COVID-19 [1]. COVID-19-related mortality in patients with hematologic malignancies is higher than in the general population, being approximately 30% in several studies performed both in the pre- and in the post-vaccine era [1,2].

These patients are at higher risk of severe COVID-19, due to the long-lasting immunodeficiency resulting from malignancy itself, anticancer treatments, or HSCT [3,4]. Moreover, there is evidence of an impaired humoral immune or cellular response after anti-SARS-CoV-2 vaccination among patients with hematologic malignancies and HSCT patients [5], and a lower post-vaccination immunogenicity [6].

Furthermore, patients with hematologic malignancies and HSCT patients may have a prolonged viral shedding [7] compared to the roughly 10-days average duration usually reported for the general population [8]. Hence, plenty of studies have demonstrated a prolonged shedding duration of active virus, up to months after symptom onset [9–12].

Currently, there are valid options for symptomatic outpatients with COVID-19 that are at a high risk for progression to severe disease. Among those, the oral combination of nirmatrelvir/ritonavir is the recommended option [13], since it has been shown to reduce the risk for hospitalization by 89% [14]. Remdesivir has a similar efficacy and is an alternative option, but its use is impractical in some outpatient settings since it requires parenteral administration over 3 days [15]. A third option is anti-SARS-CoV-2 monoclonal antibodies which have variable activity against the different SARS-CoV-2 variants. Among them, Sotrovimab was the only one that retained some activity against BA.1/BA.1.1 sub-lineages of the Omicron variant [16], but is currently no longer effective against BA.2 [17]. Molnupiravir is another possible option. However, since its lower efficacy, which was roughly 30% in reducing COVID-19-related hospitalization by 28 days [18], the COVID-19 Treatment Guidelines Panel recommended its use only when the other options are contraindicated [19]. Together with COVID-19 related hospitalization and mortality rate reduction, these drugs might also lead to a significant reduction in viral load [20].

Although clinical trials generally exclude patients with hematologic malignancies, the European Conference on Infections in Leukemia recently recommended treating patients with hematologic malignancies with mild COVID-19 with these drugs [21].

The aim of this study was to assess the impact of early therapies in reducing the hospitalization rate and the 28-days mortality due to COVID-19 in patients with hematologic malignancies in our Hospital Fondazione IRCCS Policlinico San Matteo in Pavia, Northern Italy. We also aimed to evaluate the time length of viral shedding in patients with hematologic malignancies and HSCT patients who were and were not treated with early therapies.

2. Materials and Methods

2.1. Study Design

This study was a retrospective, single-center analysis of patients with a confirmed diagnosis of COVID-19 referred to our hospital. The study was approved by our Institutional Review Board (n.prot.0031226/22).

The medical records of all the adult patients with hematologic malignancies who tested positive for real-time reverse-transcription polymerase chain reaction (RT-PCR) from nasal swabs for SARS-CoV-2 and were consequently evaluated for early treatment in our clinic, were anonymized and abstracted on standardized data collection forms. In particular, patients suffering with myeloma, Hodgkin and non-Hodgkin lymphoma, chronic and acute leukemia, paroxysmal nocturnal hemoglobinuria, amyloidosis, and myelodysplastic syndrome/myeloproliferative neoplasms were included.

Only patients with mild to moderate COVID19 diseases were considered eligible for a therapy. Specifically, they did not present with any of the following features: oxygen saturation of <94% on room air; respiratory rate of >30 breaths/min; PaO_2/FiO_2 < 300 mmHg; and lung infiltrates > 50%.

We only extracted the data of patients evaluated between 23 December 2021 and 30 of April 2022, when the vast majority of COVID-19 cases were due to the Omicron variant.

The following exclusion criteria were applied: patients hospitalized for COVID-19 and/or requiring oxygen therapy for COVID-19 at the first clinical evaluation; asymptomatic patients.

2.2. Study Setting

One of the Infectious Diseases outpatients' clinics of our hospital was allocated to the early treatment of COVID-19 outpatients from 23 December 2021. In this clinic, an infectious disease (ID) specialist was in charge of receiving daily e-mails from general practitioners

and specialists of other units who promptly notified the cases of SARS-CoV-2 positive high-risk patients, both outpatients and patients admitted for reasons other than COVID-19.

The appropriate therapy for each notified patient was chosen by the ID specialist, according to both the inclusion and exclusion criteria and the availability of each drug's pilot sheet. After signing an informed consent form, the patient was then examined and informed about the selected therapy.

Among these, ritonavir-boosted nirmatrelvir was selected as the first oral medication, but it was available only from 20 February 2022. If an intravenous (IV) drug was selected, remdesivir was administered as an IV infusion over 30 min at the recommended dosage of 200 mg for the loading dose on day 1, followed by a 100 mg maintenance dose administered on days 2 and 3. As regard with sotrovimab, it was given as a single 500 mg IV infusion, but it was used from the arrival of the Omicron BA.2 subvariant, at the end of April 2022. Patients were monitored during each infusion and observed for at least one hour after for signs and symptoms of hypersensitivity.

As a last resort, molnupiravir was administered to the individuals who were not eligible to any other drug.

2.3. Patients' Characteristics

The demographic data included sex and age. Clinical data included symptoms at presentation, comorbidities (history of cancer, heart disease, hypertension, diabetes, chronic kidney disease, lung disease, and obesity), vaccination status, and anti-spike IgG antibodies for SARS-CoV-2 (results greater than or equal to the cut-off value 50.0 AU/mL were reported as positive). Type of hematological disease; ongoing chemotherapy; type and time of HSCT if performed.

The Italian Agency of Drugs (AIFA)s guidelines for excluding patients from one treatment rather than another was strictly followed.

2.4. SARS-CoV-2 RNA Detection

Total RNA was extracted on the MGISP-960 automated workstation using the MGI Easy Magnetic Beads Virus DNA/RNA Extraction Kit (MGI Technologies, Shenzhen, China). Detection of SARS-CoV-2 RNA was performed using the SARS-CoV-2 variants ELITe MGB® kit (ELITechGroup, Puteaux, France; cat. no. RTS170ING) on the CFX96 Touch Real-time PCR detection system (BioRad, Mississauga, ON, Canada).

2.5. Outcomes

The primary outcome was to evaluate the impact of early therapies, such as remdesivir, molnupiravir, ritonavir-boosted nirmatrelvir, and sotrovimab, in preventing the hospitalization due to COVID-19 of patients with hematologic malignancies infected by SARS-CoV-2 by day 28.

In particular, we considered the progression of COVID-19 as the presence of clinical manifestations which are consistent with the categories of moderate, severe, and critical illness defined by the National Institute of Health Guidelines [22].

We also evaluated admission to the intensive care unit (ICU) of our hospital and the intra-hospital mortality by day 28.

The secondary outcomes were to evaluate the effect of the single drug in preventing the 28 days hospitalization due to COVID-19, to evaluate the length of SARS-CoV-2 viral shedding of patients receiving early therapies versus those who did not receive them, and finally, to evaluate the impact of the early therapies in patients with hematologic malignancies with negative SARS-CoV-2 antibodies at the time of evaluation.

2.6. Statistical Analysis

Data for continuous variables were presented as means and standard deviations.

Categorical variables were presented as frequencies and percentages. Comparisons between the treated and non-treated groups of patients with hematologic malignancies

were performed using chi-square tests for categorical variables and Mann–Whitney tests for non-normal continuous data.

The log-rank test was used to estimate the difference between the 28-day Kaplan–Meier hospitalization curves of patients who received and did not receive early therapies. The duration of viral shedding was calculated by using the Kaplan–Meier curves and tested by the log-rank test for survival curve comparison. When viral clearance could not be determined, the duration was censored with the last positive sample. A Cox proportional hazard model was performed controlling for sex, age, number of underlying comorbidities, and number of anti-SARS-CoV-2 vaccinations performed. A multivariable Cox proportional-hazard regression model was also performed to evaluate the impact of each drug on the hospitalization rate compared to no drugs.

Finally, a multivariable Cox proportional-hazard regression model was performed to evaluate the impact of early therapies in patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies at the time of evaluation.

The results were reported as hazard ratios (HRs) and 95% confidence intervals (CIs). Statistical analyses were conducted using R (version 4.1.2).

3. Results

Data from 88 patients were extracted. A total of 55 (62%) received early therapy and 33 (38%) did not. Demographic, clinical, and treatment characteristics are presented in Table 1.

Table 1. Demographic and clinical characteristics.

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
Sex, *n* (%)	Female	47 (53)	27 (31)	20 (23)	
	Male	41 (47)	28 (32)	13 (15)	0.41
Age, Median (IQR)		63 (49.0, 71.2)	62 (52.5, 70.0)	63 (48, 72)	0.89
Vaccination doses, Mean (sd)		2.7 (0.7)	2.6 (0.8)	2.7 (0.5)	0.69
Days from last vaccination, Mean (sd)		124.1 (65)	128.1(64.3)	116.9 (67.1)	0.51
Remdesivir, *n* (%)		-	15 (27)		-
Ritonavir-boosted Nirmatrelvir, *n* (%)		-	10 (18)		-
Sotrovimab, *n* (%)		-	15 (27)		-
Molnupiravir, *n* (%)		-	15 (27)		
Bone marrow transplantation, *n* (%)		24 (27)	18 (75)	6 (25)	0.22
Days from bone marrow transplantation, Mean (sd)		1307.4 (1793.8)	1390.3 (1981.8)	1009 (929.2)	0.68
Type of Bone marrow transplantation, *n* (%)	Autologous	20 (22)	14 (25)	6 (18)	
	Allogenic	4 (4)	4 (7)	0 (0)	0.28
Hematological disease, *n* (%)	Myeloma	26 (29)	17 (31)	9 (27)	
	Hodgkin Lymphoma	8 (9)	3 (5)	5 (15)	
	High-Grade Non-Hodgkin Lymphoma	12 (14)	10 (18)	2 (6)	
	Acute Myeloid Leukemia	4 (4)	3 (5)	1 (3)	

Table 1. *Cont.*

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
	Low-Grade Non-Hodgkin Lymphoma	16 (18)	7 (13)	9 (27)	
	Chronic Lymphocytic Leukemia	4 (4)	3 (5)	1 (3)	
	Chronic Myeloid Leukemia	8 (9)	7 (13)	1 (3)	
	MDS/MPN	3 (3)	2 (4)	1 (3)	
	Paroxysmal Nocturnal Hemoglobinuria	1 (1)	0 (0)	1 (3)	
	Acute Lymphocytic Leukemia	4 (4)	2 (4)	2 (6)	
	Amyloidosis AL	1 (1)	1 (2)	0 (0)	0.25
Immunosuppressive therapies, *n* (%)	Rituximab	20 (23)	13 (24)	7 (21)	1.00
	Obinutuzumab	5 (6)	3 (6)	2 (6)	1.00
	Methotrexate	10 (11)	5 (9)	5 (15)	0.60
	CHOP	15 (17)	12 (22)	3 (9)	0.21
	CHOEP	1 (1)	0 (0)	1 (3)	0.79
	ABVD	4 (4)	1 (2)	3 (9)	0.29
	Poli chemotherapy (VCR, Ara-C, Ida, EDX, Cisplatin, Bendamustine)	21 (24)	13 (4)	8 (24)	1.00
	VD (Bortezomib-Dexamethasone)	12 (14)	8 (14)	4 (12)	1.00
	Eculizumab	1 (1)	0 (0)	1 (3)	0.80
	Tyrosine kinase inhibitors (TKIs)	13 (15)	10 (18)	3 (9)	0.37
	Others (Daratumumab, Isatuximab, IMIDs, Brentuximab, Ab anti-PD1-PDL1)	30 (34)	17 (31)	13 (39)	0.60
Days between last therapy and examination, mean (sd)		3205.2 (11,379.2)	2902.1 (10,844.7)	3799 (12,582.5)	0.75
Positive anti SARS-CoV-2 antibodies, *n* (%)		44 (50)	20 (36)	24 (73)	<0.01
Viral decay (sd)		26.3 (21.6)	25.4 (18.0)	27.7 (24)	0.63
Comorbidities	NPL, *n* (%)	59 (69)	32 (63)	25 (78)	0.22
	CKD, *n* (%)	8 (10)	3 (7)	5 (15)	
	CVD, *n* (%)	14 (16)	8 (15)	6 (18)	0.90
	HTN, *n* (%)	34 (39)	21 (39)	13 (39)	1.00
	DM, *n* (%)	10 (11)	5 (9)	5 (15)	0.62
	LD, *n* (%)	10 (11)	7 (13)	3 (9)	0.83
	HCV, *n* (%)	2 (3)	2 (4)	0 (0)	0.70
	Obesity, *n* (%)	1 (1)	0 (0)	1 (3)	0.80

Table 1. *Cont.*

		All Patients (88)	Treated (55)	Non-Treated (33)	*p*-Value
	Smoke, *n* (%)	10 (13)	5 (12)	5 (16)	0.90
	Number of comorbidities, mean (sd)	1.5 (1.2)	1.4 (1.1)	1.7 (1.3)	0.24
Mortality, *n* (%)		2 (2)	0 (0)	2 (6)	0.27
Hospital admission, *n* (%)		12 (14)	6 (11)	6 (18)	0.52
ICU admission, *n* (%)		1 (1)	0 (0)	1 (3)	0.79
Stay at Home, *n* (%)		78 (87)	50 (91)	28 (85)	0.60
Symptoms, *n* (%)					
	Asymptomatic	10 (12)	1 (2)	9 (30)	<0.01
	Fever	39 (48)	30 (57)	9 (32)	0.06
	Cough	32 (39)	24 (45)	8 (29)	0.2
	Pharyngodinia	25 (31)	16 (30)	9 (32)	1.00
	Dyspnea	10 (13)	3 (6)	7 (25)	0.04
	Diarrhea	2 (2)	2 (4)	0 (0)	0.77
	Asthenia	15 (18)	10 (19)	5 (17)	1.00
Pneumonia		12 (14)	6 (11)	6 (21)	0.39
Oxygen therapy		11 (13)	5 (9)	6 (19)	0.33

Notes: MDS/MPN, Myelodysplastic syndrome/Myeloproliferative neoplasms; ABVD, Adriamycin/bleomycin/vinblastine/dacarbazine; VCR, vincristine; EDX, 4′-epidoxorubicin; IMIDs, immunomodulatory drugs; NPL, Neoplasia; CKD, Chronic Kidney Disease; CVD, Cardiovascular Disease; HTN, Hypertension; DM, Diabetes Mellitus; LD, lung disease; Obesity considered as Body Mass Index (BMI) > 30 kg/m^2; ICU, intensive care unit; Positive anti-SARS-CoV-2 antibodies was considered when IgG anti-trimeric SARS-CoV-2 spike protein were ≥50 AU/mL; HCV, presence of antibodies against HCV. Data are reported as absolute number and percentage and mean with standard deviation.

Most patients were vaccinated against SARS-CoV-2 (94%). However, among them, only 44 (50%) patients had positive IgG anti- SARS-CoV-2 spike protein.

Regarding the treatment, 55 (62%) patients received an early treatment for SARS-CoV-2. Fifteen (27%) were treated with remdesivir, 10 (18%) with ritonavir-boosted nirmatrelvir, 15 (27%) with sotrovimab, and 15 (27%) with molnupiravir.

Globally, the length of PCR positivity for SARS-CoV-2 on nasal swab had a mean of 26.3 (±21.6) days, 25.4 (±18.0) and 27.7 (±24.0) for the treated and untreated group, respectively. Among the treated patients, six (11%) developed COVID-19 related pneumonia, with five of them requiring oxygen therapy and hospitalization. None of the treated patients required ICU admission. Moreover, six untreated patients were hospitalized for COVID-19 related pneumonia. Among them, one was admitted to the ICU, while two died.

3.1. Impact of Early Therapies on the Outcomes

Regarding our primary outcome, treatment with any considered early therapy did not significantly reduce hospital admission by 28 days (Figure 1).

Similarly, after accounting for potential confounders, the multivariable Cox proportional-hazard regression model showed that an early treatment with any of the considered drugs did not significantly reduce the hospitalization rate (HR: 0.51; SE 0.63; $p = 0.28$) (Table 2).

Additionally, the multivariable Cox proportional-hazard regression model showed that none of the early treatments did significantly reduce the hospitalization at day 28 compared with no treatment (Table 3).

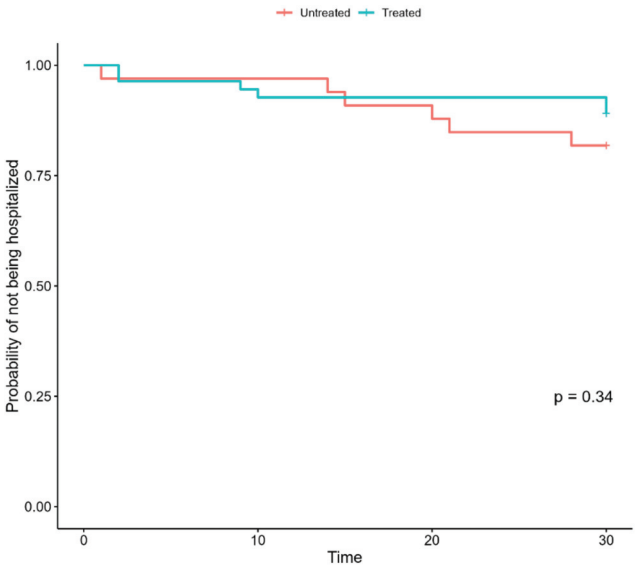

Figure 1. Kaplan–Meier curves of hospitalization in untreated and treated patients with hematologic malignancies and HSCT patients.

Table 2. Multivariate Cox regression for 28-day hospital admission.

Variable	HR	SE	*p*-Value
Treatment	0.51	0.63	0.28
Sex	0.29	0.68	0.07
Age	1.01	0.02	0.73
Number of vaccinations	1.42	0.61	0.56
Comorbidities	1.63	0.26	0.06

Table 3. Multivariate Cox regression for 28-day hospital admission considering the impact of each treatment.

Variable	HR	SE	*p*-Value
Paxlovid	0.51	1.10	0.55
Remdesivir	1.16	0.71	0.83
Molnupiravir	0.28	1.09	0.24
Sotrovimab	0.24	1.09	0.19
Sex	0.32	0.62	0.07
Age	1.03	1.41	1.41
Number of vaccinations	1.43	0.56	0.57

Finally, the multivariable Cox proportional-hazard regression model showed that patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies at the time of infection were at a significantly increased risk of hospitalization if not treated in a timely fashion with early therapies.

Specifically, after accounting for sex, age, number of vaccinations, and comorbidities, being untreated was significantly associated with an increased risk of hospitalization among patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies (Table 4) (Figure 2).

Table 4. Multivariate Cox regression for 28-day hospital admission of patients with hematologic malignancies with negative anti SARS-CoV-2 antibodies.

Variable	HR	SE	*p*-Value
Treatment	0.07	1.04	0.001
Sex	0.37	0.98	0.31
Age	1.00	0.04	0.91
Number of vaccinations	1.05	0.74	0.93
Comorbidities	1.63	0.35	0.16

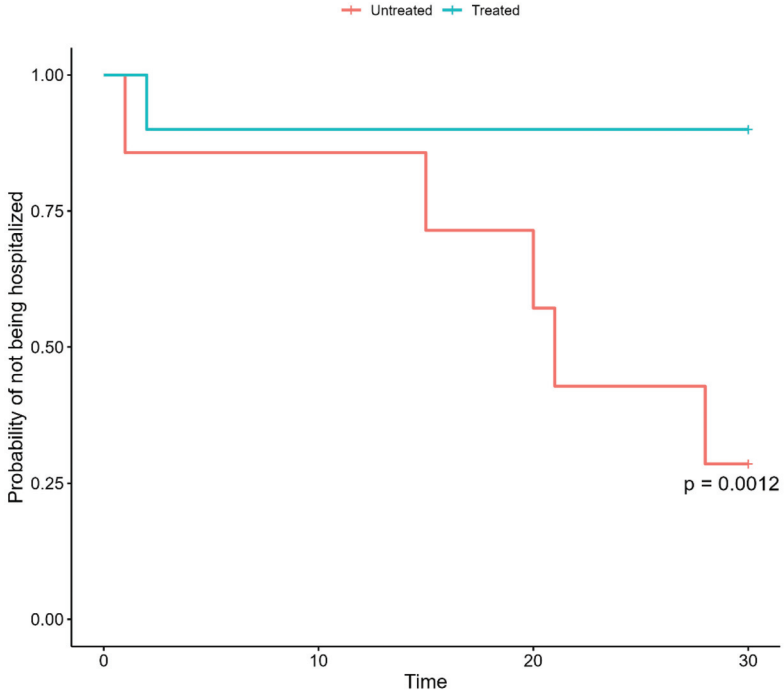

Figure 2. Kaplan–Meier curves of hospitalization in untreated and treated patients with hematologic malignancies and HSCT patients with negative anti-SARS-CoV-2 antibodies.

3.2. SARS-CoV-2 RNA Load Kinetics

In a subset of patients (49/79; 62.0%), the duration of viral load was available, and the median duration was 15 days (range 8–87 days) for untreated, 21 days (range 8–31 days) for Remdesivir, 17 days (6–46 days) for sotrovimab, and 17 days (8–27 days, log-rank test $p = 0.48$) for molnupiravir (Figure 3A). Only one patient treated with ritonavir-boosted nirmatrelvir had data on viral load duration (8 days censored). Among the untreated group, the more prolonged infection was observed in a patient with RNA detected at 87 days after first positivity, while in the treated patients' group, the more prolonged shedding was observed in one case treated with Sotrovimab with detectable RNA at 52 days after first positivity.

In addition, in a subset of patients (43/79; 54.4%) Ct values were available and used to calculate viral load decay normalized per day (Ct/day). No difference in viral load decay was observed between the groups of patients. However, the highest reduction in SARS-CoV-2 RNA was observed in untreated patients (median 1.27, range 0.50–3.25 Ct/day) as compared to Remdesivir (median 0.78, range 0.40–1.60 Ct/day),

sotrovimab (median 0.75, range 0.29–2.22 Ct/day) and Molnupiravir (median 1.00, range 0.61–1.88 Ct/day) (Figure 3B). Only one patient treated with ritonavir-boosted nirmatrelvir had data on viral load decay (2.13 Ct/day).

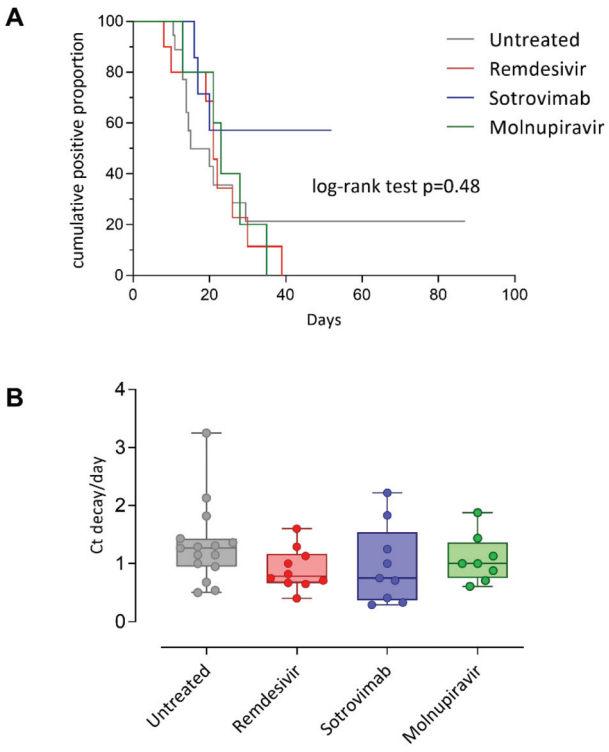

Figure 3. Kaplan–Meier curves of viral shedding duration in untreated and treated patients (**A**). SARS-CoV-2 RNA load clearance in different patients' categories (**B**).

4. Discussion

In the present study, we did not notice a significant impact of early anti-SARS-CoV-2 treatments on the COVID-19-related 28-day hospitalization rate and SARS-CoV-2 load decay in patients with hematological malignancy or HSCT. However, untreated patients with negative anti-SARS-CoV-2 antibodies had a significantly higher risk of being hospitalized than treated ones.

Patients with hematologic malignancies and HSCT might experience a relatively slow viral decay and, as a result, the duration of RT-PCR positivity in these patients was longer than that of other patients [7]. Based on previous studies, a beneficial impact of early therapies on hastening the SARS-CoV-2 viral decay was expected [14,23,24]. Interestingly, our data did not confirm this hypothesis. This result should be taken with caution since the absence of a significant effect could also be explained by a lack of statistical power due to the relatively small sample size Although a prolonged duration of RT-PCR positivity does not indicate higher severity of COVID-19 [24], the fact that the viral load in these patients is long-lasting has serious healthcare implications. In fact, RT-PCR positivity in these patients generally prevents the implementation of specific treatments for their underlying disease, and access to outpatients' care services.

In summary, the clinical and therapeutic management of hematologic malignancies and HSCT represent a major challenge for physicians. In this regard, and especially because of the constant surfacing of new SARS-CoV-2 variants of concern, we should reflect on the need of patients with hematologic malignancies or HSCT for updated vaccination

strategies, such as prompt additional vaccine doses, which might be an effective choice to enhance immunity response [25]. Even though it has been reported that the severity of the Omicron SARS-CoV-2 variant is attenuated [26,27], this is likely due to population immunity rather than to a characteristic of the virus. Therefore, despite the ongoing trend of gradually relaxing epidemic containment measures, these patients should be instructed to maintain infection control measures, such as aerosol and contact full isolation, social distancing, and wide use of masks and personal hygiene measures.

We believe that it is extremely valuable to perform real-life studies on these patients, because of their high risk of mortality and morbidity due to COVID-19 [28–30], and their low response to anti-SARS-CoV-2 vaccines [6] due to their specific illness, chemotherapy, and other immunosuppressive treatments. Our data confirm this unfortunate trend, as only slightly more than half of the subgroup of fully immunized patients with hematologic malignancies were serologically positive for IgG anti-SARS-CoV-2 spike protein. The fact that patients with hematologic malignancies who have failed to mount an adequate SARS-CoV-2 vaccine response encounter poor outcomes is well known [26], and our data support the relevance of providing a timely treatment to these patients using early therapies against COVID-19.

We have to mention some limitations of our study, such as its retrospective and monocentric nature, and the relatively small sample size. Moreover, due to the real-life experience, we did not exclude those patients treated with molnupiravir, which is less effective than the other treatments [18]. Finally, since our sample only includes patients who were infected by the Omicron variant, the generalization of our results to patient affected by other variants should be executed with caution. However, to the best of our knowledge, no previous data supporting the use of early drugs in patients with hematologic malignancies or HSCT are available. Therefore, we believe that this study fills this literature gap with real-life daily practice findings.

In conclusion, we believe that reporting these real-life data may still be the most appropriate approach to appreciate how to focus our full consideration of patients with hematologic malignancies and HSCT patients from different perspectives. However, more data are needed to understand the best way to manage the SARS-CoV-2 infection in this particularly fragile population.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board of Fondazione IRCCS Policlinico San Matteo (protocol code 0031226/22).

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

Conflicts of Interest: The authors declare no conflict of interest.

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Efficacy of Bromhexine versus Standard of Care in Reducing Viral Load in Patients with Mild-to-Moderate COVID-19 Disease Attended in Primary Care: A Randomized Open-Label Trial

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Abstract: A 28-day randomized open-label multicenter study was conducted to assess the efficacy of bromhexine plus standard of care (SOC) ($n = 98$) vs. SOC alone ($n = 93$) in 191 outpatients with mild-to-moderate COVID-19 in the primary health care setting. Bromhexine three daily doses of 10 mL (48 mg/day) were administered for seven days. The primary efficacy endpoint was the reduction of viral load estimated as the cycle thresholds (Ct) to detect ORF1ab, N Protein, and S Protein genes by RT-qPCR in saliva samples on day 4 as compared with baseline. Ct values of the three genes increased from baseline throughout days 4 to 14 ($p < 0.001$) but significant differences between the study groups were not found. Differences in the percentages of patients with low, medium, and high viral loads at 4, 7, and 14 days were not found either. In summary, treatment with bromhexine plus SCO was associated with a viral load reduction of ORF1ab, N Protein, and S Protein genes at day 4, which was not significantly different than similar viral load reductions observed with SOC alone. The present findings do not seem to favor the use of bromhexine as an antiviral in patients with COVID-19.

Keywords: COVID-19; SARS-CoV-2; bromhexine; standard of care; viral load; primary care

1. Introduction

Bromhexine is a marketed mucoactive drug currently indicated as a symptomatic treatment of upper respiratory infections. It is an old over-the-counter medication that has been extensively used for decades as a mucolytic agent, it is well-tolerated and safe. The adverse reactions related to the use of bromhexine were of low frequency ($\geq$1/1.000 to <1/100) and include vomiting, diarrhea, nausea, and upper abdominal pain. The spread of COVID-19 has stimulated huge efforts to find active treatments against SARS-CoV-2 infection, either searching for novel molecules or repurposing old drugs [1].

Cell entry of coronaviruses depends on the binding of the viral spike (S) proteins to cellular receptors and on S protein priming by proteases of host cells [2]. It has been shown that SARS-CoV-2 uses the SARS-CoV receptor angiotensin-converting enzyme 2 (ACE2) as the entry receptor and employs cellular transmembrane protease serine 2 (TMPRSS2) for S protein priming [3,4]. Therefore, TMPRSS2 inhibitors approved for clinical use blocking host cell entry might constitute a treatment option for COVID-19. A potential mechanism of action of bromhexine is related to the blockade of virus entry into the cell mediated by the TMPRSS2 receptor [5,6].

There is limited data on the potential role of bromhexine in the management of COVID-19. It is relevant to highlight that bromhexine has been initially identified as a potent inhibitor (IC50 = 0.75 μM) of the transmembrane serine protease 2 (TMPRSS2) of SARS-CoV [5], being involved also in the binding and infection (mainly via a non-endocytotic route) of SARS-CoV-2 to host cells [7]. The probability of success in identifying molecules with antiviral potential is markedly increased by including different phases of the viral replication cycle [8]. Recent studies ruled out that TMPRSS2 inhibition is responsible for the antiviral activity of bromhexine in SARS-CoV-2, as slight antiviral activity is reported in VeroE6 cells, which lack TMPRSS2 in their membranes [4]. Moreover, a multitarget approach of bromhexine to several viral and human proteins may explain its potential efficacy against SARS-CoV-2 [9].

While the assessment of bromhexine clinically in the care of patients with COVID-19 has been encouraged [10,11], only a few clinical studies have been published in the literature. In 111 hospitalized patients with confirmed COVID-19 randomized 1:1 to treatment with bromhexine (8 mg four times daily) or standard treatment lopinavir/ritonavir and interferon beta-1a, there was no difference in clinical improvement within 28 days (primary outcome) as well as in other secondary outcomes including length of intensive care unit (ICU) stay, the average time to hospital discharge, duration of supplemental oxygen, or risk of death by day 28 [12]. In contrast, in another randomized open-label study of 78 patients, the early administration of bromhexine (8 mg four times daily) for 2 weeks in addition to standard therapy reduced the need for ICU admission, intubation/mechanical ventilation, and 28-day mortality [13]. In a randomized open-label study of medical staff actively involved in the care of patients with suspected or confirmed SARS-CoV-2 infection, prophylactic treatment with bromhexine (8 mg three times daily) was associated with fewer participants who developed symptomatic COVID-19 as compared to controls, although differences in positive swab PCR test or signs of clinical infection at day 28 were not found [14].

However, the potential efficacy of bromhexine in asymptomatic post-exposure subjects or in patients with mild infection managed in the outpatient setting remains to be determined. As the infective capacity is related to the patient's viral load, if we were to achieve an antiviral therapy that reduces the viral load and acts on the patient population that has not yet developed symptoms or has developed them recently, we could impact the capability to transmit the virus early, and also delay or prevent the appearance of the first symptoms as well as the disease progression to more severe forms [15].

Therefore, the present randomized open-label clinical trial was conducted to assess the efficacy of bromhexine as compared with standard of care (SOC) to reduce the viral load in patients with mild-to-moderate COVID-19 disease attended in the primary healthcare setting. To the best of our knowledge, this is the first comparative drug repositioning study

to evaluate the benefits of an old drug in the treatment of infection caused by SARS-CoV-2 in patients with early-stage COVID-19 disease including asymptomatic subjects.

2. Materials and Methods

2.1. Study Design and Objectives

This was a phase 3, randomized, open-label, parallel group, controlled, multicenter clinical trial conducted in 19 primary healthcare centers located in the autonomous community of Madrid, Spain. The study period began on 24 February 2022 and finished on 28 July 2022. The duration of the study for each patient was 28 days.

The primary objective was to assess the efficacy of bromhexine plus SOC (active treatment) versus SOC alone (control) in reducing viral load at day 4 from baseline. Secondary objectives included the efficacy of bromhexine plus SOC versus SOC to get negative PCR from baseline. To reduce the intensity and duration of symptoms, to assess the need for medical care, admission to the hospital, and oxygen therapy, the mortality rate through day 28 from baseline, and safety of the active treatment.

The study was conducted in accordance with the principles of the Declaration of Helsinki and approved by the Medicinal Product Research Ethics Committee of Hospital Universitario Puerta de Hierro Majadahonda (Madrid, Spain) (code 21/2021, approval date 12 December 2021). The study was registered at European Union Drug Regulating Authorities Clinical Trials Database (EudraCT) (number 2021-001227-41). Written informed consent was obtained from all participants.

2.2. Participants

Eligible subjects were men or women aged 18 years or older, diagnosed with active SARS-CoV-2 infection confirmed by a positive rapid antigen detection test or a PCR test for viral RNA detection in the presence of compatible symptoms (fever, cough, shortness of breath or difficulty breathing, sore throat, body or muscle pain, fatigue, headache, chills, nasal congestion, loss of taste or smell, nausea or vomiting, and diarrhea). Symptomatic patients were required to have had one or more of the clinical manifestations within the last 72 h, the severity of which was mild or moderate. Exclusion criteria were patients living with a patient who had been enrolled in the present study and continued to be followed over the 28-day study period; patients with severe COVID-19; the presence of diseases that may be affected or interfere with the results of the study (such as active infections other than SARS-CoV-2 requiring systemic therapy, uncontrolled respiratory disorder, prior ischemic heart disease, heart failure or atrial fibrillation, severe renal failure, active or treated malignancy, immunosuppression status, expected elective surgery within 30 days after screening for the study, severe obesity); concomitant treatment with drugs with known antiviral potential; hypersensitivity or intolerance to bromhexine (or any of the excipients); pregnant or breast-feeding women; inability to understand the informed consent; ineligibility as judged by the investigators; and participation in a clinical trial within the last 30 days. All the patients needed to be informed about the study procedures and sign the informed consent form.

2.3. Randomization and Intervention

Randomization was generated by an independent technician using a web-based randomization system (http://www.randomization.com, accessed on 22 November 2022). Patients were randomized 1:1 to the active treatment or the control arm according to an allocation sequence in random blocks of 4 and 6 treatments for a total of 10 treatments for each study center. The order of blocks in each group of 10 treatments was also randomized. The allocation concealment was done by electronic database monitoring. After the patient signed the informed consent, the investigator opened the randomization envelopes and assigned the corresponding intervention.

Patients randomized to the active treatment received bromhexine, 3 daily doses of 10 mL (48 mg/day) for 7 days plus SOC therapy. Two bottles of 200 mL (16 mg per 10 mL)

were provided to each patient. Since the efficacious daily dose of the active product with viral load reduction capacity was unknown, the maximum labeled dose of the marketed product (16 mg/10 mL 3 times daily equal to 48 mg/day or 30 mL/day) for 7 days was analyzed. No bromhexine dose increase was allowed. Labeling and packing of bromhexine followed the Good Manufacturing Practice (GMP) regulations and local or national regulatory requirements.

The SOC for SARS-CoV-2 infection included acetaminophen 500 mg (1–4 times daily), non-steroidal anti-inflammatory drugs (NSAIDs), symptomatic treatment, and hydration for mild COVID-19. In moderate disease and only in case of suspicion of bacterial coinfection/superinfection, the following should be prescribed: oral azithromycin 500 mg every 24 h for 3 days plus amoxicillin 1 g every 12 h for 7 days, or amoxicillin-clavulanate 875–125 mg every 8 h for 7 days; or alternatively, levofloxacin 500 mg every 12 h on the first day and 500 mg every 24 h for 4 days. Other treatments when required included bronchodilators or inhaled corticosteroids in patients with asthma or chronic obstructive pulmonary disease (COPD), low doses of systemic corticosteroids in patients requiring oxygen therapy, and antithrombotic prophylaxis in patients immobilized or with risk factors for thrombosis [16,17].

2.4. Study Procedures

The study included a screening visit (baseline), in which eligibility criteria were confirmed, a complete medical history was taken, a SARS-CoV-2 rapid antigen test was performed, a salivary sample was collected for a SARS-CoV-2 PCR test, a peripheral fasting blood sample was drawn for laboratory analyses, the informed consent was signed, and a diary and the study medication were provided. Patients were instructed on how to take the assigned medications and to complete the diary card, in which the hospitalization criteria were described in plain language.

Telephone contacts were completed on days 1, 4, 7, and 14 after starting treatment. At the end of the study, on day 28, patients were visited at the primary care center. Saliva samples for SARS-CoV-2 PCR assay were collected on baseline and days 4, 7, and 14 at the patients' homes due to the limitation of medical visits in quarantined patients. In all telephone contacts, pulse oximetry data, heart rate, and temperature recorded by the patient with the study material supplied for that purpose were registered. Questions about the appearance of new symptoms and the severity of symptoms were assessed on a numerical rating (NRS) severity scale of 0 to 10 points (0 = no symptoms, 10 = the most severe symptoms imaginable). Symptoms recorded in the diary card as well as non-prescribed concomitant drugs were communicated to the physician during the telephone calls. Also, the investigator asked the patients if they have experienced any adverse events since the last study contact, and if any exist, recorded them on the "Adverse Event" case report form page and described the event. All adverse events were followed until their resolution or chronicity.

2.5. Viral Load

Viral load was determined by the detection of three highly conserved epitope regions within the SARS-CoV-2 pathogenic viral RNA strain, pen reading frame ORF) 1ab (ORF1ab), nucleocapsid N protein (N Protein), and spike S protein (S Protein), in saliva samples on baseline and days 4, 7, and 14 after initiation of treatment. These analyses were performed in a central laboratory (Arquimea Medical, S.L., Leganés, Madrid, Spain). Viral RNA was obtained using the chemagic™ Viral DNA/RNA 300 kit H96 from (PerkinElmer España, S.L., Tres Cantos, Madrid, Spain), and purification was carried out using the automated chemagic 360 Instrument (PerkinElmer). RT-qPCR was completed with the TagPath™ COVID-19 CE-IVD RT-PCR Kit (Thermo Fisher, Waltham, MA, USA), and detection of OFR1ab, N Protein, and S Protein was completed in the 7500 Real-Time PCR Instrument (Thermo Fisher) and QuantStudio Real-Time PCR Instrument (Thermo Fisher). The sensitivity and specificity of the platform are >99% and 99.5%, respectively. The viral load was estimated as

the number of amplification cycles (cycle thresholds, Ct) to detect genes encoding ORF1ab, N Protein, and S Protein in a single PCR reaction. An RT-qPCR for SARS-CoV-2 was considered positive in the presence of a Ct value lower than 35 for at least two of the three genes analyzed. A higher number of cycles means a lower viral load. Viral load was defined as 'high' for Ct values $\leq$ 25, 'medium' for Ct values > 25 and $\leq$30, and 'low' for Ct values $\geq$ 30.

2.6. Definitions

Asymptomatic or pre-symptomatic infection was defined in the presence of a positive diagnostic RT-qPCR test for SARS-CoV-2 in a patient without symptoms of COVID-19 disease. 'Mild' disease was defined in the presence of a positive RT-qPCR test for SARS-CoV-2 in a patient with any COVID-19-related symptoms (e.g., fever, cough, sore throat, malaise, headache, body/muscle pain, nausea/vomiting, diarrhea, loss of taste or smell) in the absence of tachypnea, shortness of breath, or abnormal findings on chest X-rays. 'Moderate' disease was defined in the presence of a positive RT-qPCR test for SARS-CoV-2 in a patient with evidence of lower respiratory tract disease as shown at physical examination (tachypnea, shortness of breath) or abnormal findings on chest X-rays, with an oxygen saturation (SpO_2) level of $\geq$94% measured by a pulse oximeter. Clinical improvement was defined as a reduction of 2 or more points in the 0–10 NRS of the severity of symptoms [16,17].

2.7. Efficacy Endpoints

The primary efficacy endpoint was the reduction in viral load (day 4 vs. baseline) in the active treatment group (bromhexine plus SOC) as compared with the control group (SOC alone). Secondary efficacy endpoints were the reduction in viral load (day 7 vs. baseline and day 14 vs. baseline) in the two study arms; the proportion of patients with a negative RT-qPCR test for SARS-CoV-2 (Ct value > 35 in at least two of three genes) in the two study arms; the time to achieve a negative viral load from baseline in the two study arms; and the comparison of the clinical efficacy in the two study arms, including reduction in the severity of each symptom (0–10 NRS score) at days 4, 7, 14, and 28 as compared with baseline; proportion of patients with clinical improvement and time to clinical improvement; proportion of patients with disappearance of each symptom at days 4, 7, 14, and 28, and time to disappearance; proportion of asymptomatic patients at days 4, 7, 14, and 28; proportion of patients requiring medical care, admission to the hospital, oxygen therapy, and development of complications related to COVID-19 disease over the study period; 28-day mortality rate; mortality rate after the end of study; and safety of bromhexine.

2.8. Statistical Analysis

The null hypothesis was established as the absence of differences in the reduction of viral load after 4 days of starting treatment as compared with baseline (prior to treatment) between the two study groups. The sample size calculation for the primary efficacy endpoint was performed for a two-sided analysis of variance (ANOVA), with fixed effects and two levels in the factor evaluated corresponding to the active treatment or the control group. A type I error was set at a two-sided 0.05 level with a minimal effect with clinical relevance of 2 log10 reductions in viral copy number as the minimal difference between the on-treatment groups. A moderate effect of 0.25 (Cohen's f) was targeted leading to an expected common standard deviation (SD) of 4 log10. Given a sample of 200 patients (100 assigned to bromhexine plus SOC and 100 assigned to SOC alone), a power of 94% was obtained to demonstrate the estimated difference (Sample Power, IBM-SPSS). The intention to treat (ITT) dataset (all randomized patients who received at least one dose of the study medication) was considered for efficacy and safety analysis.

The main analysis of the primary efficacy endpoint was measured by the Student's *t*-test for independent samples. The ANOVA for repeated measurements and a factor (Split-Plot) with Bonferroni adjustment for multiple comparisons was applied to the comparison

of viral load between the study groups at baseline, day 4, day 7, and day 14. The primary analysis was adjusted based on justified demographic and effect-modifying variables. Type I error was established at a two-sided 0.05 level. The software IBM-SPSS Version 27.0 (IBM Corp., Armonk, NY, USA) was used for the statistical analysis.

3. Results

3.1. Disposition of Patients

A total of 193 eligible patients were recruited by 19 participating centers and were randomized (99 to bromhexine plus SOC and 94 to SOC alone). However, one patient in each group was excluded because of a negative RT-qPCR test for SARS-CoV-2 at baseline. At follow-up, four patients (two in each study group) withdrew from the study, three of them because of the patients' own decisions and one because of the need for in-patient care. The final evaluable ITT population included 191 patients, 98 in the bromhexine plus SOC group and 93 in the SOC alone group. The flow chart of the study population is shown in Figure 1.

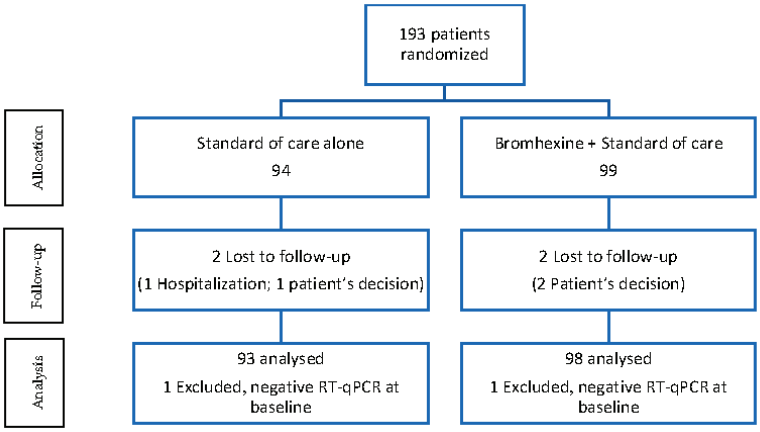

Figure 1. Flow chart of the study population. Analysis was based on the ITT dataset.

3.2. Baseline Characteristics

A total of 127 patients were women (66.5%) and 64 men (33.5%), with a mean (SD) age of 47.8 (1.1) years. Almost all patients were Caucasian (93.7%) and 6.3% Hispanic. History of previous COVID-19 was recorded in 37 patients (19.4%), with a mean time elapsed from infection to enrollment in the study of 16.3 (1.4) months. A total of 182 patients (95.3%) had been vaccinated against SARS-CoV-2 and had received a mean number of doses of 2.4 (0.06), with a mean of 5.3 (0.2) months from the last vaccination dose. As shown in Table 1 differences in demographics, BMI, and data of previous SARS-CoV-2 infection between the study groups were not found.

In relation to the severity of COVID-19, 179 patients (95.9%) presented with mild disease, 7 (3.7%) with moderate disease, and 5 (2.6%) were asymptomatic. The distribution of patients according to the severity of disease was similar in the two study groups, with 1 (1%) and 4 (4.3%) asymptomatic patients, 94 (95.9%) and 85 (91.4%) patients with mild disease, and 3 (3.1%) and 4 (4.5%) patients with moderate disease in the bromhexine plus SOC and SOC alone groups, respectively.

Table 1. Demographic and previous SARS-CoV-2 infection in the study groups.

Variables	Total Patients (n = 191)		SOC Alone (n = 93)		Bromhexine + SOC (n = 98)		p Value
	N (%)	Mean (SD)	N (%)	Mean (SD)	N (%)	Mean (SD)	
Gender							
Male	64 (33.5)		34 (36.6)		30 (30.6)		0.384
Female	127 (66.5)		59 (63.4)		68 (69.4)		
Age, years		47.8 (1.1)		48.4 (1.5)		47.2 (1.6)	0.570
Race *							
Caucasian	179 (93.7)		88 (94.6)		91 (92.9)		0.615
Hispanic	12 (6.3)		5 (5.4)		7 (7.1)		
Body mass index, kg/m^2		25.8 (0.4)		25.9 (0.6)		25.7 (0.5)	0.837
Previous COVID-19 infection							
No	154 (80.6)		75 (80.6)		79 (80.6)		0.995
Yes	37 (19.4)		18 (19.4)		19 (19.4)		
Severity of previous COVID-19 infection							
Asymptomatic *	1 (2.7)		1 (5.6)		0		0.511
Mild	22 (59.5)		12 (66.7)		10 (52.6)		
Moderate	11 (29.7)		4 (22.2)		7 (36.8)		
Severe	3 (8.1)		1 (5.6)		2 (10.5)		
Persistent	0		0		0		
Time from previous SARS-CoV-2 infection, months		16.3 (1.4)		16.0 (1.8)		16.6 (2.1)	0.831

* Fisher exact test was applied. SOC: standard of care; SD: standard deviation.

The number of symptoms ranged between 0 and 19, with a mean of 6.1 (0.3) symptoms. The distribution of severity of symptoms was similar in the two study groups, although nasal congestion was significantly more severe in the SOC alone group than in the bromhexine plus SOC group (mean 6.4 [2.1] vs. 5.3 [2.0], $p = 0.008$); ear pain was also more severe in the SOC alone group (mean 7.0 [2.7] vs. 3.8 [1.6], $p = 0.047$). The results of the physical examination were similar in the two study groups (Table 2).

Table 2. Data of physical examination in the two study groups.

Variables	Total Patients (n = 191)		SOC Alone (n = 93)		Bromhexine + SOC (n = 98)		p Value
	N	Mean (SD)	N	Mean (SD)	N	Mean (SD)	
Systolic BP, mmHg	191	123 (1.0)	93	123 (1.0)	98	124 (1.0)	0.677
Diastolic BP, mmHg	191	76 (1.0)	93	76 (1.0)	98	76 (1.0)	0.740
Respiratory rate, breaths/min	191	16 (0)	93	16 (0)	98	16 (1.0)	0.473
Oxygen saturation, %	190	97 (0)	93	97 (0)	97	97(0)	0.624
Heart rate, beats/min	190	79 (1.0)	93	78 (1.0)	97	79 (1.0)	0.579
Axillary temperature, °C	190	36.5 (0.1)	93	36.5 (0.1)	97	36.4 (0.1)	0.703

SOC: standard of care; SD: standard deviation.

A total of 126 patients (66%) were receiving treatment for medical conditions at inclusion in the study. Statistical differences between the study groups were observed in the percentage of patients treated with bronchodilators ($p = 0.033$) and receiving symptomatic treatment ($p = 0.034$), which were higher in the SOC alone group, whereas treatment for concomitant diseases was higher in the bromhexine plus SOC group ($p < 0.001$). The administration of C (cardiovascular system) products and D (Dermatological) drugs was higher in the bromhexine plus SOC group ($p = 0.002$ and $p = 0.012$, respectively). The use of R (respiratory system) drugs was higher in the SOC alone group ($p < 0.001$).

The viral load was homogeneous between the study groups, with a mean (SD) Ct value of 22.5 (0.6) for ORF1ab, 22.8 (0.6) for N Protein, and 47.1 (2.4) for S Protein. The percentages of patients with low, medium, and high viral loads were 6.1%, 25.5%, and 68.4% for the bromhexine plus SOC group and 8.6%, 19.4%, and 72% for the SOC alone group.

3.3. Efficacy Endpoints

Changes in viral load from baseline to day 4 were similar in the two study groups for the three specific SARS-CoV-2 genes (Figure 2). The mean Ct values for ORF1ab viral load were 13.54 (26.02) in the bromhexine plus SOC group as compared with 14.43 (26.94) in the SOC alone group (mean difference 0.89, 95% CI −6.67 to 8.45; $p = 0.817$). The mean Ct values of N Protein were 7.70 (18.47) in the bromhexine plus SOC group and 6.36 (17.05) in the SOC alone group, with a mean difference of -1.34 (95% CI −6.42 to 3.74; $p = 0.603$). For the S Protein, the mean Ct values were 9.74 (29.54) and 13.78 (26.81) for the bromhexine plus SOC and SOC alone groups, respectively, and a mean difference of 4.04 (95% CI −4.30 to 12.37; $p = 0.340$).

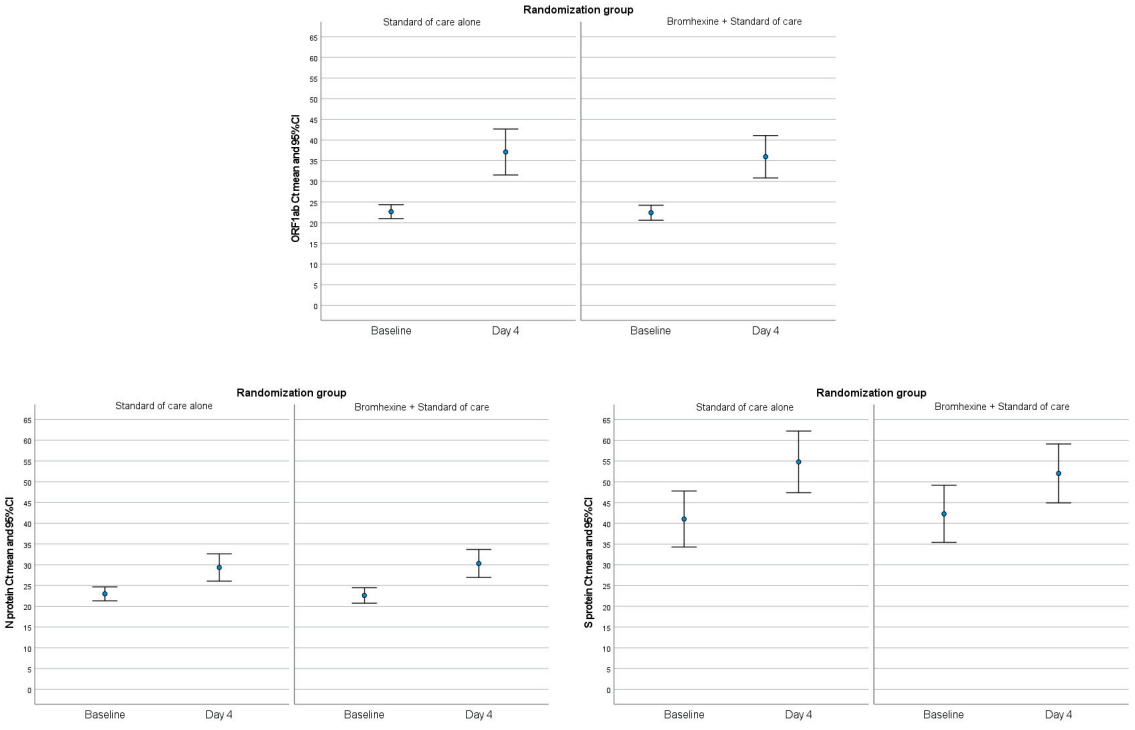

Figure 2. Reduction of viral load from baseline to day 4 of treatment for ORF1ab, N Protein, and S Protein in the two study groups. For all the comparisons Baseline versus Day 4, p value was <0.001.

In the overall study population, Ct values of ORF1ab, N Protein, and S Protein increased significantly from baseline throughout days 4 to 14 ($p < 0.001$). For the comparison of Ct values of ORF1ab between the two study groups, there were no significant differences on day 4 ($p = 0.765$), day 7 ($p = 0.431$), and day 14 ($p = 0.163$). Similar findings were obtained for Ct values of N Protein at day 4 ($p = 0.678$), day 7 ($p = 0.961$), and day 14 ($p = 0.583$), as well as for Ct values of S Protein at day 4 ($p = 0.592$), day 7 ($p = 0.450$), and day 14 ($p = 0.124$) (Figure 3).

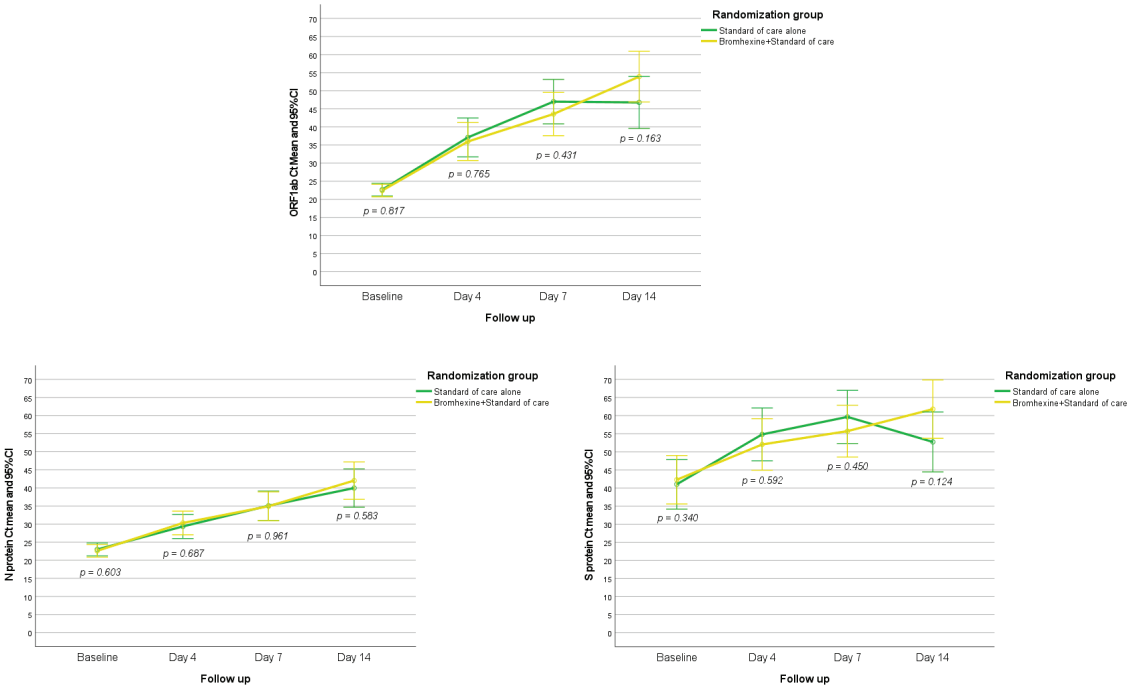

Figure 3. Evolution of Ct values of ORF1ab, N Protein, and S Protein at follow-up in the two study groups.

A sensitivity analysis performed on the primary efficacy endpoint and in the evolution of the viral load without imputation rules applied to the dataset showed no significant differences in the main efficacy results between the study groups.

No significant differences were found between bromhexine plus SOC and SOC alone in the percentage of patients with RT-qPCR positivity on day 4 (86.7% vs. 80.6%, $p = 0.254$), day 7 (74.5% vs. 65.6%, $p = 0.179$), and day 14 (53.1% vs. 61.3%, $p = 0.251$). Differences in the percentages of patients with low, medium, and high viral loads between the study groups at 4, 7, and 14 days were not found either. The median time to obtain an RT-qPCR negative result was 14 days (95% CI 12.2 to 15.8), without a significant difference between the study groups ($p = 0.565$).

No significant differences between the study groups were observed in the evolution of the vital signs that significantly improved from day 1 to day 28 ($p < 0.05$) in the oxygen saturation, heart rate ($p < 0.01$), and axillary temperature ($p < 0.001$). Also, there were no significant differences between the study groups in the severity of any of the symptoms observed throughout the study period, except for more intense dysgeusia in the SOC alone group than in the bromhexine plus SOC group (3 vs. 1.6 points, $p = 0.005$) and arthralgia (2.4 vs. 1.7 points, $p = 0.014$) on day 4. A total of 38 patients (19.9%) continued with persistent symptoms after day 28, with no differences between the study groups (Table 3).

Patients with previous SARS-CoV-2 infection showed significant lower viral load at baseline and during the follow-up compared to patients with no previous COVID-19 (Figure 4). This difference was not observed on the vaccinated versus non vaccinated patients.

Table 3. Persistent symptoms observed after 28 days of follow-up in the two study groups.

Symptom	SOC Alone Group (n = 45) N (%)	Bromhexine + SOC Group (n = 52) N (%)	Total (n = 97) N (%)
Fever	0	1 (1.9)	1 (1.0)
Cough	9 (20.0)	5 (9.6)	14 (14.4)
Odynophagia	3 (6.7)	2 (3.8)	5 (5.2)
Dyspnea	3 (6.7)	1 (1.4)	4 (4.1)
Chest pain	0	1 (1.9)	1 (1.0)
Chills	1 (2.2)	1 (1.9)	2 (2.1)
Nausea	1 (2.2)	0	1 (1.0)
Vomiting	1 (2.2)	0	1 (1.0)
Diarrhea	2 (4.4)	2 (3.8)	4 (4.1)
Abdominal pain	0	2 (3.8)	2 (2.1)
Nasal congestion	6 (13.3)	7 (12.5)	13 (13.4)
Anosmia	2 (4.4)	4 (7.7)	6 (6.2)
Dysgeusia	2 (4.4)	1 (1.9)	3 (3.1)
Headache	3 (6.7)	4 (7.7)	7 (7.2)
Myalgia	1 (2.2)	3 (5.8)	4 (4.1)
Arthralgia	1 (2.2)	3 (5.8)	4 (4.1)
Weariness	6 (13.3)	6 (11.5)	12 (12.4)
Weakness	1 (2.2)	4 (7.7)	5 (5.2)
Anorexia	1 (2.2)	1 (1.9)	2 (2.1)
Dizziness	0	2 (3.8)	2 (2.1)
Depression	0	1 (1.9)	1 (1.0)
Conjunctival congestion	0	1 (1.9)	1 (1.0)
Pale, cold skin	1 (2.2)	0	1 (1.0)
Thrombotic phenomena	1 (2.2)	0	1 (1.0)

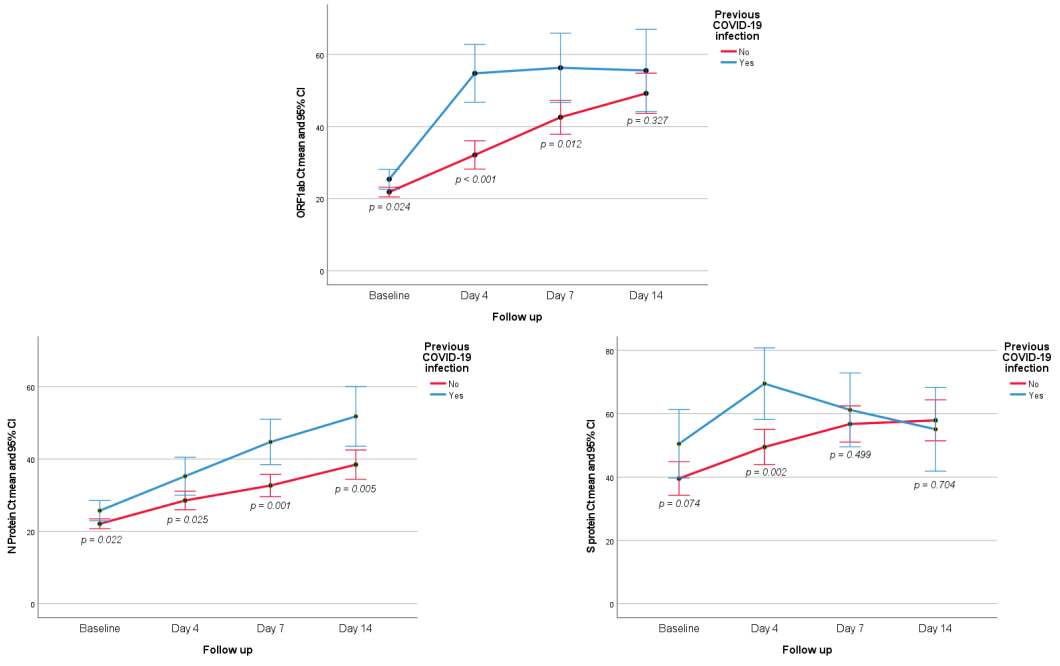

Figure 4. Evolution of ORF1ab, N Protein, and S Protein viral load according to the presence or absence of previous COVID-19 infection.

3.4. Safety Outcome

A total of 13 patients (6.8%) experienced adverse events, 8 patients in the bromhexine plus SOC group (8.2%) and 5 patients in the SOC alone group (5.4%), with no statistically significant difference ($p = 0.445$). The total number of adverse events observed was 17, 64.7% ($n = 11$) mild, and 23.5% ($n = 4$) moderate. A case of unrelated severe dizziness in one patient (5.9%) and another case of serious pulmonary thromboembolism (5.9%) were observed.

Three adverse events were considered related to bromhexine (dizziness, nausea, and pasty mouth), two possibly related (constipation and tinnitus), and one unknown (pruritus), with 11 adverse events unrelated to the study treatment (64.7%). No adverse event led to premature discontinuation of the study drug. Two moderate treatment-emergent laboratory abnormalities were observed in the bromhexine plus SOC group but were considered unrelated (leucocyte elevation, transaminase elevation).

At 12 days after the initiation of the study, one patient from the SOC alone group was required to be admitted to the hospital and oxygen therapy due to the worsening of COVID-19.

None of the patients died 28 days after completion of the study.

4. Discussion

This clinical trial explored the antiviral activity in clinical practice of an already marketed product, bromhexine, as drug repositioning in combination with standard of care. No differences were observed in the viral load at day 4 of the initiation of the study treatment in patients treated with bromhexine compared to those that only received standard of care.

Of the studies carried out in patients with COVID-19 registered in the Spanish Clinical Studies Registry (REEC) at the initiation date of the study, most of them were conducted in the hospital setting, in critically ill or moderately ill patients, and only 21.9% included patients mildly affected or asymptomatic patients. In fact, very few studies are being conducted in the outpatient setting, where there is a higher volume of patients with COVID-19. The higher proportion of studies in patients with moderate-to-severe disease is justified by the urgent need for treatments for patients at higher risk. However, the highest volume of patients infected with SARS-CoV-2 and capable of transmitting the disease are patients with mild symptoms and asymptomatic patients. These groups of patients are diagnosed, treated, and followed in primary care centers, the setting in which this study was conducted.

No previous clinical trials with bromhexine in patients with COVID-19 have been carried out in Spain, and there is limited evidence of the efficacy of this drug in the literature, the usefulness of which remains controversial [10–14]. Despite the recognition of the pharmaceutical properties of bromhexine to inhibit TMPRSS2 and its potential role in treating or preventing SARS-CoV-2 infection [10,16,18–21], expectations of efficacy in clinical practice appeared to be disappointing, which are consistent with findings of the present study.

In this randomized clinical study, the active treatment group (bromhexine plus SOC) was compared with a control group of SOC alone. A sample of 191 patients was included, 66.5% were women, with a mean age of 47.8 years, which is in agreement with overall data recorded in Spain with the most affected age range during the pandemic being between 50 and 59 years, with 55% of women [22]. The eligibility criteria established in the study limited the recruitment rate, since the groups of patients at higher risk of developing COVID-19 (e.g., older age, cardiovascular disease, COPD, cancer, immunosuppression, and other conditions) were excluded. The large majority of patients had mild disease, which accounted for a high mean number of clinical symptoms of 6.1 at initial presentation. Overall baseline data, including vital signs and distribution of symptoms, was similar in the two study groups, except for nasal congestion and ear pain, which were more severe in the SOC alone group. The prescription of bronchodilators and symptomatic treatments was more frequent in the SOC alone group, but differences for specific drugs were not found.

Viral load at baseline was similar in the two study groups as well as the percentages of patients with low, medium, and high viral loads. It was not possible to obtain the translation from Ct values to the number of viral copies, so comparison to viral loads reported in other studies is not possible. However, in other studies of bromhexine in hospitalized patients [12,13] or medical personnel [14], viral loads were not measured. In a protocol for systemic review and meta-analysis to assess the efficacy and safety of bromhexine hydrochloride tablets in treated pediatric COVID-19, assessment of viral load was not included among the types of outcome measures [23].

In relation to the primary efficacy endpoint of a reduction in the viral load from baseline to day 4, there were no differences between the study groups for none of the specific genes of the SARS-CoV-2 pathogenic viral RNA strain. In all three ORF1ab, N Protein, and S Protein genes, statistically significant reductions in viral loads were found from baseline to any time point of the follow-up for the overall study population, but differences at days 4, 7, and 14 between the study groups were not observed and these findings were confirmed in the sensitivity analysis. On the other hand, the percentage of patients with positive RT-qPCR results on days 4, 7, and 14 were similar in the two study groups, as was the percentage of patients classified into the groups of low, medium, and high viral loads. Patients with previous SARS-CoV-2 infection showed lower viral loads than patients without a history of COVID-19 disease, but differences between vaccinated and non-vaccinated patients were not found.

Other clinical data included the lack of differences between the study groups in the evolution of vital signs, overall improvement of severity of symptoms, and percentage of patients with persistent symptoms after day 28.

Regarding safety outcomes, a few patients reported adverse events without differences between the study groups. Most adverse events were of mild intensity and unrelated to treatment. In three cases (dizziness, nausea, and pasty mouth), adverse events were considered to be related to the use of bromhexine, and 2 cases (constipation and tinnitus) were possibly related. None of the patients discontinued the study because of any adverse event. One patient in the SOC alone group required in-patient care and oxygen therapy, with a successful recovery.

The open-label design is a limitation of the study. Although the primary efficacy endpoint was a laboratory variable on which a placebo effect is unlikely to occur, the inclusion of a control arm was important to determine whether there were differences in the evolution of the viral load when patients received the active medication, as well as to compare variables not considered in the study design that could influence the primary or secondary efficacy endpoints. In fact, we observed differences in the administration of concomitant drugs, with the use of bronchodilators and symptomatic treatments more frequently among patients in the SOC alone group. The relationship between these therapies and the reduction of viral load is unknown. In addition, the effect of bromhexine on the evolution of symptoms could not be evaluated due to the limited sample size. The SARS-CoV-2 virus variant responsible for the infection suffered by the study patients was not analyzed. The most frequent variant at the time of study completion in Spain was Omicron (100%) BA.5 and derivatives [24], but it has not been studied whether the mechanism of action of bromhexine might differ as per virus variant. So, it is unknown if the results could have been different if the study should be completed earlier in the pandemic.

5. Conclusions

In this study, treatment with bromhexine plus SCO was associated with a viral load reduction of ORF1ab, N Protein, and S Protein genes at day 4, which was not significantly different than similar viral load reductions observed with SOC alone. The present findings do not seem to provide arguments in favor of using bromhexine for treating patients with mild-to-moderate COVID-19 disease managed in the primary care setting although it can be used as a supplementary agent in addition to the standard treatment to reduce symptoms in these patients.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Medicinal Product Research Ethics Committee of Hospital Universitario Puerta de Hierro Majadahonda (Madrid, Spain) (code 21/2021, approval date 12 December 2021). The study was registered at European Union Drug Regulating Authorities Clinical Trials Database (EudraCTnumber 2021-001227-41).

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

Data Availability Statement: Study data are available from the corresponding author upon reasonable request.

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Conflicts of Interest: A. Martínez and C. Gil are employees of the sponsor Agencia Estatal Consejo Superior de Investigaciones Científicas, M.P. (CSIC); B. Soler López was contracted to carry out the design, monitoring, statistical analysis and management of the publications derived from the study. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Cellular and Humoral Responses to Recombinant and Inactivated SARS-CoV-2 Vaccines in CKD Patients: An Observational Study

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Abstract: Background: It remains unclear what B cell and humoral responses are mounted by chronic kidney disease (CKD) patients in response to recombinant and inactivated SARS-CoV-2 vaccines. In this study, we aimed to explore the cellular and humoral responses, and the safety of recombinant and inactivated SARS-CoV-2 vaccines in CKD patients. Methods: 79 CKD and 420 non-CKD individuals, who completed a full course of vaccination, were enrolled in the study. Adverse events (AEs) were collected via a questionnaire. Cellular and humoral responses were detected at 1, 3, and 6 months, including IgG antibody against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (anti-RBD-IgG), neutralizing antibodies (NAbs), the positive rate of NAbs and anti-RBD-IgG, RBD-atypical memory B cells (MBCs) (CD3 − CD19 + RBD + CD21 − CD27−), RBD-activated MBCs (CD3 − CD19 + RBD + CD21 − CD27+), RBD-resting MBCs (CD3 − CD19 + RBD + CD21 + CD27+), and RBD-intermediate MBCs (CD3 − CD19 + RBD + CD21 + CD27−). Results: We found no differences in the positivity rates of NAbs (70.89% vs. 79.49%, $p = 0.212$) and anti-RBD IgG (72.15% vs. 83.33%, $p = 0.092$) between the CKD and control groups. A total of 22 CKD individuals completed the full follow-up (1, 3, and 6 months). Significant and sustained declines were found at 3 months in anti-RBD IgG (26.64 BAU/mL vs. 9.08 BAU/mL, $p < 0.001$) and NAbs (161.60 IU/mL vs. 68.45 IU/mL $p < 0.001$), and at 6 months in anti-RBD IgG (9.08 BAU/mL vs. 5.40 BAU/mL, $p = 0.064$) and NAbs (68.45 IU/mL vs. 51.03 IU/mL, $p = 0.001$). Significant differences were identified in MBC subgroups between CKD patients and healthy controls, including RBD-specific atypical MBCs (60.5% vs. 17.9%, $p < 0.001$), RBD-specific activated MBCs (36.3% vs. 14.8%, $p < 0.001$), RBD-specific intermediate MBCs (1.24% vs. 42.6%, $p < 0.001$), and resting MBCs (1.34% vs. 22.4%, $p < 0.001$). Most AEs in CKD patients were mild (grade 1 and 2) and self-limiting. One patient with CKD presented with a recurrence of nephrotic syndrome after vaccination. Conclusions: The recombinant and inactivated SARS-CoV-2 vaccine was well-tolerated and showed a good response in the CKD cohort. Our study also revealed differences in MBC subtypes after SARS-CoV-2 vaccination between CKD patients and healthy controls.

Keywords: SARS-CoV-2 vaccine; safety; cellular response; CKD; humoral responses

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1. Introduction

Coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an infectious disease that has extensively impacted human health worldwide. Several studies have reported that chronic kidney disease (CKD) is a significant risk factor for hospital admission and mortality following infection with COVID-19 [1,2]. Among those infected with SARS-CoV-2, patients undergoing dialysis,

with a history of organ transplantation, and with an estimated glomerular filtration rate (eGFR) < 30 mL/min/1.73 m^2 appear to have higher likelihood for worse outcomes [3]. Immunological dysfunction, which is a feature of CKD that is exacerbated by decreased eGFR, is likely of multi-factorial origins including chronic inflammation, endothelial cell dysfunction, uremia, malnutrition, and cytokine deregulation. Vaccination is a critical component of the defense against infection. Individuals with CKD benefit from vaccinations against hepatitis B, influenza, pneumococcal disease, and herpes zoster [4]. Several studies have demonstrated good responses to mRNA vaccines in non-dialysis dependent patients, hemodialysis and peritoneal dialysis patients. Current clinical evidence also supports that mRNA vaccines are safe in CKD patients [5,6].

There are areas of potential concern, however. Memory B cells (MBCs), which maintain long-lasting immunity, are an important component of the humoral and cellular response to SARS-CoV-2 [7]. However, few studies have tested the MBCs response to the SARS-CoV-2 vaccine [8]. This makes it difficult to evaluate the duration of protective immunity resulting from the vaccination. Thus, further research is necessary to explain if and whether these cell populations affect the protective responses and incidence of adverse reactions following vaccination.

Inactivated and recombinant SARS-CoV-2 vaccines have been widely used in many countries around the world, including China. However, few observational studies have focused on the safety and efficacy of these vaccines in CKD populations [9]. The MBCs response characteristics of CKD patients to SARS-CoV-2 vaccination also remain unknown. In this observational study, we report on the antibody levels and MBCs' responses to SARS-CoV-2 inactivated and recombinant vaccines in CKD patients with and without hemodialysis.

2. Methods

2.1. Participants

Healthy individuals, those with non-dialysis-dependent CKD, and those undergoing hemodialysis were recruited into this observational study between 1 August 2021 and 31 December 2021 from the Second Affiliated Hospital of Chongqing Medical University, China. CKD participants had to meet the clinical diagnostic criteria of the KDIGO guidelines for CKD. According to the preliminary results, the positive rate of antibody in the non-CKD group and the CKD group was 86% and 65%, respectively. With 10% of the loss of follow-up involved in calculating, more than 75 cases were required for each the non-CKD group and the CKD group. Recombinant and inactivated vaccine recipients who received the SARS-CoV-2 vaccine within 3 months were enrolled; vaccine subtypes were used as an independent variable. The following were exclusionary: (1) history of COVID-19 or positive SARS-CoV-2 nucleic acid amplification test; (2) close contact with SARS-CoV-2 infected individuals; (3) current pregnancy; (4) did not complete the full-course of vaccination. The study was approved by the Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University and conformed to the ethical guidelines of the Declaration of Helsinki (Ratification No. 111/2021). All participants provided written informed consent before participation. The study was registered at ClinicalTrials.gov NCT05043246 and the follow ups are ongoing.

2.2. Data Collection

Electronic questionnaires and e-cases were used to obtain patient demographic, adverse events, and clinical data. The questionnaire of adverse events is shown in Supplementary Materials S5. Information about the patient's gender, age, time of vaccination to sample collection, body mass index, type of vaccine, comorbidities, etc. were collected. Time intervals after the full course of vaccination were defined as 1 month (=21–45 days), 3 months (=76–105 days), and 6 months (=165–195 days).

2.3. SARS-CoV-2 Antibody Test

Plasma samples were collected to detect IgG antibodies against the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein (anti-RBD-IgG) and neutralizing antibodies (NAbs) using capture chemiluminescence immunoassays (MAGLUMI X8, Snibe, Shenzhen, China) according to the manufacturer's instructions. The manufacturer of the kit (130219017M, 130619017M, MAGLUMI X8, Snibe, Shenzhen, China) reported that the anti-S-RBD-IgG tests have a 100% sensitivity and 99.6% specificity for the diagnosis of COVID-19, while the manufacturer of the kit (130219027M, 130619027M,MAGLUMI X8, Snibe, Shenzhen, China) reported that NAbs tests have a 100% sensitivity and 100% specificity. The cut-off values were 4.33 BAU/mL for Anti-RBD-IgG and 60.75 IU/mL for NAbs. The detailed procedures used to process each kit are shown in Supplementary Materials S1.

2.4. Detection of SARS-CoV-2 Specific B Cells by Flow Cytometry

For SARS-CoV-2 specific B cell detection, biotinylated SARS-CoV-2 Spike RBD protein (40592-V08H2-B, Sino Biological, Beijing, China) was mixed with Streptavidin-BV421 (405225, Biolegend, San Diego, CA, USA) at a 4:1 molar ratio for one hour at 4 °C to obtain the antigen probe. According to the manufacturer's instructions, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized (sodium heparin) fresh whole blood (the time from arm to cell isolation was <4 h) by Histopaque (10771, Sigma-Aldrich, MI, USA) density gradient centrifugation. After washing with FACS buffer (PBS+2% FBS [FSD500, Excell Bio, Shanghai, China]), approximately 0.5×10^6 PBMCs were incubated with the antigen probe (biotinylated SARS-CoV-2 Spike RBD protein-Streptavidin-BV421) or incubated with Streptavidin-BV421 alone as a negative control, for 30 min at 4 °C and the following conjugated antibodies: PerCP/Cyanine5.5 conjugated anti-human CD3 (1:50, 300430, Biolegend, San Diego, CA, USA), APC conjugated anti-human CD19 (1:50, 302212, Biolegend), Alexa Fluor® 700 conjugated anti-human CD21 (1:50, 354918, Biolegend), and PE conjugated anti-human CD27 (1:50, 356406, Biolegend). After staining, cells were washed and resuspended in 200μL of FACS buffer. Sample data were then acquired by flow cytometry (Beckman Coulter, CytoFLEX, Brea, CA, USA) and analyzed using FlowJo (Treestar, 10.0.7r2). Lymphocytes were sorted by utilizing FSC and SSC gated channels. The gating strategy for RBD-specific B cells was based on the negative control. RBD-specific memory B cells (MBCs) were divided into four subsets based on the expression of CD27 and CD21. The cell populations were identified by using the following strategy: RBD-specific B cells (CD3 − CD19 + RBD+), RBD-specific MBCs (CD3 − CD19 + RBD + CD27+), RBD-atypical MBCs (CD3 − CD19 + RBD + CD21 − CD27−), RBD-activated MBCs (CD3 − CD19 + RBD + CD21 − CD27+), RBD-resting MBCs (CD3 − CD19 + RBD + CD21 + CD27+), and RBD-intermediate MBCs (CD3 − CD19 + RBD + CD21 + CD27−). The full gating strategy for the target cell populations is shown in Supplementary Materials S2. Examples of flow plots are presented in Supplementary Materials S3.

2.5. Statistical Analysis

Antibody and MBCs levels were compared between the CKD and healthy control groups. As a subgroup analysis, we contrasted the responses of CKD patients receiving inactivated vaccine and dialysis-dependent CKD patients versus healthy controls. Due to differences in baseline and sample sizes between the CKD and control groups, 1:1 Propensity Score Matching (PSM) was utilized to screen and reduce potential bias arising from baseline differences [10]. Categorical variables were analyzed by using Chi-square or Fisher's precision probability tests. Independent samples *t*-tests were used to compare normally distributed continuous variables, while the Mann–Whitney test was used for non-normally distributed data. A p-value < 0.05 was considered statistically significant. Data analysis was performed by using IBM SPSS 25.0 (Armonk, NY, USA). Data were visualized using Graphpad 7.0.

3. Results

3.1. Characteristics of Participants

We recruited 79 CKD individuals, including 22 with dialysis-dependent CKD and 57 with non-dialysis-dependent CKD. 420 non-CKD individuals were enrolled as controls. In the baseline comparison, the proportions of gender and vaccine type were found to differ between the CKD and control groups and thus 1:1 PSM was used. The prevalence of diabetes, hypertension, and cardiovascular disease was higher in the CKD group. However, diabetes, hypertension, and cardiovascular disease were not included in the PSM model, as the prevalence was too low in the controls. Subgroup analyses of the inactivated vaccine group and the hemodialysis CKD patient group were conducted. The baseline characteristics for the overall CKD patient and healthy control groups are shown in Table 1. The baseline characteristics for the inactivated vaccine and healthy control groups are shown in Table 2. The hemodialysis and healthy control group characteristics are shown in Table 3. Due to China's vaccination policy against COVID-19, we were only able to collect 17 healthy unvaccinated cases from the community. We compared NAbs levels between the unvaccinated and vaccinated groups. These data are shown in Supplementary Materials S4.

Table 1. Baseline characteristics of the CKD patients and healthy controls.

Variables	CKD Group	Control Group	*p* Value	Control Group *	*p* Value *
Age (years)	47.25 ± 14.31	47 (34–58)	0.682	42 (25–53)	0.051
≤60	64/79	334/420	0.763	67/79	0.526
>60	15/79	86/420		12/79	
Gender (male, (*n*%))	53/79 (67%)	218/420 (52%)	0.013	47/79 (59%)	0.409
BMI (kg/m^2)	24.70 ± 4.25	25.04 (20.55–27.33)	0.381	23.85 ± 3.79	0.102
<24	36/79	151/420	0.105	45/79	0.152
≥24	43/79	269/420		34/79	
Acquisition time (months)					
<3	61/79	330/420	0.788	59/79	0.71
≥3	18/79	90/420		20/79	
Vaccines					
Recombinant vaccine (*n*%)	16/79	177/420	<0.001	18/79	0.15
Inactivated vaccine (*n*%)	63/79	243/420		61/79	
Comorbidities					
Diabetes	11/79	15/420	<0.001	0/79	<0.001
Hypertension	35/79	36/220	<0.001	4/79	<0.001
Cardiovascular diseases	14/79	3/420	<0.001	0/79	<0.001

* Presented as value after 1:1 Propensity Score Matching. Categorical variables were analyzed by using the Chi-square or Fisher's precision probability tests. Independent samples *t*-tests were used to compare normally distributed continuous variables, while the Mann–Whitney tests were used for non-normally distributed data. *p*-values < 0.05 were considered statistically significant. BMI, Body Mass Index.

Table 2. Baseline characteristics of CKD patients receiving inactivated vaccine and healthy controls.

Variables	CKD Group	Control Group	*p* Value	Control Group *	*p* Value *
Age (years)	48.60 ± 14.28	49 (34–60)	0.642	48 (36–64)	0.918
≤60	51/63	184/243	0.287	43/63	0.102
>60	12/63	59/243		20/63	
Gender (male, (*n*%))	41/63	127/243	0.068	42/63	0.814
BMI (kg/m^2)	24.56 (21.22–27.23)	24.96 ± 3.55	0.293	25.65 ± 3.02	0.064
<24	29/63	152/243	0.017	17/63	0.026
≥24	34/63	91/243		46/63	
Acquisition time (months)					

Table 2. *Cont.*

Variables	CKD Group	Control Group	*p* Value	Control Group *	*p* Value *
<3	48/63	166/243	0.224	53/63	0.264
≥3	15/63	77/243		10/63	
Diabetes	11/63	8/243	<0.001	0/63	<0.001
Hypertension	35/63	24/243	<0.001	3/63	<0.001
Cardiovascular diseases	14/63	2/243	<0.001	0/63	<0.001

* Presented as value after 1:1 Propensity Score Matching. Categorical variables were analyzed by using the Chi-square or Fisher's precision probability tests. Independent samples *t*-tests were used to compare normally distributed continuous variables, while the Mann–Whitney tests were used for non-normally distributed data. *p*-values < 0.05 were considered statistically significant. BMI, Body Mass Index.

Table 3. Baseline characteristics of the hemodialysis CKD patients and healthy controls.

Variables	CKD Group	Control Group	*p* Value
Age (years)	48.00 ± 14.16	47 (34–58)	0.604
≤60	19/23	334/420	1
>60	4/23	86/420	
Gender (male, (*n*%))	14/23 (61%)	218/420 (52%)	0.402
BMI (kg/m^2)	22.16 ± 4.38	25.04 (20.55–27.33)	0.007
<24			0.008
≥24			
Acquisition time (months)			
<3	21/23	330/420	0.189
≥3	2/23	90/420	
Vaccines			
Recombinant vaccine (*n*%)	3/23	177/420	0.006
Inactivated vaccine (*n*%)	20/23	243/420	
Comorbidities			
Diabetes	1/23	15/420	0.58
Hypertension	0/23	36/220	0.243
Cardiovascular diseases	0/23	3/420	1

Categorical variables were analyzed by using the Chi-square or Fisher's precision probability tests. Independent samples *t*-tests were used to compare normally distributed continuous variables, while the Mann–Whitney tests were used for non-normally distributed data. *p*-values < 0.05 were considered statistically significant. BMI, Body Mass Index.

3.2. SARS-CoV-2 Vaccination in CKD Patients

Using 1:1 PSM, 79 healthy individuals were included in the healthy control group. Humoral immunity was assessed by comparing Anti-RBD IgG and NAbs in the CKD and control groups. The positivity rates of NAbs (70.89% vs. 79.49%, $p = 0.212$) and anti-RBD IgG (72.15% vs. 83.33%, $p = 0.092$) were found to not differ between groups (Figure 1B,D). Additionally, there was no significant difference in anti-RBD IgG levels between the CKD and the control groups (13.70 BAU/mL vs. 18.96 BAU/mL, $p = 0.089$), while NAbs levels were lower in the CKD group (96.39 IU/mL vs. 127.58 IU/mL, $p = 0.046$) (Figure 1A,C).

MBCs are recognized as a crucial component of cellular and humoral responses in virological immunity. We next assessed the frequency of RBD-specific MBCs in each group. There were no differences in RBD-specific MBCs levels between the CKD and control groups (Supplementary Materials S4). MBCs were divided into four subsets according to CD21 and CD27 expression [11]. Intermediate MBCs and resting MBCs with the non-plasmablast population express CD21+; there are two related subsets, including a CD21− CD27+ population with plasmablast-like features, and a CD21− CD27− population, which are deemed to be atypical memory B cells. The frequencies of RBD-specific atypical MBCs (60.5% vs. 17.9%, $p < 0.001$) and RBD-specific activated MBCs (36.3% vs. 14.8%, $p < 0.001$) were higher in the CKD group (Figure 1E,G). The frequencies of RBD-specific intermediate MBCs (1.24% vs. 42.6%, $p < 0.001$), and resting MBCs (1.34% vs. 22.4%, $p < 0.001$) were higher in the control group (Figure 1F,H).

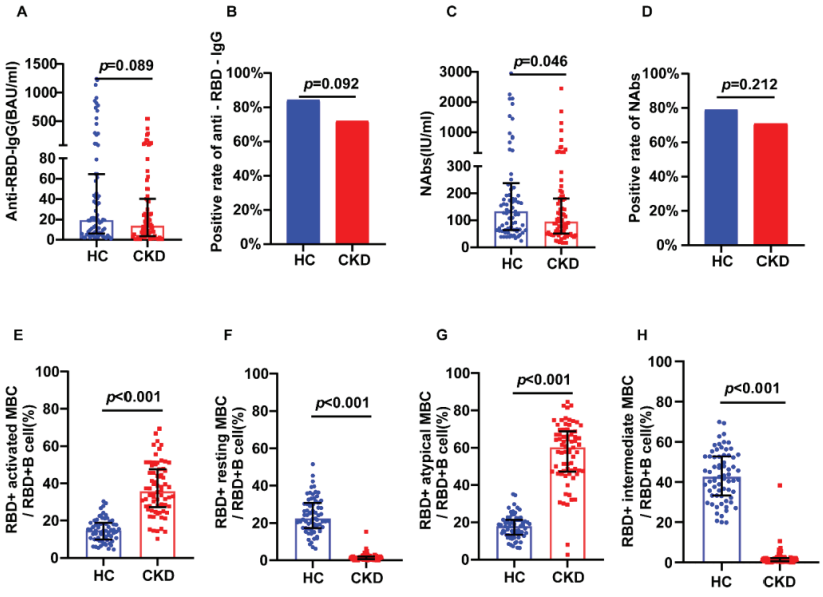

Figure 1. Humoral immune responses following vaccination in CKD patients and healthy controls. (**A**) The serum anti-RBD-IgG levels. (**B**) The seropositivity rates of anti-RBD-IgG. (**C**) The Serum NAbs levels. (**D**) The seropositivity rates of Nabs. (**E**) The frequency (percentage of RBD-specific B cells) of RBD-specific activated MBCs. (**F**) The frequency (percentage of RBD-specific B cells) of RBD-specific resting MBCs. (**G**) The frequency (percentage of RBD-specific B cells) of RBD-specific atypical MBCs. (**H**) The frequency (percentage of RBD-specific B cells) of RBD-specific intermediate MBCs responses. The IQR are indicated by error bars. anti-RBD-IgG, spike receptor-binding domain IgG antibody; CKD chronic renal disease; HC healthy controls; IQR interquartile range; MBCs memory B cells; NAbs neutralizing antibodies.

22 individuals with CKD completed the full follow-up from 1 month to 6 months after vaccination. We found a sustained reduction between 1 and 3 months for anti-RBD IgG (26.64 BAU/mL vs. 9.08 BAU/mL, $p < 0.001$), NAbs (161.60 IU/mL vs. 68.45 IU/mL, $p < 0.001$), and positive rate of NAbs (95.45% vs. 63.64%, $p = 0.021$) and anti-RBD IgG (77.27% vs. 31.82%, $p = 0.006$). We also found a reduction between 3 and 6 months for anti-RBD IgG (9.08 BAU/mL vs. 5.40 BAU/mL, $p = 0.064$) and Nabs (68.45 IU/mL vs. 51.03 IU/mL, $p = 0.001$) (Figure 2).

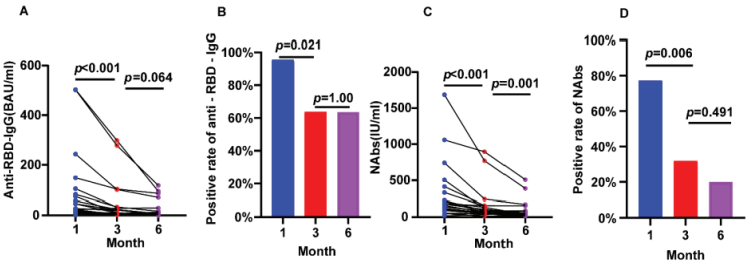

Figure 2. Longitudinal changes of humoral immune responses following immunization with vaccines in CKD patients. (**A**) The change of serum anti-RBD-IgG levels. (**B**) The change of seropositivity rates of anti-RBD-IgG. (**C**) The change of serum NAbs levels. (**D**) The change of seropositivity rates of NAbs. The IQR are indicated by error bars. anti-RBD-IgG, spike receptor-binding domain IgG antibody; CKD chronic renal disease; NAbs neutralizing antibodies.

3.3. Inactivated SARS-CoV-2 Virus Vaccination in CKD

Using 1:1 PSM, 63 healthy individuals were included as healthy controls. In the subgroup that received the inactivated vaccine, we found no significant differences in anti-RBD IgG (12.61 BAU/mL vs. 11.49 BAU/mL, $p = 0.469$) or NAbs (88.29 IU/mL vs. 98.42 IU/mL, $p = 0.188$) levels in the CKD versus the control group (Figure 3A,C). The positivity rates of NAbs (60.32% vs. 78.33%, $p = 0.0634$) and anti-RBD IgG (68.25% vs. 79.03%, $p = 0.1717$) were not different between groups (Figure 3B,D). The CKD group showed higher levels of RBD-specific atypical MBCs (57.75% vs. 18%, $p < 0.001$) and RBD-specific activated MBCs (38.05% vs. 15.2%, $p < 0.001$) than controls (Figure 3E,G), and showed lower RBD-specific intermediate MBCs (1.28% vs. 40.65%, $p < 0.001$) and resting MBCs (1.20% vs. 24.2%, $p < 0.001$) than controls (Figure 3F,H).

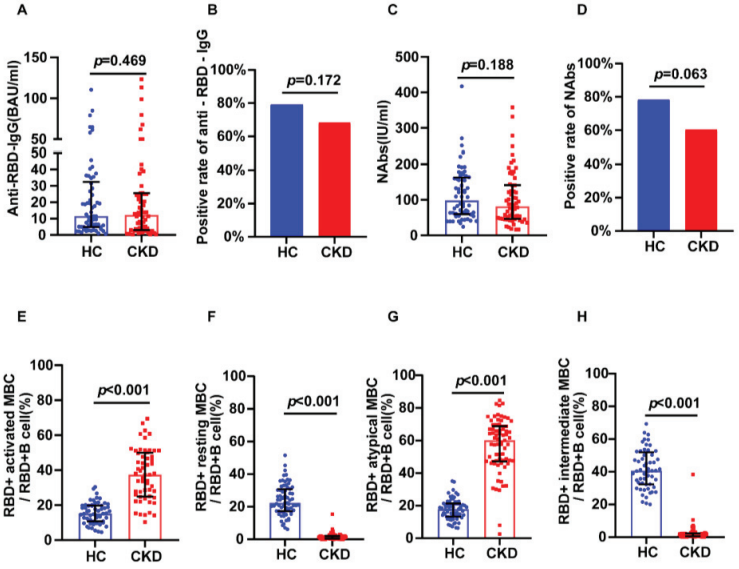

Figure 3. Humoral immune responses following immunization with inactivated vaccines in CKD patients and healthy controls. (**A**) The serum anti-RBD-IgG levels. (**B**) The seropositivity rates of anti-RBD-IgG. (**C**) The serum NAbs levels. (**D**) The seropositivity rates of Nabs. (**E**) The frequency (percentage of RBD-specific B cells) of RBD-specific activated MBCs. (**F**) The frequency (percentage of RBD-specific B cells) of RBD-specific resting MBCs. (**G**) The frequency (percentage of RBD-specific B cells) of RBD-specific atypical MBCs. (**H**) The frequency (percentage of RBD-specific B cells) of RBD-specific intermediate MBCs. The IQR are indicated by error bars. anti-RBD-IgG, spike receptor-binding domain IgG antibody; CKD chronic renal disease; HC healthy controls; IQR interquartile range; MBCs memory B cells; NAbs neutralizing antibodies.

3.4. SARS-CoV-2 Vaccination in Hemodialysis Patients

Hemodialysis patients were expected to display a lower vaccine response. Due to the heterogeneity of the included groups, 1:1 PSM might have excessively reduced the sample size. Therefore, in this subgroup analysis, we did not perform sample matching. The positivity rate of anti-RBD IgG (69.57% vs. 89.1%, $p = 0.0132$) was lower in hemodialysis patients, while the positivity rate of NAbs (69.57% vs. 79.89%, $p = 0.2854$) was not significantly different between hemodialysis patients and controls (Figure 4B,D). The levels of anti-RBD IgG (16.75 BAU/mL vs. 24.51 BAU/mL, $p = 0.011$) were lower in the hemodialysis group, while the levels of NAbs (95.18 IU/mL vs. 130.21 IU/mL, $p = 0.061$) were not different between groups (Figure 4A,C). The CKD group showed higher levels of RBD-specific atypical MBCs (61.7% vs. 19.5%, $p < 0.001$) and RBD-specific activated MBCs (33.9% vs. 15.7%, $p < 0.001$) than controls (Figure 4E,G), and showed fewer RBD-specific intermediate MBCs

(1.89% vs. 41%, $p < 0.001$) and resting MBCs (1.07% vs. 21.4%, $p < 0.001$) than controls (Figure 4F,H).

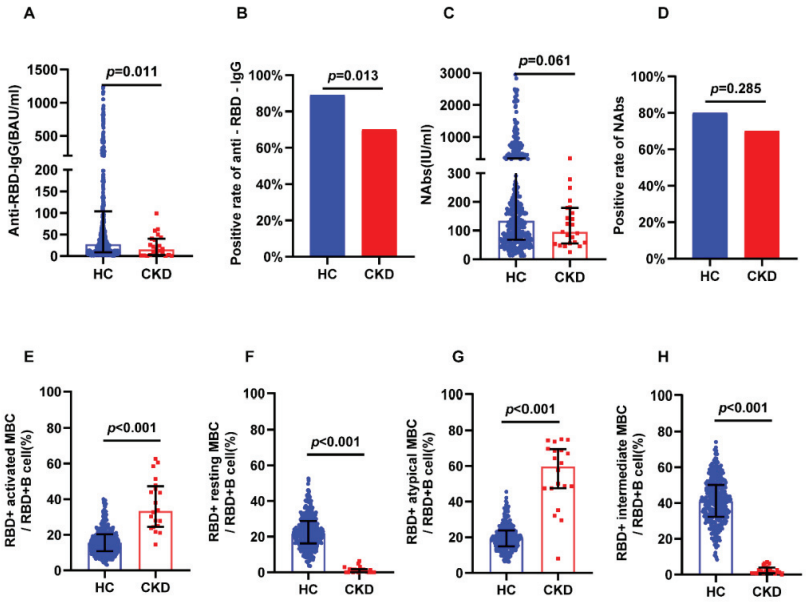

Figure 4. Humoral immune responses following immunization with vaccines in dialysis CKD patients and all healthy controls. (**A**) The serum anti-RBD-IgG levels. (**B**) The seropositivity rates of anti-RBD-IgG. (**C**) The serum NAbs levels. (**D**) The seropositivity rates of Nabs. (**E**) The frequency (percentage of RBD-specific B cells) of RBD-specific activated MBCs. (**F**) The frequency (percentage of RBD-specific B cells) of RBD-specific resting MBCs. (**G**) The frequency (percentage of RBD-specific B cells) of RBD-specific atypical MBCs. (**H**) The frequency (percentage of RBD-specific B cells) of RBD-specific intermediate MBCs. The IQR are indicated by error bars. anti-RBD-IgG, spike receptor-binding domain IgG antibody; CKD chronic renal disease; HC healthy controls; IQR interquartile range; MBCs memory B cells; NAbs neutralizing antibodies.

3.5. Safety of SARS-CoV-2 Vaccination in CKD

There was a difference in the overall incidence of AEs between the CKD and healthy control groups (6.3% vs. 13.1%, $p = 0.09$) (Table 4). Most recorded AEs in the CKD group were mild (grades 1 and 2) and self-limiting; these included fatigue, slight fever, and nausea. Proteinuria occurred in two patients with CKD, both of whom had preexisting chronic glomerulonephritis. One of the patients progressed to nephrotic syndrome and required immunosuppressive therapy. In the healthy control group, we did not find any moderate or severe AEs (grades 3 and 4).

Table 4. Adverse events after SARS-CoV-2 vaccination.

	CKD Patients (n = 79)	Controls (n = 420)	p Value
Overall adverse events	5	55	0.09
Local adverse events			
Pain	/	27	0.021
Redness	/	4	0.384
Rash	/	7	0.248
Systemic adverse events			

Table 4. *Cont.*

	CKD Patients (n = 79)	Controls (n = 420)	p Value
Fatigue	1	6	0.9102
Dizziness	/	3	0.451
Diarrhea	/	1	0.664
Laryngeal pain	/	/	/
Cough	/	1	0.664
Chest distress	/	/	/
Chest pain	/	/	/
Chill	/	/	/
Proteinuria	2	/	0.001
Elevated blood pressure	/	/	/
Fever	1	1	0.185
Inappetence	/	/	/
Muscle pain	/	2	0.541
Nausea	1	4	0.806
Palpitation	/	/	/
Pruitus	/	/	/
Grade 3 and 4 adverse events	1	/	0.022

Categorical variables were analyzed by using Chi-square or Fisher's precision probability tests. p-values < 0.05 were considered statistically significant.

4. Discussion

In this study, anti-RBD-IgG and NAbs were used to comprehensively analyze the humoral immune response to SARS-CoV-2 vaccination. We report that the positive rate of NAbs and anti-RBD IgG were 70.89% and 72.15%, respectively, in CKD patients following two doses of the vaccine. In our subgroup analysis for the CKD population using inactivated vaccines, we achieved a similar result. In the hemodialysis subgroup, the positive rate of anti-RBD IgG was significantly lower than in healthy controls. In additional analyses of antibody levels, NAbs levels were lower in the CKD group and hemodialysis subgroup, and anti-RBD IgG levels were lower in the CKD group. These results are consistent with studies assessing responses to COVID19 vaccines. In data from Israel's largest healthcare organization, a 74% protection rate against the subsequent development of severe disease was reported after two doses of the BNT162b2 mRNA vaccine [12]. Chung et al. reported that 94.16% of maintenance dialysis patients without prior SARS-CoV-2 infection achieved a positive antibody response after two doses of the ChAdOx1 nCoV-19 Vaccine [13]. The RECOVAC immune-response study reported a high seroconversion rate in participants with CKD G4/5 (100%) and dialysis (99.4%), which was similar to controls. A factor analysis of several studies showed that older age and immunosuppressive treatment were risk factors for reduced vaccine response rates [6].

In the follow-up cohort, we found a rapid decrease in antibody levels over time. Quiroga et al. reported a sustained decline of anti-spike antibody titers at 1, 3 and 6 months in CKD patients following two doses of the BNT162b2 mRNA vaccine [14]. Zhang et al. reported a longitudinal analysis of T cell, B cell, and antibody responses to four different SARS-CoV-2 vaccines in humans and concluded that mRNA vaccines were associated with substantially reduced antibodies at 6 months, but memory T cell and B cell levels remained fairly stable [15]. Our study shows a low immune response to vaccines in the CKD population. Nevertheless, it does not dismiss the importance of vaccination in protecting CKD individuals, who are more vulnerable to COVID-19 sequelae.

MBCs are antigen exposed cells with the ability to generate a more rapid and effective immune response during secondary antigen exposure. RBD-specific MBCs were not found to be significantly different between CKD and healthy controls. As a subset of MBCs, CD21 + CD27 + MBCs play a central role in humoral immune responses and can rapidly differentiate into antibody-secreting plasma cells. CD21 + CD27− intermediate MBCs, a naïve subset with RBD-specific surface Ig receptors, can be activated following ligation

of their cognate antigens through the antigen-specific B cell receptors (BCRs), and enter into germinal centers to undergo affinity maturation [16,17]. We found significantly lower CD21+ CD27+ MBCs and CD21+ CD27− MBCs in CKD patients. We are still uncertain how such changes impact the immune memory of cells in CKD patients. Two poorly understood subsets of MBCs are CD21− CD27+ activated MBCs or the plasmablast-like subset and the CD21− CD27− population or atypical MBCs [16,17]. We found an expansion of atypical CD21− CD27− MBCs and activated CD21− CD27+ MBCs in CKD compared to the healthy control group following vaccination. This shows a discrepancy in cellular immunological mechanisms between CKD patients and healthy controls. Currently, the function of atypical MBCs remains unclear. Atypical MBCs, considered a subset of MBCs, are usually seen at high frequencies in chronic diseases [18]. Several studies have reported remarkable increases of CD21− CD27− atypical B cells in chronic infectious diseases, such as malaria, HIV-AIDS, tuberculosis (TB), and several autoimmune conditions [19,20]. A previous study showed that atypical memory B cells are short-lived activated cells that may represent a precursor plasma cell (PC) population [18].

Several studies into rheumatic diseases have reported that atypical MBCs may be a pathogenic immune factor of disease-causing antibodies. Chunmei et al. reported that greater numbers of atypical MBCs were associated with high disease flares and disease-specific autoantibodies such as anti-Smith (Sm) antibodies. Atypical MBCs are also found to infiltrate kidneys in lupus nephritis and to be closely associated with disease activity and renal dysfunction [21]. Cloé et al. demonstrated that TLR9 signaling in HCV-associated atypical memory B cells triggers Th1 and rheumatoid factor autoantibody responses. It appears that ongoing chronic inflammation promotes the generation of these MBCs [22]. However, in infectious diseases, evidence suggests that atypical memory B cells are positive alternatives that participate in mounting defenses against pathogens. Christine S et al. reported that in acute febrile malaria, specific atypical MBCs and activated MBCs up-regulate similar intracellular signaling cascades to stimulate differentiation into antibody-secreting cells and the up-regulation of molecules that mediate B-T cell interactions. With the T follicular helper cells and staphylococcal enterotoxin B, atypical MBCs can differentiate into CD38+ antibody-secreting cells in vitro [23]. Similar supporting evidence has been found in single-cell sequencing studies; that atypical B cells are part of a broader alternative lineage that is abundant even in healthy individuals, and that they are a critical component of the humoral immune response [16]. Kathryn A et al. reported that SARS-CoV-2 infection results in the production of more atypical MBCs than when the immune system is primed by mRNA vaccines [24]. Atypical memory B cells appear to be an important cell subset for developing humoral immunity in response to the SARS-CoV-2 vaccine. More research is needed to explore their pros and cons.

Most of our CKD patients showed a good tolerance after vaccination. Most adverse events were mild and self-limiting. Importantly, in the CKD group, two individuals presented with recurrent proteinuria, one of whom progressed to nephrotic syndrome and required immunosuppressive therapy. A small number of case reports have reported de novo or recurrent glomerulonephritis following SARS-CoV-2 vaccination; the proteinuria gradually improved without any medication, suggesting that immune activation by the vaccine is unlikely to elicit a marked progression of glomerulonephritis.

Our study is the first in which healthy people were utilized as controls and focused on the cellular and humoral responses to inactivated and recombinant SARS-CoV-2 vaccination in CKD patients. These data are important for clinical risk-benefit decision-making. We also evaluated the subtypes of MBC responses to explore the underlying antibody responses. There were some limitations. First, only hemodialysis and non-dialysis dependent patients were included; renal transplant recipients and peritoneal dialysis patients were not enrolled. Second, it was a low sample size study, and only 22 patients completed the 6-month antibody test follow-up. Due to the lack of follow-up data for healthy controls, only the CKD group levels were available for comparison. Third, although baseline matching was performed by 1:1 PSM, the prevalence of cardiovascular disease, hypertension, and diabetes

were significantly higher in the CKD group than in the healthy control group, which may have introduced bias.

In conclusion, we analyzed the antibody response, B cell response, and safety profile of recombinant and inactivated SARS-CoV-2 vaccines in patients with CKD and healthy controls. After completing a full vaccination course, we found that the recombinant and inactivated anti-SARS-CoV-2 vaccines were well tolerated and showed good responses in majority of the CKD population. Nevertheless, we found a decrease in antibody levels at 3 months post-vaccination. We also found differences in MBCs subtypes after SARS-CoV-2 vaccination between CKD patients and healthy controls. The differences in subgroups of MBCs between CKD patients and healthy individuals deserves further study. Based on our findings, we believe that it is essential to develop a vaccination strategy that is appropriate for people with CKD. Additionally, the B-cell signatures of CKD patients will be an important inspiration for revealing the pathological immunological mechanisms of the CKD population.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12031225/s1, S1: MAGLUMI® SARS-CoV-2 S-RBD IgG II (CLIA); S2: The full gating strategy of flow cytometry for target cell populations; S3: Examples of flow plots of figures; S4: Neutralizing antibody levels in healthy people before and after vaccination; S5: Questionnaire on Adverse Reactions to COVID-19 vaccination; S6: Original data.

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A Clinical Prediction Rule for Thrombosis in Critically Ill COVID-19 Patients: Step 1 Results of the Thromcco Study

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Abstract: The incidence of thrombosis in COVID-19 patients is exceptionally high among intensive care unit (ICU)-admitted individuals. We aimed to develop a clinical prediction rule for thrombosis in hospitalized COVID-19 patients. Data were taken from the Thromcco study (TS) database, which contains information on consecutive adults (aged $\geq$ 18) admitted to eight Spanish ICUs between March 2020 and October 2021. Diverse logistic regression model analysis, including demographic data, pre-existing conditions, and blood tests collected during the first 24 h of hospitalization, was performed to build a model that predicted thrombosis. Once obtained, the numeric and categorical variables considered were converted to factor variables giving them a score. Out of 2055 patients included in the TS database, 299 subjects with a median age of 62.4 years (IQR 51.5–70) (79% men) were considered in the final model (SE = 83%, SP = 62%, accuracy = 77%). Seven variables with assigned scores were delineated as age 25–40 and $\geq$70 = 12, age 41–70 = 13, male = 1, D-dimer $\geq$ 500 ng/mL = 13, leukocytes $\geq 10 \times 10^3/\mu L = 1$, interleukin-6 $\geq$ 10 pg/mL = 1, and C-reactive protein (CRP) $\geq$ 50 mg/L = 1. Score values $\geq$28 had a sensitivity of 88% and specificity of 29% for thrombosis. This score could be helpful in recognizing patients at higher risk for thrombosis, but further research is needed.

Keywords: thrombosis; COVID-19; risk prediction model; clinical prediction rule

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1. Introduction

There is sufficient clinical evidence indicating that coronavirus disease 2019 (COVID-19) is associated with thrombotic complications, increasing disease severity [1,2]. The incidence is exceptionally high in critically ill individuals admitted to intensive care units (ICUs), in whom both venous thromboembolism (VTE) and pulmonary embolism (PE) have been observed in more than 20% of patients, especially during ancestral Delta and Omicron variants, a trend that seemed to decrease with the new variants [1,3–5]. In hospitalized individuals, the incidence is greater when assessed according to screening than by clinical diagnosis [6,7]. For instance, when systematic computer tomography pulmonary angiogram is performed in all hospital-admitted patients, higher rates of thromboembolism are observed [8]. However, systematic thrombosis screening is not currently indicated in COVID-19 individuals, and other predictive tools must be developed.

Before the ongoing pandemic, the Geneva and Wells scores were the most used to predict PE and deep vein thrombosis (DVT) in the general population, respectively [9,10]. Still, in COVID-19 individuals, their efficacy has not been proven [11]. Therefore, other predictive scores have been adapted to respond to the need for early thrombosis identification [12,13]. However, their application has been hampered by their low sensitivity and specificity, the use of variables hardly used outside of a few limited settings, and a lack of validation in clinical settings [13].

Early identification of predictive factors for thrombosis could improve clinical decision making to treat and reduce the morbidity and mortality in COVID-19 subjects. Hence, there is a need to systematically assess the risk of thrombosis in hospitalized COVID-19 patients and develop methodical diagnostic protocols. Therefore, the present study aimed to develop a clinical prediction rule for thrombosis in hospitalized COVID-19 population.

2. Materials and Methods

2.1. Study Design and Ethics

We conducted a cross-sectional retrospective observational study with a clinical prediction rule for thrombosis in hospitalized COVID-19 patients that required ICU admission. To do so, we developed a scoring system based on the recommendations of Zhang et al. [14]. We also considered the Transparent Reporting of a Multivariable Prediction Model for Individual Prognosis or Diagnosis (TRIPOD) guidelines [15].

Our study was approved by the Ethics Committee of La Paz University Hospital.

2.2. Source of Data

The present investigation is part of the Thromcco Study Project (TSP), a multicenter retrospective database that contains the de-identified data of hospitalized patients admitted to the ICUs of the following Spanish hospitals: La Paz University Hospital in Madrid, Germans Trias I Pujol Hospital in Barcelona, University Hospital in Guadalajara, University Hospital in Burgos, Parc Taulí University Hospital in Sabadell, Clinical University Hospital in Valencia, Clinical University Hospital in Valladolid, and Son Espases University Hospital in Palma de Mallorca. We managed the data-collecting process by creating the study database in the REDcap clinical data repository, a secure web application for managing hospital databases that provide a standard for data collection among all involved medical institutions. Access to this repository was authorized for the professionals in charge of the data management of every participating hospital, who had at their disposal a database replication-blinded to other hospitals' information. Only authorized data analysts (KLRC, SCM, and EM) could access all database instances.

2.3. Participants

Consecutive hospitalized COVID-19 patients aged $\geq$18 years who were admitted to the ICUs of the participating hospitals between March 2020 and October 2021 were studied. All ICU-admitted subjects had a confirmed reverse-transcription polymerase chain reaction (RT-PCR) test positive for severe acute respiratory syndrome coronavirus 2 (SARS-CoV2). Patients were followed from hospital admission (index date) to hospital discharge or death.

2.4. Variables

The Thromcco database comprises 478 variables composed of hospital and ICU records collected retrospectively. To perform this study, we selected the following variables: sociodemographic data (age, sex, race, and smoking habit), body mass index (BMI), blood type, previous comorbidities (hypertension, diabetes, obesity (divided into categories as class 1: BMI 30–34.9, class 2: BMI 35–39.9, and class 3: BMI >40), asthma, chronic obstructive pulmonary disease (COPD), and ischemic and valvular heart disease), length of hospital and ICU stays, number of venous doppler ultrasounds of the lower limbs performed, anticoagulant regimen received (prophylactic, intermediate, and therapeutic), blood components transfused (red cells, fresh-frozen plasma, and platelets), and requirements of

invasive and noninvasive mechanical ventilation, tracheotomy, or prone positions. We also included the blood test results (D-dimer, fibrinogen, leucocytes, lymphocytes, platelets, ferritin, C-reactive protein (CRP), and interleukin 6 (IL6)), prothrombin time (PT), procalcitonin, creatinine, lactate dehydrogenase (LDH), aspartate dehydrogenase (AST), and alanine transaminase*(ALT)) that were collected at admission and on days 1, 2, 5, and 10 of hospitalization. Adverse outcomes such as sepsis and death were also gathered.

2.5. Statistical Analysis and Predictors

The primary outcomes of our study were venous thrombosis, DVT, PE, and catheter-related thrombosis. The secondary outcomes was arterial thrombosis, considered when a stroke or myocardial infarction occurred. Only thrombotic events registered during hospitalization were studied. If patients had more than one admission to the ICU, only the first one was considered.

Patients from the TS database with large proportions of missing data (>30% of selected variables) were excluded. To determine the factors predictive for thromboembolism, samples were randomly split into a training set, including 70% of patients, and a test set, considering the remaining 30%. Diverse logistic regression model configurations were performed, including demographic data, pre-existing conditions, and blood tests collected during the first 24 h of hospitalization. Once we obtained a model with statistically significant predictors (p-value < 0.05) and overall accuracy above 70% (training set), this model was validated through computations of accuracy and performance using the remaining 30% of patients (test set). A receiver operating characteristic (ROC) curve was generated in conjunction with the area under the curve (AUC) to assess the discriminative ability of the final model.

Once the model that better predicted thrombosis was obtained, we developed a scoring system for thrombosis risk stratification following the recommendations of Zhang and colleagues. The numeric and categorical variables included in the model were converted to factor variables, giving a score to the values obtained. This score is named the Thromcco Study (TS) score.

To establish predictive cut-off values, subjects that did not present a thrombotic event during hospitalization were considered the control group (n = 60). Thus, considering the prevalence of thrombosis in our sample (20.1%), we calculated the sensitivity (SE) and specificity (SP) of the score and its positive and negative predictive values (PPV and NPV, respectively). In addition, we evaluated the capacity of the TS score as a tool to indicate an imaging test to detect DVT by determining the doppler ultrasounds of the lower limb veins that would be needed to diagnose one case of thrombosis. Only the subset of subjects with a doppler ultrasound was considered for this last analysis.

Categorical variables are reported as count data by frequency, while continuous variables are reported as mean ± standard deviation or median and interquartile range (IQR). Patients' characteristics were compared between subjects with and without thrombosis using chi-square or Fisher's exact test (categorical variables) and Mann–Whitney or Kruskal–Wallis tests (continuous variables), setting the significance level to 0.05.

The statistical analysis of this study was performed in R version 4.1.3 (17 March 2022).

3. Results

Out of 2055 subjects with COVID-19 registered in the Thromcco database, only 299 patients were considered for the final TS score development and analysis. This final data subset resulted from diverse model configurations that only included patients with complete medical records.

3.1. Patient Characteristics

The median age of participants was 62.4 years (interquartile range (IQR), 51.5–70), and most of them were men (79.9%). Hypertension (40.8%), obesity (35.8%), and diabetes (22.7%) were the most common chronic comorbidities at baseline (at COVID-19 diagnosis).

Subjects were hospitalized for a median of 28 days (IQR 18–42 days), of which 14 (IQR 7–28 days) stayed in the ICU. The median time from hospital admission to ICU admission was two days (IQR 0–5 days). Blood test results during the first 24 h of hospital admission showed elevated median levels of D-dimer (1676, IQR 779–4084), fibrinogen (719, IQR 608–861), ferritin (974, IQR 482.1–1634), CRP (126, IQR 69.6–207.6), PT (12.9, IQR 11.9–15.6), and IL6 (71.3, IQR 36.5–167.8). During ICU admission, 70.9% of subjects required invasive mechanical ventilation (IMV) and 47.2% required a tracheotomy; during the whole hospitalization, 15.4% developed sepsis, and 29% died due to COVID-19 (Table 1).

Table 1. Patients' characteristics, n = 299.

Age, Years, Median	62.4 (IQR 51.5–70)
Sex	% (n)
Female	28.1 (84)
Male	79.9 (215)
Race	
Caucasian	51.6 (154)
Latin American	11 (33)
Asian	0.3 (1)
African	1 (3)
Arabic	2.7 (8)
Unknown	33.4 (100)
Smoking habit	3.3 (10)
Comorbidities at hospital admission	
Hypertension	40.8 (122)
Diabetes	22.7 (68)
Asthma	3 (9)
COPD	4 (12)
Ischemic heart disease	6.7 (20)
Valvular heart disease	1 (3)
Auricular fibrillation	3 (9)
Obesity	36.1 (108)
Class 1	23.4 (70)
Class 2	8.7 (26)
Class 3	4 (12)
Hospitalization	Median (IQR)
Days from COVID-19 symptoms onset to hospital admission	7 (5–9)
Length of hospital stay, days	28 (18–42)
Time from hospital admission to ICU admission, days	2 (0–5)
Length of ICU stay, days	14 (7–28)
Blood tests results	
D-dimer	1676 (779–4084)
Fibrinogen	719 (608–861)
Leucocytes	8.2 (IQR 5.6–12.4)
Lymphocytes	0.6 (0.4–0.95)
Platelets	201 (147–259)
Ferritin	974 (482.1–1634)
C-reactive protein	126 (69.6–207.6)
Prothrombin time (PT)	12.9 (11.9–15.6)
IL6	71.3 (36.5–167.8)
Creatinine	0.83 (0.68–1.23)
Procalcitonin	0.25 (0.13–0.75)
Lactate dehydrogenase	493.2 (315.5–734.5)
Aspartate dehydrogenase	49 (30.1–80.2)
Alanine transaminase	37.6 (22–71.2)

Table 1. *Cont.*

Doppler ultrasounds of the lower limb veins	77.5 (232)
Thrombosis	20.06 (60)
Deep vein thrombosis (DVT)	10.6 (31)
Pulmonary embolism (PE)	3.67 (11)
DVT + PE	5.01 (15)
Stroke + DVT	0.33 (1)
Stroke	0.66 (2)
Anticoagulant therapy received	% (*n*)
Prophylactic-dose anticoagulation	44.1 (132)
Intermediate-dose anticoagulation	9.03 (27)
Therapeutic-dose anticoagulation	23.4 (70)
Bleeding	5 (15)
Transfusions	
Transfusion of blood components	30.4 (91)
Platelet's transfusion	7 (21)
Fresh-frozen plasma transfusion	5 (15)
ICU	
Noninvasive mechanical ventilation	58.5 (175)
Invasive mechanical ventilation	70.9 (212)
Tracheotomy	47.2 (141)
Prone positions	59.2 (177)
Sepsis	15.4 (46)
Deaths	29 (87)

The incidence of thrombosis was 20.06% (n = 60). DVT accounted for 78.3% of cases (n = 47), of which 32% (n = 15) also presented a PE. Compared with the control group (n = 239), subjects with thrombosis were older (63.2 years (IQR 53–72) vs. 60 years (IQR 51–65.9), p = 0.043), had more extended hospital and ICU stays (35.5 days (IQR 25–53) vs. 27 days (IQR 17–37) in hospital, p = 0.013; and 27.5 days (IQR 15–40) vs. 12 days (IQR 7–24) in the ICU, p = 0.001), needed more blood and platelets transfusions (50% vs. 25.5% p = 0.000; and 13.3% vs. 5.4%, p = 0.013, respectively), and more commonly developed sepsis (33.3% vs. 17.9%, p = 0.002). Moreover, in the ICU, they required more IMV (88.3% vs. 66.5% p = 0.001), tracheotomy (60% vs. 43.9%, p = 0.028), and prone positions (81.6% vs. 53.5%, p = 0.000). Furthermore, without statistical significance, the mortality rate was higher in patients with thrombosis than in those without it (38.3% vs. 26.7%, p = 0.078) (Table 2).

Table 2. Bivariate analysis between subjects with and without thrombosis.

	Thrombosis ***n*** **= 60 ***	**No Thrombosis** ***n*** **= 239**		
	Median (IQR)	**Median (IQR)**	**Median Difference (95% CI)**	***p*-Value**
Age, years	60 (51–65.9)	63.2 (53–72)	−2.3 (−5.5–0.95)	0.043
Blood test results				
D-dimer	1859.5 (1151–5970)	1605 (772–3335)	280 (−2039.3–2600.8)	0.786
Fibrinogen	813 (567–1020)	781 (625–903)	27.2 (−38.1–92.6)	0.410
Leucocytes	7.85 (5.2–12.1)	7.30 (5.32–10.3)	0.39 (−1.50–0.79)	0.527
Lymphocytes	0.77 (0.47–1.2)	0.70(0.40–1.0)	−0.02 (−0.17–0.10)	0.511
Platelets	239 (173–283)	210 (160–274)	2.6 (−24.9–30.21)	0.851
Ferritin	1006.5 (528–1573.2)	925.4 (474–1634)	138.5 (−624.6–901.8)	0.721
C-reactive protein	120.1 (64.7–277.4)	128.7 (82.4–206.7)	7.32 (−26.5–41.1)	0.668
Prothrombin time	13.1 (12–16.1)	13.4 (11.9–58)	3.5 (−10.9–3.07)	0.268

Table 2. *Cont.*

	Thrombosis $n = 60$ * Median (IQR)	No Thrombosis $n = 239$ Median (IQR)	Median Difference (95% CI)	*p*-Value
IL6	95.2 (42–238.2)	71.1 (38–167.8)	10.5 (−73.3–94.4)	0.803
Creatinine	0.86 (0.62–1.01)	0.82 (0.69–1.2)	0.07 (−0.11–0.25)	0.479
Procalcitonin	0.25 (0.14–1.08)	0.22 (0.09–0.53)	−0.045 (−0.137–0.030)	0.303
Lactate dehydrogenase	392 (325–557)	384 (301–559)	−12 (−63.9–39)	0.665
Aspartate dehydrogenase	49.5 (32.5–70.8)	43.5 (29.8–74)	−7.7 (−15.000–13.0)	0.995
Alanine transaminase	43 (29–75)	41 (24.8–72.5)	−3.0 (−17.0–9.0)	0.566
Hospitalization				
Days from COVID-19 onset to hospital admission	6 (4–7)	7 (IQR 5–10)	−1.5 (−3.8–1.1)	0.139
Length of hospital stay, days	35.5 (25–53)	27 (17–37)	10 (2.1–17.9)	0.013
Length of ICU stay	27.5 (15–40)	12 (7–24)	12.8 (5.8–19.9)	0.001
	% (*n*)	% (*n*)	Crude OR (95% CI)	
Gender				
Male	82 (49)	69 (166)	1.95 (0.96–3.98)	0.060
Female	18 (11)	31 (73)		
Race				
Caucasian	56.6 (34)	50.2 (120)		
Latin American	13.3 (8)	10.4 (25)	0.88 (0.36–2.14)	0.819
Lifestyle habits				
Smoker	3.3 (2)	3.3 (8)	0.97 (0.18–4.45)	0.914
Previous comorbidities				
Hypertension	50 (30)	38.4 (92)	1.4 (0.81–2.6)	0.203
Diabetes mellitus	21.6 (13)	23 (55)	0.82 (0.41–1.6)	0.578
Asthma	0 (0)	3.7 (9)	0.79 (0.74–0.84)	0.127
COPD	5 (3)	3.7 (9)	1.3 (0.35–5.1)	0.712
Ischemic heart disease	3.3 (2)	7.5 (18)	0.42 (0.09–1.8)	0.386
Valvular heart disease	1.6 (1)	0.83 (2)	2.0 (0.17–22.5)	0.491
Auricular fibrillation	1.6 (1)	3.3 (8)	0.48 (0.06–3.9)	0.693
Obesity	38.3 (23)	35.1 (84)	0.96 (0.53–1.75)	0.911
Class 1	25 (15)	23 (55)	0.95 (0.48–1.86)	0.894
Class 2	11.6 (7)	7.9 (19)	1.28 (0.50–3.32)	0.598
Class 3	1.6 (1)	4.6 (11)	0.31 (0.04–2.55)	0.257
Bleeding	16.3 (8)	3.8 (7)	4.9 (1.7–14.5)	0.004
Transfusions				
Transfusion of blood components	50 (30)	25.5 (61)	2.9 (1.62–5.23)	0.000
Platelet's transfusion	13.3 (8)	5.4 (13)	2.6 (1.05–6.78)	0.032
Fresh-frozen plasma transfusion	8.3 (5)	4.1 (10)	2.0 (0.68–6.33)	0.188
ICU management				
Noninvasive mechanical ventilation	60 (36)	58.1 (139)	1.09 (0.60–1.97)	0.764
Invasive mechanical ventilation	88.3 (53)	66.5 (159)	3.8 (1.65–8.76)	0.001
Tracheotomy	60 (36)	43.9 (105)	1.9 (1.06–3.38)	0.028
Prone positions	81.6 (49)	53.5 (128)	3.8 (1.79–8.18)	0.000
Sepsis	33.3 (20)	17.9 (43)	3.06 (1.46–6.39)	0.002
Deaths	38.3 (23)	26.7 (64)	1.70 (0.93–3.07)	0.078

* The cases of thrombosis were distributed as follows: 31 cases of deep vein thrombosis (DVT); 26 of pulmonary embolism (PE) (24 of them peripheric and 2 central PE); 3 cases of stroke. A total of 15 cases of PE and 1 case of stroke also presented DVT. Nine cases of DVT also presented catheter-related thrombosis. No cases of acute myocardial infarction were found in this sample.

3.2. Risk Prediction Model

The model showed, with an SE of 83% and an SP of 62%, that age; sex; levels of D-dimer, leucocytes, and IL6 collected at admission; and levels of CRP collected during the first 24 h of hospitalization could predict thrombosis with an accuracy of 77% (95% CI 69.9–84.0%) (Figure 1).

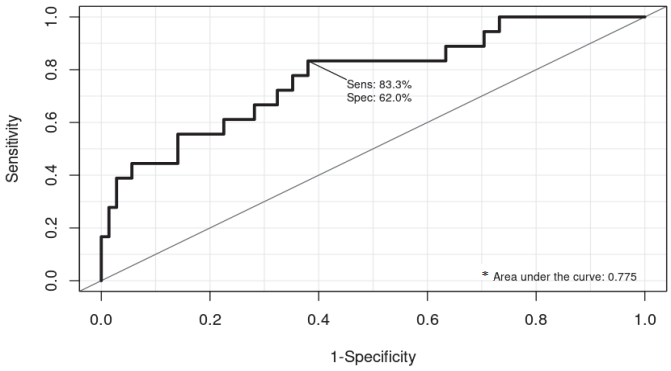

Figure 1. ROC curve and area under the curve (AUC) that assessed the discriminative ability of the final model. * Area under the curve = 0.775; 95% CI 0.6994, 0.8402.

The TS score, according to the factors included in the model, is shown in Figure 2. As can be observed, the overall TS score could range between 12 and 30 points, with age ranging between 41 and 70 and D-dimer values ≥ 500 ng/mL, the factors with the highest score values.

TS SCORE

Factor	Score
Male	1
Age 25–40	12
Age 41–70	13
Age ≥ 71	12
Leukocytes ≥ 10 × 10^3/µL	1
D dimer ≥ 500 ng/mL	13
IL6 ≥ 10 pg/mL	1
CRP ≥ 50 mg/L	1

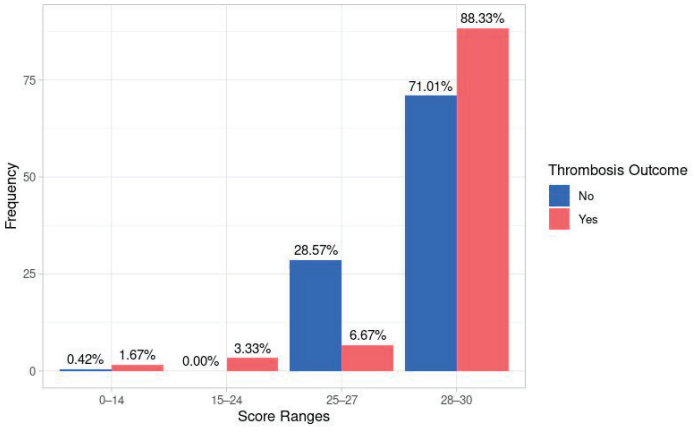

Figure 2. TS score and frequency of thrombosis according to cut-off values of 0–14, 14–24, 25–27, and 28–30.

Compared with the control group, the median TS score was higher in subjects with thrombosis (29, IQR 28–29 vs. 28, IQR 27–29, $p = 0.001$) (Figure 2). In addition, the frequency of thromboembolisms was proportional to a TS score increase. Thus, a TS score ≥28 had an SE for thrombosis of 88.3% (95% CI 78.7–94.8%) and an NPV of 91% (95% CI 83.2–96%); on the contrary, the SP (29.3%, 95% CI 23.8–35.3%) and PPV (23.8%, 95% CI 18.6–29.8%) were low.

A TS score ≥28 was associated with higher requirements for IMV (74.3% vs. 61.0%, OR 1.8, 95% CI 1.06–3.19, $p = 0.27$) and prone position (70.3% vs. 47.3%, OR 2.6 95% CI 1.52–4.55) compared with subjects with TS score vales ≤28.

Finally, during the hospital stay, 232 doppler ultrasounds were performed, and 47 cases of DVT were identified. We calculated that if a TS score ≥28 was considered before performing these tests, only 178 doppler ultrasounds of the lower limb veins would be indicated, which is a decrease of 23% in the number of tests performed. However, in contrast, only 41 cases of DVT (SE = 87.2%) would be diagnosed.

4. Discussion

The high incidence of adverse outcomes associated with thrombosis in COVID-19 individuals highlights the need to develop prediction models to identify patients at higher risk. In our study, subjects with thrombosis experienced worse outcomes, such as more extended hospital and ICU stays, higher rates of sepsis, and increased requirements for IMV, tracheotomy, and prone positions than individuals without thrombosis. Interestingly, the results of our study suggest that the TS score could predict thrombosis in hospitalized COVID-19 individuals within the first 24 h of admission with high sensitivity. In addition, despite the lack of statistical significance in comparing the mortality rates between patients with and without thrombosis a significant association was determined between a TS score $\geq$28 and IMV and prone position. This finding points to the impact of thromboembolism on the progression and severity of COVID-19 and suggests the possible additional utility of this score to identify subjects at higher risk of worse outcomes.

To the best of our knowledge, this is the first study that developed a clinical prediction rule for thromboembolism in severe COVID-19 patients admitted to an ICU. However, due to the characteristics of our sample, it is still being determined whether the TS score is valid for predicting thrombosis in less severe COVID-19 individuals. Other predictive scores, such as the 3D past score, which was performed in the inpatient COVID-19 population, has a similar sensitivity for thrombosis; however, the rate of ICU-admitted patients was not reported, and its relationship with other adverse outcomes was not studied [16].

As observed in other populations [16,17], D-dimer level elevation would be essential to reach a significant TS score; however, other blood tests must be considered to reach significance. For instance, regardless of age, men with D-dimer elevation must have one or two altered factors to reach a predictive cut-off point. In contrast, women must have two or three other abnormal parameters to reach a TS score $\geq$28.

Our predictive model found that well-known risk factors for thrombosis and hypercoagulability, such as LDH, fibrinogen, or lymphocyte levels, were not statistically significant [3,18]. However, the relationships between thrombosis and IL6 and CRP, which were included in the score, have been previously explored. For instance, Farouk et al. reported that IL6 levels at admission were related to DVT [19]. Similarly, Smilowitz found that the association between CRP levels and adverse outcomes was consistent in patients with low and high D-dimer levels [20].

Interestingly, our results demonstrated that if the TS score is considered when indicating a doppler ultrasound, the number of tests performed could considerably decrease, which could also decrease the related costs. Nonetheless, it must be noted that in the TS database, the reason for performing this test was not registered; thus, it needs to be clarified whether most of the tests were performed due to clinical suspicion of DVT or due to screening.

Limitations of the study need to be considered. For instance, the TS database includes retrospective records with a significant number of patients with incomplete information. Although we did not impute missing values to build a better model that predicted VTE individually, the exclusion of subjects with >30% of missing data could have led to bias. In addition, we considered the prevalence of thrombosis found in our sample; however, the PPV and the NPV of the score must be adapted to the prevalence of thrombosis in different COVID-19 populations. On the other hand, the blood test results that were considered in the final model were primarily taken in subjects admitted during the first waves of COVID-19, but currently, parameters such as IL6 and CRP are not routinely collected at admission. Thus, the TS score may not be feasible. Finally, this study lacked a validation cohort, so the following steps must include narrow and broad validation of the score in different patient samples and clinical environments that include larger and prospective cohorts.

5. Conclusions

The initial evaluation of COVID-19 subjects could play a fundamental role in the early identification of factors predictive for thrombosis. The TS could be an effective tool in clinical decision making for hospitalized COVID-19 population; however, further validation studies must be performed.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of La Paz University Hospital (protocol code HULP: PI-4114, approved on 28 April 2020).

Informed Consent Statement: Patient consent was waived due to the retrospective characteristics of this study.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors declare no conflict of interest.

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Characterizing and Predicting Post-Acute Sequelae of SARS CoV-2 Infection (PASC) in a Large Academic Medical Center in the US

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Abstract: Background: A growing number of Coronavirus Disease-2019 (COVID-19) survivors are affected by post-acute sequelae of SARS CoV-2 infection (PACS). Using electronic health record data, we aimed to characterize PASC-associated diagnoses and develop risk prediction models. Methods: In our cohort of 63,675 patients with a history of COVID-19, 1724 (2.7%) had a recorded PASC diagnosis. We used a case–control study design and phenome-wide scans to characterize PASC-associated phenotypes of the pre-, acute-, and post-COVID-19 periods. We also integrated PASC-associated phenotypes into phenotype risk scores (PheRSs) and evaluated their predictive performance. Results: In the post-COVID-19 period, known PASC symptoms (e.g., shortness of breath, malaise/fatigue) and musculoskeletal, infectious, and digestive disorders were enriched among PASC cases. We found seven phenotypes in the pre-COVID-19 period (e.g., irritable bowel syndrome, concussion, nausea/vomiting) and sixty-nine phenotypes in the acute-COVID-19 period (predominantly respiratory, circulatory, neurological) associated with PASC. The derived pre- and acute-COVID-19 PheRSs stratified risk well, e.g., the combined PheRSs identified a quarter of the cohort with a history of COVID-19 with a 3.5-fold increased risk (95% CI: 2.19, 5.55) for PASC compared to the bottom 50%. Conclusions: The uncovered PASC-associated diagnoses across categories highlighted a complex arrangement of presenting and likely predisposing features, some with potential for risk stratification approaches.

Keywords: Coronavirus Disease-2019 (COVID-19); post-acute sequelae of SARS CoV-2 (PASC, long COVID, post-COVID conditions); phenome-wide association study; phenotype risk score; electronic health records

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1. Introduction

Coronavirus Disease-2019 (COVID-19) has posed unprecedented challenges to the public health and healthcare system. As of 30 September 2022, 96,158,524 confirmed COVID-19 cases were in the US [1]. Studies suggest that 20 to 40% of patients with a history of COVID-19 may be affected by post-acute sequelae of COVID-19 (PASC) [2–4]—also termed post COVID conditions (PCC), [5,6], long COVID [7], post-acute COVID-19 syndrome (PACS) [8], chronic COVID-19 syndrome [9], and long haul COVID-19 [10]. PASC is an aggregate term for a highly heterogeneous group of post-COVID-19 problems, including persistent symptoms of acute infection (e.g., cough, fatigue, loss of smell [11–13]), new chronic disorders, (e.g., chronic lung or neurologic disease [3,14–21]), and late post-COVID

complications (e.g., autoimmune complications). COVID-19 vaccinations could decrease the risk for PASC by 13%–22% [22,23]; however, with a massive number of breakthrough infections and a relaxation of mitigation measures throughout the world, the high prevalence of PASC during an ongoing pandemic could present a tremendous burden for healthcare systems worldwide.

Several demographic factors, preexisting conditions, and biomarkers have been associated with PASC. For example, severe acute COVID-19, female gender, older age, preexisting diabetes, or the experience of specific symptoms during the acute COVID-19 phase, including fatigue, headache, hoarse voice, etc., were reported to increase the risk for PASC [24–27]. A previous investigation reported an immunoglobulin (Ig) signature, based on total IgM and IgG3, as a predictor for PASC [28], while another study identified a series of features, including the rate of healthcare utilization, patient age, dyspnea, and other diagnosis and medication information, to predict PASC [29]. Another study identified four risk factors: type 2 diabetes, the presence of SARS-CoV-2 RNA, Epstein–Barr virus, and specific auto-antibodies [30]. Together, these studies highlight the possibility and the need to uncover and understand PASC risk factors to identify and protect vulnerable groups. Furthermore, a better understanding of PASC might allow the identification of PASC subtypes and their specific risk profiles. However, the novelty of this condition and the sparsity of studies so far have hampered the development of risk-prediction models for PASC.

In our current study, we aim to fill this gap by identifying predisposing diagnoses of PASC through phenome-wide association studies (PheWAS) of the pre-COVID-19 and acute-COVID-19 time periods and then use the identified pre-existing conditions to develop and evaluate integrated and usable phenotype risk scores (PheRS) [31] to predict PASC [32,33]. To do this, we leverage a cohort of over 60,000 patients with a history of COVID-19 cared for at Michigan Medicine (MM), a large academic medical center in the Midwestern US, between March 2020 and August 2022. This cohort includes 1724 patients that were subsequently diagnosed with PASC using diagnostic codes or clinical problem lists. With its rich retrospective EHR data that includes socioeconomic status (SES), demographics, and other relevant variables, this cohort offers a unique opportunity to study PASC.

2. Materials and Methods

2.1. Study Cohort

The study included Michigan Medicine (MM) patients with a recorded COVID-19 diagnosis or a positive real-time reverse transcriptase chain (RT-PCR) test for SARS-CoV-2 infection performed/recorded at MM between 10 March 2020, and 31 August 2022. Diagnoses were recorded at clinic visits and hospital encounters. RT-PCR testing data were collected for routine screening at hospital admission, before procedures, and for employee screening. Tests included both symptomatic and asymptomatic individuals.

For each subject, the date of their first COVID-19 diagnosis or RT-PCR positive test, whichever came first, was considered the index date. Dates were regarded as protected health information and operationalized as days since birth; however, the quarter of the year of the index date was obtained. To allow sufficient follow-up time for diagnosing PASC, we limited the analysis to patients with encounters at least two months after being COVID-19 positive and stratified them in PASC cases (had a recorded PASC diagnosis) and PASC controls (had no recorded PASC diagnosis).

PASC diagnoses were either based on an entry of PASC in the diagnosis section of the EHR database's Problem Summary List (PSL, Table S1) or on observations of the ICD-10-CM (International Classification of Diseases codes, tenth edition with clinical modifications) U09.9 ("Post COVID-19 condition, unspecified") or B94.8 ("Sequelae of other specified infectious and parasitic diseases"). The CDC recommended the latter as a temporary alternative to the PASC-specific U09.9 code, which was implemented on 1 October 2021 [34]. PSL diagnoses represent active and resolved patient problems entered by healthcare providers. The age at the first observed ICD- or PSL-based PASC diagnosis

was considered the age of onset of PASC. PASC cases (see definition below) without a prior positive test were excluded because the timepoint of the test was crucial for defining the pre-COVID-19 and acute-COVID-19 time periods (Figure 1).

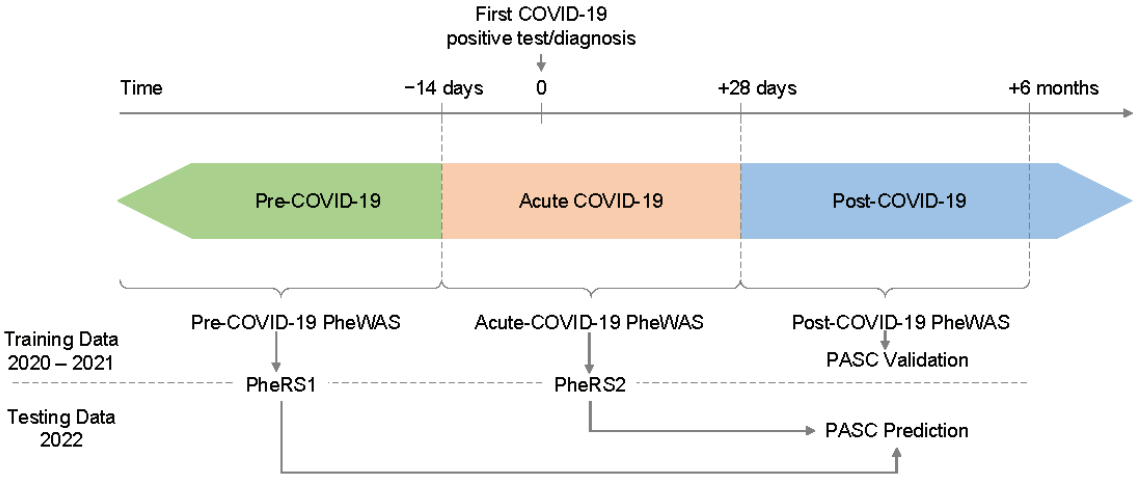

Figure 1. Schematic on study design. Three time periods were defined relative to the 1. positive COVID-19 test or diagnosis (index date): pre-COVID-19 until −14 days, acute-COVID-19 from −14 to +28 days, and post-COVID-19 from +28 days onwards. The post-COVID-19 PheWAS is used to validate features of PASC cases compared to COVID-19 cases without PASC diagnoses. The pre-COVID-19 and acute-COVID-19 PheWAS on the training data (index date in 2020–2021) inform on phenotype risk scores (PheRS) that will be used to predict PASC in the testing data (index date in 2022).

We also categorized PASC patients based on ICD10 diagnoses concurrently recorded with their first PASC diagnosis and mapped them to 29 phenotype concepts previously reported as common PASC symptoms [3]. In addition, we manually mapped detailed PSL diagnoses to these 29 concepts (Tables S1 and S2).

2.2. Definition of Demographics, Socioeconomic Status, and Other Covariates

To examine and adjust for confounding by patient characteristics, socioeconomic status, and other variables, we obtained the following data for each participant: age, self-reported gender, self-reported race/ethnicity, neighborhood disadvantage index (NDI) without proportion of Black (coded as quartiles, with larger quartiles representing more disadvantaged communities) [35,36], and population density measured in persons per square mile (operationalized as quartiles).

Additional covariates included vaccination status, the Elixhauser comorbidity score [37,38], COVID-19 severity (non-severe (not hospitalized) and severe (hospitalized or deceased)), healthcare worker (HCW) status, the timespan of records in the EHR before and after the COVID-19 test/diagnosis, the timespan of records in the EHR before 2020 (referred to as "pre-pandemic" time period). These timespans were based on the first or last recorded encounter in the EHR data. Additional details and definitions of these covariates can be found in Appendix A and Table S3.

We assumed completely at random missingness of the covariates included in our adjusted analyses and performed complete case analyses for each adjustment.

2.3. Time-Restricted Phenomes

We constructed each subject's medical phenome by extracting available ICD9 and ICD10 codes from the EHR and mapping them to 1813 broader phenotype concepts (Phe-

Codes) using the R package "PheWAS" [39,40]. In short, individuals with ICD codes that map to a specific PheCode were coded as "1", then individuals with ICD codes that map to the PheCode's specific exclusion criteria were coded as missing, and finally, all remaining individuals were coded as "0" for that particular PheCode (further details are described elsewhere [40]). We created three time-restricted phenomes relative to the index date: post-COVID-19 (+28 days to +6 months), pre-COVID-19 (predating −2 weeks), and acute COVID-19 (−14 and +28 days; Figure 1).

2.4. Matching

To minimize confounding when we compare PASC (case) versus no PASC (control), we matched each PASC case to up to 10 PASC controls using the R package "MatchIt" [41]. Nearest neighbor covariate matching was applied for age at index date, pre-COVID-19 years in EHR, and post-COVID-19 years in EHR without applying a caliper. Exact matching was used for sex, primary care visit at Michigan Medicine within the last two years (yes/no), race/ethnicity, and year quarter of the index date. We retained the case–control matching throughout all analyses.

2.5. Statistical Analysis

2.5.1. PASC-Associated PheCodes in Post COVID-19 Period

To characterize diagnoses enriched in COVID-19 patients with PASC, we also conducted PheWAS to identify phenotypes associated with PASC in the post-COVID-19 period (at least 28 days after the COVID-19 index date, see Figure 1) using Firth bias-corrected logistic regression by fitting the following model for each PheCode of the post-COVID-19 period phenome:

$$\begin{aligned} logit\,(P(PheCode \;&= 1 \mid PASC,\, Covariates)) \\ &= \beta_0 + \beta_{PASC}\text{PASC} + \beta_{Covariate\ 1}\ \text{Covariate 1} + \beta_{Covariate\ 2}\ \text{Covariate 2} \\ &+ \ldots + \beta_{Covariate\ p}\ \text{Covariate p} \end{aligned} \tag{1}$$

where covariates were pre-COVID-19 Elixhauser Score (AHRQ), NDI, population density, healthcare worker status (HCW), vaccination status, and severity, details are summarized in Appendix A and Table S3.

2.5.2. Pre-Disposing PheCodes

We conducted PheWAS to identify PheCodes pre-disposing to PASC using either PheCodes from the pre-COVID-19 period or PheCodes from the acute-COVID-19 period. We ran Firth bias-corrected logistic regression by fitting the following model for each PheCode of the corresponding time-restricted phenome:

$$\begin{aligned} logit\,(P(PASC \;&= 1 \mid Phecode\ is\ present,\, Covariates)) \\ &= \beta_0 + \beta_{PheCODE}\text{PheCODE} + \beta_{Covariate\ 1}\ \text{Covariate 1} \\ &+ \beta_{Covariate\ 2}\ \text{Covariate 2} + ... + \beta_{Covariate\ p}\ \text{Covariate p} \end{aligned} \tag{2}$$

We applied a similar set of covariate adjustments as before (Table S3).

The phenomes were split into a training set (index dates in 2020 and 2021) and a testing set (index date in 2022). This choice was to retain the true spirit of future prediction using past data. The training set was used to identify predisposing PheCodes in phenome-wide association studies (PheWAS), while the testing set was used to evaluate prediction models based on the PheWAS results.

To evaluate the robustness of effect sizes of predisposing PheCodes, we performed several sensitivity analyses: (1) females only, (2) males only, (3) index date in 2020, (4) index date in 2021, (5) non-severe outcomes (not hospitalized), (6) severe outcomes (hospitalized or deceased), (7) recorded within two years before the index date, and (8) pre-pandemic (before 2020). For the acute-COVID-19 PheWAS, we excluded PASC cases whose first

recorded PASC diagnosis was observed less than 28 days after the index date. The sample sizes of the complete case analyses for various analyses are listed in Table S4.

PheWASs were restricted to PheCodes observed at least five times among cases and among controls. For all PheWAS, we excluded PheCode 136 "Other infectious and parasitic diseases" as it included the ICD-10 code "B94.8" which was used to record a PASC diagnosis.

For each PheWAS, we applied a Bonferroni correction adjusting for the number of analyzed PheCodes (Table S4). In Manhattan plots, we present –log10 (p-value) corresponding to tests for association of the underlying phenotype. Directional triangles on the PheWAS plot indicate whether a trait was positively (pointing up) or negatively (pointing down) associated.

We also tested for differences between effect sizes of three subgroup comparisons (non-severe vs. severe outcome, female vs. male, and index date in 2020 vs. 2021) using the following t-statistics:

$$t = \frac{\beta_A - \beta_B}{\sqrt{SE(\beta_A)^2 + SE(\beta_B)^2}} \tag{3}$$

where β_A and β_B are the subgroup-specific beta-estimates with corresponding standard errors $SE(\beta_A)$ and $SE(\beta_B)$.

2.5.3. Phenotype Risk Scores (PheRS)

PheRS Generation

To generate the phenotype risk score or PheRS, we first screened the PheWAS for PheCodes that were phenome-wide significant at a Bonferroni corrected threshold in a one-at-a-time analysis in terms of their association with PASC (after adjusting for covariates). Next, we ran a joint multivariate model with all phenome-wide significant PheCodes using ridge penalized logistic regression (R Package "glmnet" [42,43]) to obtain the adjusted coefficients/weights per PheCode from the training data before calculating the PheRS in the testing data. More specifically, we weighted the presence of PheCodes with their adjusted coefficients from the multivariate ridge penalized logistic regression and calculated the PheRS as the weighted sum. For subject j, the PheRS was of the form $\text{PheRS}_j = \sum_i \hat{\beta}_i \ PheCode_{ij}$ where the sum extends over all included PheCodes, $\hat{\beta}_i$ are the adjusted ridge regression coefficients for PheCode i from the multivariate model, and $PheCode_{ij}$ denotes the presence/absence (coded as 1 and 0) of a PheCode i in subject j. We used Ridge regression because it has been shown to offer good performance when there is multicollinearity between features, and when prediction is the goal [44].

PheRS Evaluation

To evaluate each of the PheRS, we fit the following Firth bias-corrected logistic regression model adjusting for age, gender, race/ethnicity, Elixhauser Score, population density, NDI, HCW, vaccination status, pre-COVID19 years in EHR and severity using a complete case analysis:

$$\begin{aligned} logit\,(P(PASC \ &= 1 \mid PheRS,\ Covariates)) \\ &= \beta_0 + \beta_{PheRS}\text{PheRS} + \beta_{Covariate\ 1}\text{Covariate 1} \\ &+ \beta_{Covariate\ 2}\ \text{Covariate 2} + ... + \beta_{Covariate\ p}\text{Covariate p} \end{aligned} \tag{4}$$

For each PheRS, we assessed the following performance measures relative to the PASC status: (1) overall performance with Nagelkerke's pseudo-R^2 using R packages "rcompanion" [45], (2) accuracy with Brier score using R package "DescTools" [46]; and (3) ability to discriminate between PASC cases and matched controls as measured by the area under the covariate-adjusted receiver operating characteristic (AROC; semiparametric frequentist inference) curve (denoted AAUC) using R package "ROCnReg" [47]. Firth's bias reduction method was used to resolve the problem of separation in logistic regression (R package "brglm2") [48].

To also evaluate models with both predictors (PheRS1-Ridge + PheRS2-Ridge), we combined them by first fitting a logistic regression with the predictors in the training set to obtain the linear predictors that we used to obtain the combined score in the testing data.

Unless otherwise stated, analyses were performed using R 4.2.0 [49].

3. Results

3.1. Patient Characteristics

Among 63,675 patients with a history of COVID-19 who were seen in MM at least two months after their first record of COVID-19, 1724 (2.7%) received a PASC diagnosis. The PASC prevalence within three months of a COVID-19 infection ranged from 0.18% (Q3 of 2020) to 1.8% (Q3 of 2021). The most PASC cases were observed in Q4/2021 (n = 134), coinciding with the second peak of positive tests at MM (Table 1; Figure S1).

We observed that PASC cases compared to controls were on average older at their index date (mean age 47.9 versus 41.7 years), had a slightly longer timespan covered in the pre-test EHRs (11.7 versus 10.4 years), were more likely female (64.5% versus 56.7%), more likely to have received primary care at MM in the last two years (60.7% versus 46.4%) and showed different distributions across the year quarters over time (Table 1). To adjust for these observed differences, we performed nearest neighbor matching (age at index date, pre-test years in EHR, post-test years in EHR) and exact matching (gender, primary care at MM, race/ethnicity, quarter of year at COVID-19 index date). All significant differences in covariates became non-significant after matching (Table 1).

3.2. PASC Symptoms/Post-COVID-19 PheWAS

When categorizing 1362 PASC cases with concurrent diagnoses based on 29 previously reported symptoms [3] (362 of the 1724 cases had no concurrent diagnoses, Tables S1 and S2), the 10 most common diagnoses were: shortness of breath (34.3%), anxiety (30.6%), malaise and fatigue (28.5%), depression (27.2%), sleep disorders (25.4%), asthma (23.6%), headaches (21.4%), migraine (13.8%), cough (13.0%) and joint pain (12.6%) (Table S5).

In the post-COVID-19 PheWAS of 1256 cases versus 12,492 matched controls, all 29 PASC symptoms were enriched among PASC cases (OR > 1), and 27 reached phenome-wide significance ($p < 0.05/960$ tested PheCodes; $p < 5.2 \times 10^{-5}$) while 2 were not significant (Table S6). In addition to PASC-related phenotypes (e.g., shortness of breath: OR = 9.03 [7.77, 10.50], $p = 2.94 \times 10^{-181}$; malaise and fatigue: OR = 6.17 [5.33, 7.14], $p = 2.32 \times 10^{-132}$; and cardiac dysrhythmias: OR = 2.75 [2.37, 3.18], $p = 3.95 \times 10^{-41}$), many additional diagnoses were enriched in PASC cases, among others musculoskeletal disorders (e.g., costochondritis: OR = 6.88 [95%: 3.05, 14.8], $p = 6.72 \times 10^{-8}$), infectious diseases (e.g., septicemia: OR = 2.31 [1.66, 3.16] $p = 2.67 \times 10^{-7}$), and digestive disorders (e.g., gastroesophageal reflux disease (GERD): OR = 1.72 [1.50, 1.99], $p = 5.10 \times 10^{-14}$) (Figure 2, File S1A).

3.3. Pre-COVID-19 PheWAS

Of the 1724 individuals, 163 had incomplete covariate data. The 1561 remaining individuals were split into a training set (1212 individuals whose 1. positive test/diagnosis was recorded before 2022) and a testing set (349 individuals whose 1. positive test/diagnosis was recorded in 2022; also see flowchart in Figure S2). To identify potential PASC-predisposing conditions, we performed a PheWAS using the pre-COVID-19 phenome, comparing 1212 PASC cases versus 11,919 matched controls. Among 1405 tested PheCodes, 7 reached phenome-wide significance ($p < 3.56 \times 10^{-5}$): irritable bowel syndrome (IBS; OR = 1.78 [1.44, 2.18], $p = 4.00 \times 10^{-8}$), concussion (OR = 1.95 [1.51, 2.49], $p = 1.24 \times 10^{-7}$), nausea and vomiting (OR = 1.45 [1.26, 1.67], $p = 2.90 \times 10^{-7}$), shortness of breath (OR = 1.51 [1.29, 1.76] 3.38×10^{-7}), respiratory abnormalities (OR = 1.39 [1.22, 1.59], $p = 1.10 \times 10^{-6}$), allergic reaction to food (OR = 1.94 [1.42, 2.60], $p = 1.66 \times 10^{-5}$) and general circulatory disease (OR = 1.52 [1.24, 1.85], $p = 3.30 \times 10^{-5}$; Figure 3, File S1B).

Table 1. Characteristics of patients with a history of COVID-19, stratified into patients with a PASC diagnosis (cases) and without observed PASC diagnosis (controls). Case–control matching was based on nearest neighbor matching (age at index date, pre-test years in EHR, post-test years in EHR) and exact matching (gender, primary care at MM, race/ethnicity, quarter of year at COVID-19 index date).

	COVID-19 Patients with PASC Diagnosis	COVID-19 Patients without PASC Diagnosis			
		Unmatched	*p* Value *	Matched	*p* Value *
n	1724	61951		17205	
Age at index date; mean (SD)	47.88 (18.85)	41.67 (22.14)	<0.001	47.12 (18.94)	0.110
Pre-test years in EHR; mean (SD)	11.70 (7.47)	10.41 (7.49)	<0.001	11.67 (7.37)	0.870
Post-test years in EHR; mean (SD)	1.07 (0.56)	0.93 (0.55)	<0.001	1.05 (0.55)	0.445
Female; *n* (%)	1112 (64.5)	35713 (57.6)	<0.001	11089 (64.5)	0.989
Primary care at MM; *n* (%)	1047 (60.7)	28773 (46.4)	<0.001	10435 (60.7)	0.969
Race/ethnicity; *n* (%)			0.151		0.990
Caucasian/Non-Hispanic	1273 (73.8)	44822 (72.4)		12730 (74.0)	
African American/Non-Hispanic	199 (11.5)	7020 (11.3)		1990 (11.6)	
Other/Non-Hispanic or Hispanic	175 (10.2)	6593 (10.6)		1746 (10.1)	
Other/unknown ethnicity	77 (4.5)	3516 (5.7)		739 (4.3)	
Quarter of year at index date; *n* (%)			<0.001		1.000
2020/1	27 (1.6)	588 (0.9)		263 (1.5)	
2020/2	57 (3.3)	1697 (2.7)		555 (3.2)	
2020/3	64 (3.7)	2617 (4.2)		640 (3.7)	
2020/4	273 (15.8)	13317 (21.5)		2730 (15.9)	
2021/1	236 (13.7)	7063 (11.4)		2360 (13.7)	
2021/2	241 (14.0)	5475 (8.8)		2410 (14.0)	
2021/3	168 (9.7)	4088 (6.6)		1680 (9.8)	
2021/4	282 (16.4)	10853 (17.5)		2820 (16.4)	
2022/1	268 (15.5)	10887 (17.6)		2680 (15.6)	
2022/2	100 (5.8)	5008 (8.1)		1000 (5.8)	
2022/3	8 (0.5)	358 (0.6)		67 (0.4)	
Neighborhood Deprivation Index (%)			0.003		0.350
Quartile 1	631 (36.6)	22679 (36.6)		6629 (38.5)	
Quartile 2	401 (23.3)	13028 (21.0)		3708 (21.6)	
Quartile 3	325 (18.9)	11330 (18.3)		3203 (18.6)	
Quartile 4	253 (14.7)	9235 (14.9)		2444 (14.2)	
Missing	114 (6.6)	5679 (9.2)		1221 (7.1)	
Population density (%)			0.002		0.128
Quartile 1	413 (24.0)	15218 (24.6)		4417 (25.7)	
Quartile 2	491 (28.5)	17796 (28.7)		5013 (29.1)	
Quartile 3	551 (32.0)	18123 (29.3)		5229 (30.4)	
Quartile 4	155 (9.0)	5135 (8.3)		1325 (7.7)	
Missing	114 (6.6)	5679 (9.2)		1221 (7.1)	
Elixhauser Score AHRQ; mean (SD)	4.52 (12.97)	3.75 (10.72)	0.003	4.01 (11.36)	0.077

* *p*-value of differences between COVID-19 patients with a PASC diagnosis and COVID-19 patients without a PASC diagnosis. Abbreviations: EHR, electronic health records; MM, Michigan Medicine; AHRQ, Agency for Healthcare Research and Quality

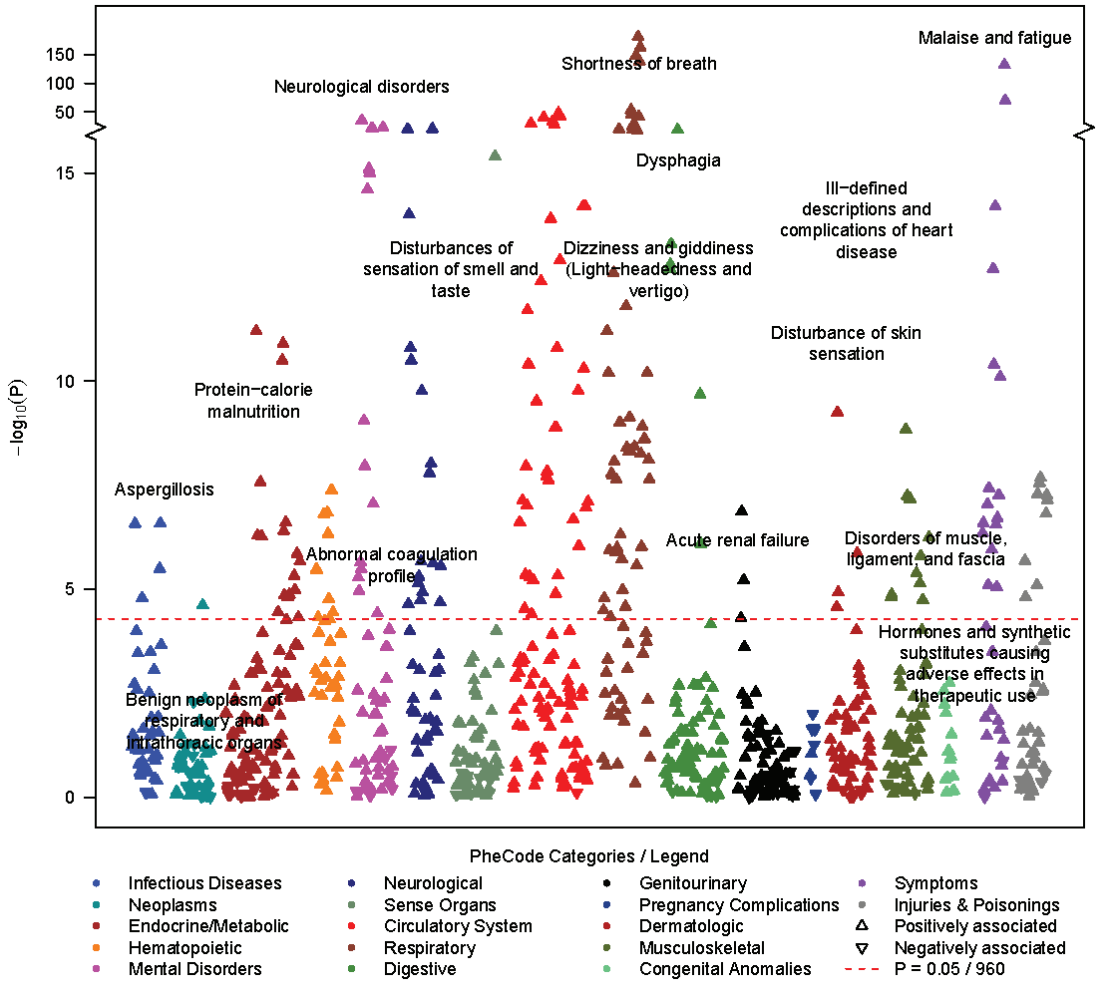

Figure 2. PheWAS on symptoms that occurred between 28 days and 6 months after the first COVID-19 test (outcome: post-COVID-19 symptoms/phecodes; predictor: PASC diagnosis yes/no). Among PheCodes that reached phenome-wide significance (red dashed line, $p \leq 0.05/960 = 5.2 \times 10^{-5}$), only the strongest association per PheCode category was labeled. The analysis was adjusted using the following covariates: age at index date, gender, race/ethnicity, Elixhauser Score AHRQ, population density (quartiles), NDI (quartiles), health care worker status, vaccination status, post-test years in EHR, and severity. Summary statistics can be found in File S1.

Additional sensitivity analyses indicated robust associations across various settings (females only, males only, 2020 only, 2021 only, non-severe outcome, severe outcomes, within two years before the index date, or before the pandemic, Figure S3A–G, File S1D–F).

3.4. *Acute-COVID-19 PheWAS*

To uncover PASC-predisposing acute-COVID-19 symptoms, we screened 664 phenotypes of the acute-COVID-19 phenome, comparing 874 cases with 8671 controls. To not identify actual PASC symptoms compared to pre-PASC symptoms, we excluded cases whose PASC diagnosis was recorded less than 28 days after their index date and only retained their matched controls. A total of 69 phenotypes was significantly associated

with PASC ($p < 7.54 \times 10^{-5}$) and included, among others, 22 respiratory phenotypes (e.g., shortness of breath, respiratory failure/insufficiency/arrest, dependence on a respirator or supplemental oxygen, and cough), 13 circulatory system phenotypes (orthostatic hypotension, hypotension), 7 neurological phenotypes (e.g., sleep disorder, migraine, pain), 6 digestive phenotypes (e.g., GERD, IBS), 5 mental health phenotypes (e.g., anxiety, depression), and other symptoms (e.g., malaise and fatigue, myalgia and myositis) (Figure 4, File S1C).

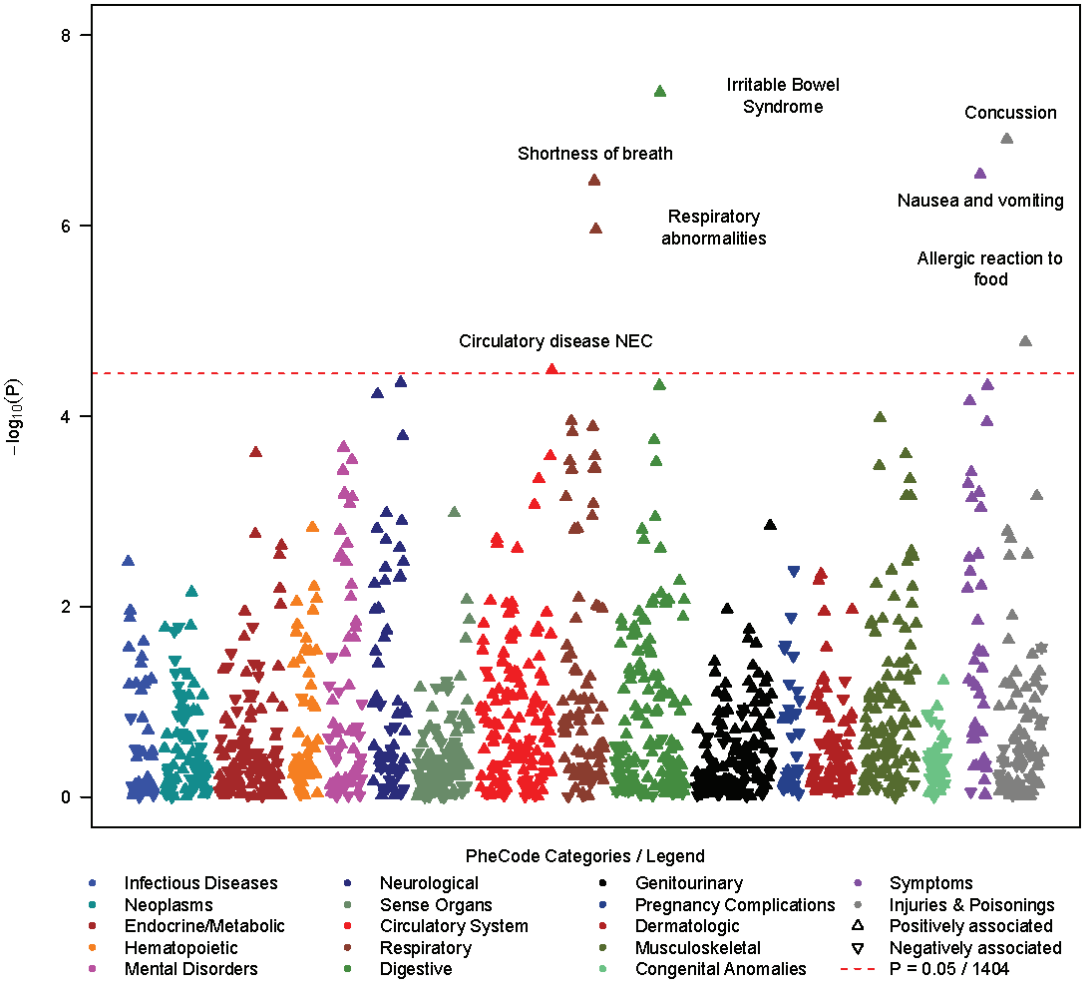

Figure 3. PheWAS on symptoms that occurred at least 14 days before the first positive COVID-19 test (outcome: PASC diagnosis yes/no; predictors: PheCodes). Among PheCodes that reached phenome-wide significance (red dashed line, $p \leq 0.05/1404 = 3.56 \times 10^{-5}$), only the strongest association per PheCode category was labeled. The analysis was adjusted using the following covariates: age at index date, gender, race/ethnicity, Elixhauser Score, population density (quartiles), NDI (quartiles), health care worker status, vaccination status, pre-test years in EHR, and severity. Summary statistics can be found in File S1.

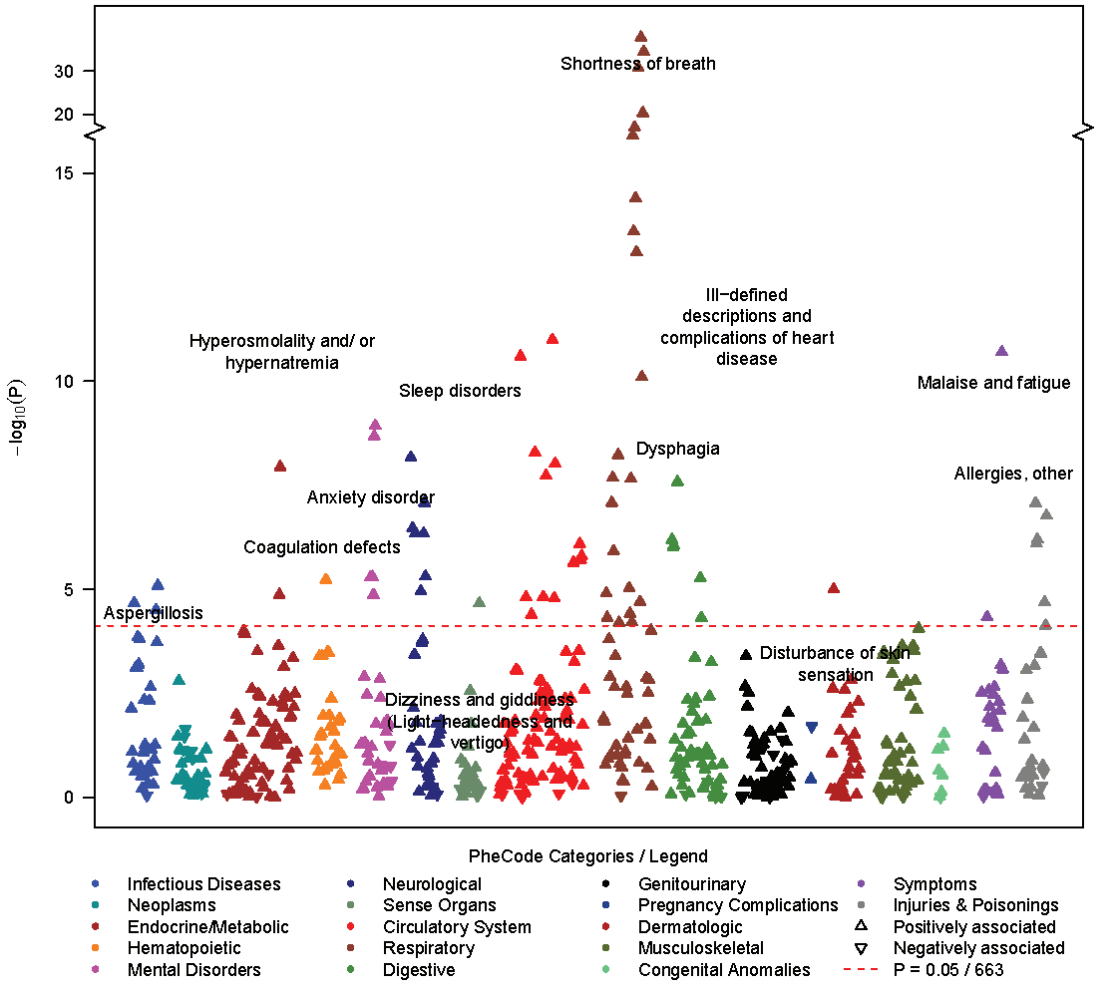

Figure 4. Acute-COVID-19 PheWAS on symptoms that occurred between −14 and +28 days relative to testing positive for COVID-19 (outcome: acute-COVID-19 symptoms/PheCodes; predictor: PASC diagnosis yes/no). Among PheCodes that reached phenome-wide significance (red dashed line, $p \leq 0.05/663 = 7.5 \times 10^{-5}$), only the strongest association per PheCode category was labeled. The analysis was adjusted using the following covariates: age at index date, gender, race/ethnicity, Elixhauser Score AHRQ, population density (quartiles), NDI (quartiles), health care worker status, vaccination status, post-test years in EHR, and severity. Summary statistics can be found in File S1.

Our sensitivity analyses indicated robust associations across various settings (females only, males only, 2020 only, 2021 only, non-severe outcomes, severe outcomes) where most associations remained nominally significant in each sub-analyses or had overlapping confidence intervals in their sensitivity analyses. However, effect sizes were not as consistent (Figure S4A–AK, File S1G–I). Noteworthily, the effect size for shortness of breath differed significantly between index dates in 2020 and 2021 (2020: OR = 2.20 [1.60, 2.99], $p = 7.8 \times 10^{-7}$ compared to 2021: OR = 4.59 [3.62, 5.81], $p = 9.37 \times 10^{-37}$; $P_{Difference} = 0.000234$), though they were significantly associated with PASC in both years (Figure S4AA, File S1C,I). Despite low numbers of individuals with severe outcomes (160 PASC cases and 150 controls), 6 of the 69 significantly associated phenotypes (as-

pergillosis, bacterial pneumonia, MRSA pneumonia, hyperosmolality and/or hypernatremia, septic shock, and voice disturbances) only had sufficient observations among the subset with severe outcomes but among the non-severe outcome subset (724 PASC cases and 6799 controls; Table S4 and File S1C,G). This suggested that these phenotypes might be hospital-acquired complications. None of the 49 significantly associated phenotypes that were tested among individuals with non-severe outcomes and individuals with severe outcomes showed significant effect size differences ($P_{difference} \geq 0.001$ [0.05/49 tests]). All phenotypes with nominal effect size differences between non-severe and severe outcomes ($P_{difference} < 0.05$) were all strongly and positively associated in individuals with non-severe outcomes, thus unlikely to merely represent hospital-acquired complications (File S1G).

3.5. Comparison of "Pre-PASC" Associated PheCode across Three PheWAS

To investigate whether the associated "pre-PASC" phenotypes of the pre- and acute-COVID-19 periods ("pre-PASC" phenotypes) are associated with novel PASC symptoms or if they become long-term features that manifest as PASC, we explored their frequencies and their association signals across all three PheWAS (Figure S5). Interestingly, almost all associated "pre-PASC" phenotypes were also significantly enriched in the post-COVID-19 PheWAS, except for "allergic reaction to food" of the pre-COVID-19 PheWAS and "candidiasis" and "inflammation and edema of the lung" in the acute-COVID-19 PheWAS. However, their ORs were all positive (File S1A–C). While we observed similarities between pre-existing conditions and presenting PASC features, further analyses using rigorous causal inference methods are needed to evaluate their causal role in developing PASC. The current analysis is merely correlative and a prediction exercise.

3.6. Developing Phenotype Risk Scores for Predicting PASC

The pre- and acute-COVID-19 PheWASs indicated pre-disposing conditions for PASC. To study whether these conditions might be helpful in predicting PASC among patients with a history of COVID-19, we generated two PheRSs: a pre-COVID-19 PheRS "PheRS1" and an acute-COVID-19 PheRS "PheRS2". We avoided overfitting by using PheWAS results and PheRS weights obtained from individuals with index dates in 2020 or 2021, while the evaluations were performed in individuals with index dates in 2022 (Figures 1 and S2 and File S1J). To limit the impact of potential hospital-acquired complications of an acute-COVID-19 infection, we excluded the six phenotypes that were only tested/observed in the individuals with severe outcomes (see "acute-COVID-19 PheWAS" above).

We found that PheRS1 and PheRS2 could discriminate cases and controls, yet only with low accuracy (AAUC < 0.7). PheRS1 performance was comparable in the complete testing data ($AAUC_{PheRS1}$ = 0.548 [95% CI: 0.516, 0.580]) and the testing data that were reduced to PASC cases that had at least 28 days between their index date and the PASC diagnosis ($AAUC_{PheRS1}$ = 0.555 [95% CI: 0.496, 0.612]). PheRS2 was only analyzed in the latter data ($AAUC_{PheRS2}$ = 0.605 [95% CI: 0.549, 0.663]) but performed better than PheRS1, which was also evident from its pseudo-R^2 which was almost five-fold higher (0.0116 and 0.0547, respectively). A combination score further improved the discrimination of cases and controls, but its accuracy remained low ($AAUC_{Combined}$ = 0.615 [0.561, 0.670]; Table 2).

We also explored if PheRSs based on additional suggestively associated PheCodes (defined as $p < 1 \times 10^{-3}$) could further improve the prediction of PASC but found their individual or combined predictive ability slightly worse compared to the PheRSs that were based on phenome-wide significant hits (e.g., $AAUC_{Combined}$ = 0.601 [0.548, 0.658]; Table S7).

While the use for individual-level prediction seemed very limited, we found that PheRS1 and PheRS2 could significantly enrich PASC cases in their top 10% and top 10–25% risk bins compared to the lower 50% of their distributions (Table 3). For example, individuals in the top 10% of PheRS1 were 2.5 times (OR = 2.48 [95% CI: 1.24, 4.97]) and in the top 10% of PheRS2 4.1 times more likely to obtain a PASC diagnosis (OR: 4.10 [2.28, 7.40]). Moreover, both PheRSs combined improved enrichment also in the top 10–25% risk

bin (OR: 2.91 [1.73, 4.90]), identifying a fourth of all COVID-19 cases with substantially increased risk for PASC (Table 3, Figure 5).

Table 2. PheRS Evaluation in the testing data (COVID-19 positive in 2022). PheRS1 was based on the significant hits of the PheWAS with the pre-COVID-19 training data (1256 cases and 11,674 controls; COVID-19 positive in 2020/2021) while PheRS2 was based on the significant hits of the PheWAS with the acute-COVID-19 training data (874 cases and 8144 controls; COVID-19 positive in 2020/2021 and at least 28 days between first COVID-19 and first PASC diagnosis). Underlying weights can be found in File S1J and Table S8.

Predictor	Testing Data		AAUC [a] (95% CI)	Pseudo-R^2 [b]	Brier Score
	n Cases	*n* Controls			
PheRS1	349	3248	0.548 (0.516, 0.580)	n/a [c]	n/a [c]
PheRS1	123	1154	0.555 (0.496, 0.612)	0.0116	0.0857
PheRS2			0.605 (0.549, 0.663)	0.0547	0.0823
PheRS1 and PheRS2			0.615 (0.561, 0.670)	0.0553	0.0824

[a] Adjusted for age at index date, gender, race/ethnicity, Elixhauser Score, population density, NDI, health care worker status, vaccination status, pre-test years in EHR, and severity; [b] Nagelkerke (Cragg and Uhler)); [c] not applicable, only useful in evaluating multiple models predicting the same outcome on the same dataset.

Table 3. PheRS-based risk stratification in the testing data. Analysis is based on patients with a history of COVID-19 in 2022 with at least 28 days between the first COVID-19 and the first PASC diagnosis; 123 cases and 1154 controls.

PheRS	Upper Risk Bin	%Cases in Risk Bin	%Cases in Lower 50%	OR (95% CI) [a]	*p*
PheRS1	25–50%	10.0	7.8	1.48 (0.91, 2.42)	0.12
	10–25%	12.1		1.86 (1.06, 3.25)	0.029
	≥10%	13.6		2.48 (1.24, 4.97)	0.011
	≥25%	12.7		2.10 (1.29, 3.43)	0.0029
PheRS2	25–50%	8.1	6.6	1.26 (0.76, 2.08)	0.38
	10–25%	12.6		2.13 (1.25, 3.62)	0.0053
	≥10%	21.6		4.10 (2.28, 7.40)	2.7×10^{-6}
	≥25%	16.5		2.92 (1.85, 4.59)	3.9×10^{-6}
PheRS1 and PheRS2	25–50%	8.3	6.2	1.36 (0.82, 2.28)	0.23
	10–25%	15.2		2.91 (1.73, 4.90)	5.8×10^{-5}
	≥10%	19.4		3.94 (2.10, 7.42)	2.1×10^{-5}
	≥25%	17.0		3.48 (2.19, 5.55)	1.5×10^{-7}

[a] Enrichment of PASC cases in risk bin compared to lower 50%; adjusted for age at index date, gender, race/ethnicity, Elixhauser Score, population density, NDI, health care worker status, vaccination status, pre-test years in EHR, and severity.

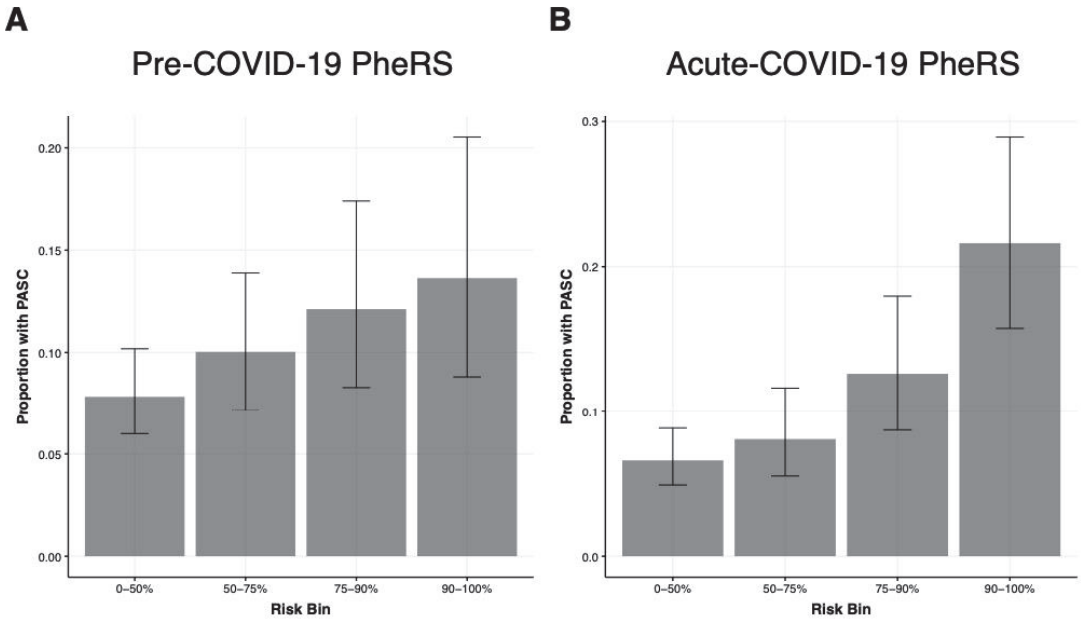

Figure 5. PheRS-based risk stratification in the testing data. The proportion of PASC cases among different PheRS bins is shown for (**A**) the pre-COVID-19 PheRS (PheRS1) and (**B**) the acute-COVID-19 PheRS (PheRS2). The analysis is based on patients with history of COVID-19 in 2022 with at least 28 days between the first COVID-19 and first PASC diagnosis; 123 cases and 1154 controls. Risk bins correspond to selected ranges of the PheRS distributions. Vertical lines represent confidence intervals for binomial proportions [46].

4. Discussion

In this study, we used data from a relatively large cohort of patients with history of COVID-19 from Michigan Medicine. We applied a PheWAS approach across time-restricted phenomes to identify phenotypes that may predispose to PASC. We found seven phenotypes (IBS, concussion, nausea and vomiting, shortness of breath, respiratory abnormalities, allergic reaction to food, and general circulatory disease) of the pre-COVID-19 period and 69 phenotypes (predominantly respiratory and circulatory symptoms) of the acute-COVID-19 period to be significantly enriched among PASC cases. Most of them were also observed enriched among PASC cases in the post-COVID19 period indicating that some of these phenotypes might have become longer-lasting or even chronic conditions. When incorporating these findings into PheRSs, we found that both the pre-COVID-19 PheRS and the acute-COVID-19 PheRS could predict PASC only with low accuracy among patients with a history of COVID-19, even when combined.

Possible explanations could be the random variation due to the small number of PASC cases, or differences due to different waves of coronavirus variants, the effect of vaccines, and changes in treatment and care of severe cases. Temporal trends in PASC diagnosis and management make this forward-looking prediction exercise much harder. We noted differences in the feature distributions between the training and testing sets, e.g., "nausea and vomiting" among the pre-COVID-19 features or "anxiety" among the acute-COVID-19 features, showed less pronounced differences between PASC cases and "No PASC" controls in the testing set (File S1J,K). However, both combined PheRSs could identify a quarter of patients with a history of COVID-19 in the testing cohort with a 3.5-fold increased risk of PASC (95% CI: 2.19, 5.55) compared to the bottom 50%. This observation highlighted the clinical utility of existing EHR data on pre-existing and acute COVID-19 symptoms for risk

stratification and the identification of a large group of vulnerable individuals who might benefit from stricter protective measures or earlier interventions.

A comparison of our findings with previous studies confirmed many pre-existing conditions that are predisposed to PASC. For example, in the pre-COVID-19 period PheWAS, we identified several respiratory symptoms that predisposed to PASC, including shortness of breath and other respiratory abnormalities. These findings are consistent with previous works [15,27,50]. The literature on IBS as a pre-disposing diagnosis for PASC seems sparse; however, there might be a connection between gut microbiota and the clinical course of COVID-19 [51] and mediation of risk factors effects for COVID-19 [52,53]. Similarly, little seems to be known of concussion as a pre-disposing diagnosis for PASC; yet, pre-existing cognitive risk factors such as mild traumatic brain injury were reported as enriched among cognitive PASC cases compared to non-cognitive PASC patients [54]. Future studies are needed to substantiate our findings and investigate how pre-disposing diagnoses relate to PASC. In addition to the results from the pre-COVID-19 period conditions, our findings from the acute-COVID-19 period also accord with previous studies. Among the 69 PASC-associated phenotypes, the majority were respiratory symptoms and in line with earlier reports (e.g., cough [55,56], dyspnea [57], respiratory insufficiency [58]). Additionally, the identified muscle-related symptoms, including myalgia, malaise, and fatigue, were supported by previous PASC studies [59,60]. Similar to a previous study, we found circulatory diseases to play an essential role as a predisposing factor for PASC [61]. While not all observed associations were previously reported, our sensitivity analyses indicated overall robustness across various settings [62,63].

An overlap between the enriched symptoms in the three periods implies the possibility of PASC being recurring symptoms of pre-existing conditions [17]. The difference in subsiding rate between cases and controls in some symptoms (e.g., respiratory symptoms) potentially indicates the development of chronic conditions [9,64].

There are several limitations to our analysis. First, we focused on predisposing diagnoses and performed matching, incl. on age, gender, and race/ethnicity, to adjust for potential confounding; however, these demographic characteristics were previously implicated as pre-disposing factors [65–67]. So, while matching and adjusting for these covariates might have effectively increased the power to identify pre-existing phenotypes that increase the risk for PASC, we disregarded these demographic factors as PASC predictors. Future studies are needed to evaluate the combined contributions of these variables in more comprehensive prediction models. Second, although a clinical diagnosis of PASC was used, many reported symptoms are non-specific to PASC, and defining PASC consistently across the time period of this study is nearly impossible [68]. The uncertainty around the definition of PASC is reflected in an initial lack of CDC-approved ICD10 codes. For example, the code "U09.9" ("Post COVID-19 condition, unspecified") was first introduced in October 2021, while it was recommended to also accompany this new code with existing codes for specific conditions and/or identified symptoms [69]. Before the approval of this code, the CDC encouraged providers to use an alternative but COVID-19-unrelated code, namely "B94.8" ("Sequelae of other specified infectious and parasitic diseases") [70]. The use of PSL diagnoses enabled us to detect PASC cases before any CDC recommendations were implemented. This covers the period of March 2020 to October 2021, a pre-vaccination period where PASC incidence was possibly higher. In addition, the various descriptions in the PSL diagnoses we used to define PASC cases (see Supplementary Table S1) reflect the developing language and awareness of PASC, e.g., "Post-COVID-19 syndrome", "COVID-19 long hauler" and "Multiple persistent symptoms after COVID-19". Furthermore, many of the PASC-related PSL diagnoses offered specific information about the underlying conditions and symptoms.

The performed post-COVID-19 PheWAS validated our definition of PASC in that it identified many of the established PASC symptoms. Yet, the awareness about PASC only recently increased and still might lead to an underdiagnosis of PASC [71,72]. For example, we only observed 2.7% PASC-diagnosed patients in our COVID-19 positive cohort, which

is far lower than PASC studies from the US, which estimated a prevalence between 19% and 35% [73]. As a result, our predictions of PASC might be overly conservative. The available diagnosis codes for PASC lacked specificity to stratify PASC cases into PASC subtypes reliably. Future studies that incorporate natural language processing of clinical notes and that have larger sample sizes will likely improve the identification of PASC cases and subtypes [74]. Third, the analysis was restricted to the patients with a history of COVID-19 who were also seen at MM during the pre-COVID-19 and post-COVID-19 periods; due to this selection bias, both cases and controls might be less healthy and older compared to randomly chosen individuals with a history of COVID-19 [75].

Moreover, it has been reported that around 15%–40% of the confirmed COVID-19 population were asymptomatic [76,77]. Using data from a health system caused our cohort to be enriched for symptomatic COVID-19 patients, while asymptomatic COVID-19 cases may be underrepresented. Such biases and omissions might limit the generalizability to the overall population. Although this study included a large size of COVID-19 patients, attention might be given to expanding and diversifying the collection and analysis of data.

Our study used a clinical definition of PASC. In addition to the commonly used ICD code U09.9 ("Post COVID-19 condition, unspecified") or B94.8 ("Sequelae of other specified infectious and parasitic diseases"), we applied the information from the EHR internal problem list database (PSL, Table S1) to categorize PASC patients, which enabled us to collect patients whose diagnosis were recorded even before official ICD-10 recommendations/codes became available. The post-COVID-19 period PheWAS validated our PASC definition in that we enriched diagnoses consistent with subtypes of PASC that were previously reported (e.g., shortness of breath, neurological disorders, malaise, fatigue, and dysphagia) [3,74,78]. Furthermore, given the benefit of rich retrospective EHR data, we could adjust for essential confounders in our models, including race, Elixhauser comorbidity score, vaccination status, etc., that might have affected PASC outcomes. We expect that our approach and the resulting prediction models will improve over time with increasing sample sizes and, by doing so, will likely facilitate earlier detection of PASC cases or improve risk stratification. Furthermore, a better characterization of PASC mechanisms might inform on distinct PASC forms that differ in their profiles of pre-existing conditions.

5. Conclusions

PASC represents a worldwide public health challenge affecting millions of people. While effective therapies for PASC are still in development [79–82], prediction and risk models can help to identify individuals at increased risk for PASC and its subcategories more reliably and potentially inform preventive or therapeutic efforts.

The present research aimed to identify PASC pre-disposing diagnoses from the pre- and acute-COVID-19 medical phenomes and to explore them as predictors for PASC. We identified known and potentially novel associations across various disease categories in both phenomes. These phenotypes, when aggregated into PheRSs, have predictive properties for PASC, especially when considered for risk stratification approaches. Future studies might consider applying more complex non-linear models such as machine learning to improve prediction models. The next opportunity will be to incorporate additional, more complex data such as laboratory measurements or medication data into such prediction models, as they have proven relevant for PASC but have yet to be fully investigated [2,83,84]. The presented PheRS framework can also be adapted to explore alternative outcomes such as survival and, by doing so, offer comprehensive insights into the long-term consequences of COVID-19.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12041328/s1, Figure S1. The proportion of clinically documented PASC within 3 months of testing positive and the number of total unmatched COVID-19-positive individuals by year quarter when they were tested positive/diagnosed for COVID-19 for the first time; Figure S2. Overview flowchart showing the sample filtering and analysis setup; Figure S3. Forest plots of the PreCOVID-19 Sensitivity analyses; Figure S4. Forest plots of the Acute

COVID-19 Sensitivity analyses; Figure S5. Comparison of PheCode prevalence during pre-COVID-19, acute/short-COVID-19, and post-COVID-19 periods in cases and controls; Table S1. PASC Problem list; Table S2. PASC symptom and concurrent symptom mapping; Table S3. Covariate summary and missingness in the unmatched and matched cohort; Table S4. Main and sensitivity PheWAS; Table S5. Concurrent diagnoses on day of the first PASC diagnosis; Table S6. Enrichment 29 known PASC symptoms among post-COVID-19 diagnoses in PASC cases compared to "No PASC" controls; Table S7. PheRS Evaluation in the testing data (COVID-19 positive in 2022); Table S8. Weights for combining PheRS1 and PheRS2 or PheRS1* and PheRS2*; File S1A. Post-COVID-19 (6 months) PheWAS; File S1B. Pre-COVID-19 PheWAS; File S1C. Acute-COVID-19 PheWAS; File S1D. Sensitivity Analysis "Severity" Pre-COVID-19; File S1E. Sensitivity Analysis "Gender" Pre-COVID-19; File S1F. Sensitivity Analysis "Year of infection" Pre-COVID-19; File S1G. Sensitivity Analysis "Severity" Acute-COVID-19; File S1H. Sensitivity Analysis "Gender" Acute-COVID-19; File S1I. Sensitivity Analysis "Year of Infection" Acute-COVID-19; File S1J. PheRS Weights.

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Data Availability Statement: Data cannot be shared publicly due to patient confidentiality. The data underlying the results presented in the study are available from the University of Michigan Precision Health Analytics Platform at https://precisionhealth.umich.edu/tools-resources/data-access-tools/ (last accessed: 3 February 2023) for researchers who meet the criteria for access to confidential data.

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Appendix A

Appendix A.1. Neighborhood Disadvantage Index (NDI)

The neighborhood disadvantage index (NDI) without the proportion of Black includes four census indicators (proportion of female-headed families with children, the proportion of households with public assistance income or food stamps; the proportion of families with income below the federal poverty level; the proportion of population age 16+ unemployed). We did not include measures of racial distribution within this index.

Appendix A.2. Pre- and Post-COVID-19 Years in EHR

For each individual, we defined pre-COVID-19 years in EHR as the time between the first recorded EHR entry and the first positive COVID-19 test or diagnosis and post-COVID-19 years in EHR as the time between the first positive COVID-19 test or diagnosis and the last recorded EHR entry.

Appendix A.3. Vaccination Status

We created a covariate to capture the vaccination status at the index date coded as "unvaccinated", "after 1. vaccination", "after full vaccination" and "after booster" using records of vaccinations for patients who received a vaccination at MM or who have a recorded vaccination record in the Michigan Care Improvement Registry (MCIR). Michigan's immunization providers are required to report COVID vaccination to MCIR within 24 h of administration, meaning the EHR vaccination record should be nearly complete. Among the matched case–control cohort, 11,925 individuals had at the date of their first positive test or COVID-19 diagnosis no documented vaccination and thus were considered unvaccinated. It is possible although unlikely that they may have been vaccinated elsewhere and these records were not available. A total of 7004 individuals had at least one documented dose of a COVID-19 vaccine. According to FDA's vaccination guideline [85], we categorized 6000 individuals as fully vaccinated in the primary series, meaning documentation of two doses of Moderna or Pfizer-BioNTech vaccine, or a single dose of Janssen vaccine at least 21 days before the corresponding test date [86–88]. A subset of 1646 of the fully vaccinated patients was further classified as being boosted, i.e., they received at least 1 additional vaccination at least 21 days after completing the primary series. The remaining 1004 vaccinated patients who did not complete the primary series were considered "partially vaccinated".

Appendix A.4. COVID-19 Severity

The covariate for COVID-19-related outcome severity was dichotomized as "severe", i.e., either hospitalization or intensive care unit (ICU) admission within one month after a positive SARS-CoV-2 RT-PCR test result or COVID-19 diagnosis, or death within two months after a positive RT-PCR test or COVID-19 diagnosis. Data on hospitalizations, ICU admissions, and death were obtained from Michigan Medicine's EHR databases as well as the Michigan Death Registry. The remaining individuals were considered "non-severe" COVID-19-related outcomes and included non-hospitalized, symptomatic, or asymptomatic COVID-19 cases.

Appendix A.5. Elixhauser comorbidity score

The Elixhauser comorbidity score developed by the Agency for Healthcare Research and Quality (AHRQ) was calculated to comprehensively characterize patients' pre-existing comorbidity conditions using ICD9 and ICD10 codes and the R package "comorbidity" [37,38].

Appendix A.6. Healthcare Worker (HCW) Status

Healthcare worker (HCW) status was defined based on documented participation in an HCW survey or a SARS-CoV-2 PCR test order for HCW.

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Impact of Age and Sex Interaction on Post-Acute Sequelae of COVID-19: An Italian Cohort Study on Adults and Children

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Abstract: Identifying factors predisposing individuals to post-acute sequelae of COVID-19 (PASC) would allow for the timely treatment of those vulnerable. Attention on the role of sex and age is growing, but published studies have shown mixed results. Our objective was to estimate the effect modification of age on sex as a risk factor for PASC. We analyzed data from two longitudinal prospective cohort studies on adult and pediatric subjects positive to SARS-CoV-2 infection that were enrolled between May 2021 and September 2022. Age classes (≤5, 6–11, 12–50, >50 years) were based on the potential role of sex hormones on inflammatory/immune and autoimmune processes. A total of 452 adults and 925 children were analyzed: 46% were female and 42% were adults. After a median follow-up of 7.8 months (IQR: 5.0 to 9.0), 62% of children and 85% of adults reported at least one symptom. Sex and age alone were not significantly associated to PASC, but their interaction was statistically significant (p-value = 0.024): the risk was higher for males aged 0–5 (females vs. males HR: 0.64, 95% CI: 0.45–0.91, $p = 0.012$) and for females aged 12–50 (HR: 1.39, 95% CI: 1.04–1.86, $p = 0.025$), especially those in the cardiovascular, neurological, gastrointestinal and sleep categories. Further research on PASC with regard to sex and age is warranted.

Keywords: post-acute sequelae; COVID-19; sex hormones; age

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1. Introduction

Two years into the COVID-19 pandemic, considerable advances have been made in the understanding of acute COVID-19, its management and treatment, and effective vaccines have been developed in a historically short timeframe [1]. However, as the health crisis becomes less threatening, the impact of the disease is far from over. In fact, increasing numbers of people report prolonged symptoms after recovery from COVID-19, a condition often called long COVID, post-COVID-19 condition, or post-acute sequelae of COVID-19, (PASC) among other names [2,3]. Prevalence rates of PASC in adults and children reported in reviews and meta-analyses vary greatly, ranging from zero up to 70%, depending on the study design and methodological quality [4–6], SARS-CoV-2 variants and vaccination status [7–9], the definition used and considered symptoms [10,11], and the follow-up duration [12,13]. To date, the mechanisms causing PASC are still poorly understood, and the treatments and outcomes are still unknown [14]. Comprehensive research is therefore urgently needed with regard to this condition, including the identification of factors that can predispose an individual to its development, or instead have a protective effect [15,16]. This knowledge will enable the prompt identification of vulnerable subjects who can be closely monitored and promptly provided with the necessary care and support and given

sufficient resources [17]. In this regard, growing attention is given to the potential role of sex and age as determinants of long COVID risk [18].

Although women exhibit a lower risk for severe acute infection and lower mortality rates than males, growing evidence suggests that they are at an increased risk of developing PASC compared to men [18–20]. A meta-analysis conducted by some of the authors of this paper [21], which included 20 studies and 13,340 adult patients (age range 40–70 years), found a statistically significant association of female sex with any symptoms (OR 1.52; 95% CI 1.27–1.82), with mental health symptoms (OR 1.67, 95% CI 1.21–2.29), and with fatigue (OR 1.54, 95% CI 1.32–1.79).

Age is also being considered as a determinant of long-term consequences of COVID-19. Concerning the adult population, some literature reviews have suggested that risk of developing long COVID increased with increasing age [2,22], but evidence on this point is contradictory. For instance, a large matched cohort study [23] including 486,149 adults with confirmed SARS-CoV-2 infection and 1,944,580 controls found that risk of reporting symptoms at $\geq$12 weeks after infection increased along a gradient of decreasing age. In the pediatric population, evidence seems to suggest that older age is associated with greater risk. In this regard, the retrospective cohort study by Kostev et al. [24], including 6568 children and adolescents, found that patients 13–17 years of age were more likely to be diagnosed with PASC compared with those aged $\leq$5 years (RR = 3.14). Similarly, in a prospective study by Osmanov et al. [25] on 518 patients $\leq$18 years old hospitalized with confirmed COVID-19, older age was associated with persistent symptoms: compared with children <2 years of age, those 6–11 years of age had an OR = 2.57 (95% CI 1.29–5.36) for persistent symptoms, and those 12–18 years of age had an OR = 2.52 (95% CI 1.34–5.01).

Although sex and age have been investigated as separate determinants of long COVID, it is important to evaluate whether interactions between these two demographic parameters may influence risk. To the best of our knowledge, no such analysis has yet been performed.

Therefore, the objective of our study was to explore whether PASC risk changes according to sex and age variations, in a population including adults and children, using standardized follow-up data collection protocols developed by the International Severe Acute Respiratory and Emerging Infection Consortium (ISARIC) Global Adult and Pediatric COVID-19 follow-up working groups [26]. In particular, we classified age groups in such a way as to verify the hypothesis of a sex hormone effect.

2. Materials and Methods

This study is reported based on the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) checklist for cohort studies [27].

2.1. Study Design, Population and Setting

This work analyzed data from two longitudinal prospective cohort studies on adult and pediatric subjects, conducted in the ISARIC framework, the methods of which were described in previous papers [28,29]. The analysis herein reported has the main objective of investigating whether an interaction between age and sex exists that modulates long COVID risk.

Only individuals with positive polymerase chain reaction (PCR) confirmed SARS-CoV-2 infection were included in this study. Adults were patients hospitalized for COVID-19 who had been discharged to home approximately 3 months before and who had provided their informed consent. Children were subjects under 18 years of age with symptom onset within approximately 1–3 months and the consent of their parent, caregiver or guardian to participate in the study. The adult and pediatric subjects were identified from electronic medical records and the Local Health Information System at the host institutions.

Given the lack of a clinical definition of PASC at the time the ISARIC study protocols were designed, in this study we considered any post-acute symptoms reported by the patient at the time of follow-up that were not explained by underlying conditions and were not present before the COVID-19 infection [26].

The studies were coordinated by the University Hospital of Parma, a 1044 bed facility with a catchment area of >400,000 inhabitants located in northern Italy, an area severely hit by the first wave of the COVID-19 epidemic [30]. Subjects were enrolled from 3 May 2021 to 26 September 2022 at the University Hospital of Parma (adults and children) and other pediatric centers across Italy (Naples, Palermo, Catanzaro, Bari, Milan, Ferrara, Rome, Piacenza, Genoa).

The studies were approved by the Area Vasta Emilia Nord (AVEN) Local Ethics Committee. The adult study took place on 13 April 2021 (protocol no. 196/2021/OSS/AOUPR), and the pediatric study took place on 30 November 2021 (protocol no. 952/2021/OSS/AOUPR). Participant/parental consent was sought during hospital discharge or by telephone interview during follow-up, and then confirmed at the first hospital accessed.

2.2. Data Collection

For the initial and follow-up assessments, we adopted the Tier 1 ISARIC Long-term Follow-up Study Case Report Form (CRF) for adults, and version 1.3 of the COVID-19 Pediatric Case Control Follow-up form for pediatric subjects, both developed by the ISARIC Global COVID-19 follow-up working group and independently translated into Italian.

The follow-up was planned to track PASC at 3-to-6-month intervals for up to 3 years.

Study data were collected and managed adopting REDCap electronic data capture tools (Vanderbilt University, Nashville, TN, USA) hosted at the University Hospital of Parma [31,32]. Italian translations of the abovementioned forms were used in the form of surveys which the enrolled subjects were asked to complete with the supervision of senior academic researchers and trained physicians.

The initial survey data collection was performed by a team of trained physicians or medical students by direct, face to face interview or by telephone interview. After the second interview, subjects were asked to complete the survey on their own through a web link sent by email. Participants unable to complete the survey online were assisted by telephone from the same trained medical students or physicians who had performed the first interview.

2.3. Outcome Measures

The main outcome measures were to describe the relationship and estimate the interaction between the two sexes (female vs. male) and four age classes ($\leq$5, 6–11, 12–50, >50 years) in determining PASC during the follow-up period. The two lower age classes were identified based on data by Osmanov et al. [25], which indicate a greater risk for older individuals compared to those 0–5 years. The third cut-off was selected following recent evidence suggesting that women under the age of 50 exhibit a greater prevalence of PASC [33]. Symptoms were categorized into ten manifestations: musculoskeletal, cardiovascular, respiratory, neurological or cognitive dysfunction, dermatological, gastrointestinal, sensory, sleep, fatigue, and poor appetite or weight loss (Table S1). Symptom categorization was based on previously published literature and on discussions within the ISARIC working group [15,25].

2.4. Statistical Analysis

Descriptive statistics used included mean and standard deviation (SD), and median and interquartile range (IQR) for continuous variable frequency (percentage) for categorical variables. Reporting rates of symptoms among age classes and between males and females were compared using the Pearson chi square or Fisher's exact tests. The non-parametric test by Cuzick et al. [34] was adopted to test for trends in reporting rates across age classes.

The outcomes were measured in terms of event occurrence, considering an event to be the first symptom recorded in the observation period of any subject, and the association in terms of hazard ratio. The cumulative symptom-free survival was estimated with the Kaplan–Meier method, considering the time from the first survey to the first symptom recorded; censoring was applied to the last available follow-up time point (last survey

compiled) in the absence of symptoms. A log-rank test was used to test for differences among age classes or between males and females. Hazard ratios were calculated using a Cox proportional hazards regression model, and proportionality was checked using Schoenfeld residuals. Median follow-up time was calculated by adopting the reverse Kaplan–Meier method [35]. Forest plots were used to summarize the hazard ratios of each post-COVID-19 manifestation on females compared to males stratified by age classes. We performed tests for the interaction between sex and age, adding the specific interaction term within the Cox models.

A subpopulation treatment effect pattern plot methodology (STEPP) [36] was used to explore and display the cumulative reporting rate of post-COVID symptoms in females compared to males and along the continuous scale of age by using overlapping subject subgroups.

To determine the robustness of our assessments, we decided to perform two post-hoc sensitivity analyses. Firstly, we reanalyzed data of subjects aged ≥18 year (adults hospitalized) and <18 (pediatric outpatients) separately. Secondly, we reanalyzed the data excluding the first survey on pediatric subjects, which was completed closer to symptom onset for children than for adults.

Due to the exploratory nature of subgroup analyses, we did not apply any adjustment for multiplicity. We included all participants for whom the variables of interest were available in the final analysis, without imputing missing data. The results are shown with 95% confidence intervals (95% CI), and statistical significance was considered for two-sided p values < 5%. All analyses were performed using STATA version 17.0 (StataCorp, College Station, TX, USA).

3. Results

A total of 2603 adults and 2545 children were eligible for the study; 1222 (47%) and 1826 (72%) could be contacted, and 518/1222 (42%) and 1018/1826 (56%) were enrolled. Overall, 452/518 (87%) adults and 925/1018 (91%) children completed at least the initial survey and were included in the analyses (Figure 1).

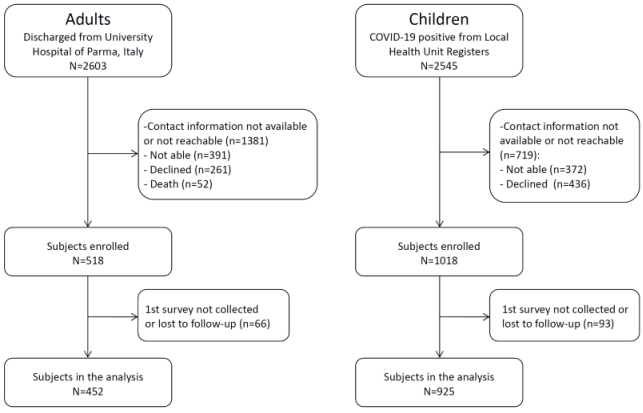

Figure 1. Flow-chart diagram of the cohorts.

The timings of the initial and the follow-up surveys are shown in Table S2; in adults the first survey was taken at a median of 3.3 months from hospital discharge, and children (or parents) were initially interviewed after a median of 2.4 months from the first COVID-19 symptom. The main demographics of the enrolled subjects are shown in Table 1: 46% were female, 43% were adults, and 48% were children; the median age in adults was 59 years (IQR, 50–68), while it was 8 years (IQR, 6–11) in children.

Table 1. Demographics.

	Adults (*n* = 452)	Children (*n* = 925)	All (*n* = 1377)
Sex, *n* (%)			
Male	257 (57)	482 (52)	739 (54)
Female	195 (43)	443 (48)	638 (46)
Age, years			
mean (SD)	58.4 (13.7)	8.0 (4.2)	24.6 (25.2)
median (IQR)	59 (50–68)	8 (6–11)	11 (7–50)
min–max	19–86	0–17	0–86
Age classes, years			
0–5	-	230 (25)	230 (17)
6–11	-	531 (57)	531 (39)
12–17	-	164 (18)	164 (12)
18–50	115 (25)	-	115 (8)
51–64	172 (38)	-	172 (13)
65+	165 (37)	-	165 (12)

Abbreviations: SD, standard deviation; IQR, interquartile range.

During the observation period, 62% (575/925) of children and 85% (383/452) of adults reported at least one symptom (Fisher's exact test $p < 0.001$). During the first survey, 263/925 (28%) children and 319/452 (71%) adults reported more than one symptom. Furthermore, in the second survey, 34% (157/458) of children and 64% (226/351) of adults reported persistent symptoms.

The categorized reported symptoms are summarized in Table S3 by sex and age group. The most common symptoms reported in children were respiratory (36%), neurological (27%), fatigue (20%), and gastrointestinal (19%); in adults, neurological (66%), fatigue (64%), musculoskeletal pain (63%) and respiratory (57%) symptoms were the most common.

Another variable collected in the survey was anti-COVID-19 vaccination status. This data is shown in Table S4: 360 (80%) of the hospitalized adults and 198 (21%) of the pediatric outpatients were vaccinated with at least one dose.

Overall, the subjects were followed for a median of 7.8 months (IQR: 5.0 to 9.0). Time to first reported post COVID-19 symptom analysis showed that females (any age) did not have a significantly higher risk than males (Log-rank $p = 0.289$, Figure S1). Similarly, no difference among age classes was observed (Log-rank $p = 0.273$, Figure S2).

The subgroup analysis, which was conducted to investigate the interaction between sex and age, showed significantly different risk estimates in females vs. males by age class (p-value for sex × age interaction = 0.024). Specifically, the difference was statistically significant in the age class of 0–5 years of age, where the risk was higher for males (HR: 0.64, 95% CI: 0.45–0.91, $p = 0.012$, Figure 2A) and in subjects 12–50 years of age, while the risk was higher for females (HR: 1.39, 95% CI: 1.04–1.86, $p = 0.025$, Figure 2C); no difference was observed in subjects aged 6–11 year (HR: 1.09, 95% CI: 0.88–1.35, $p = 0.436$, Figure 2B) and in subjects >50 years of age (HR: 1.19, 95% CI: 0.94–1.50, $p = 0.155$, Figure 2D).

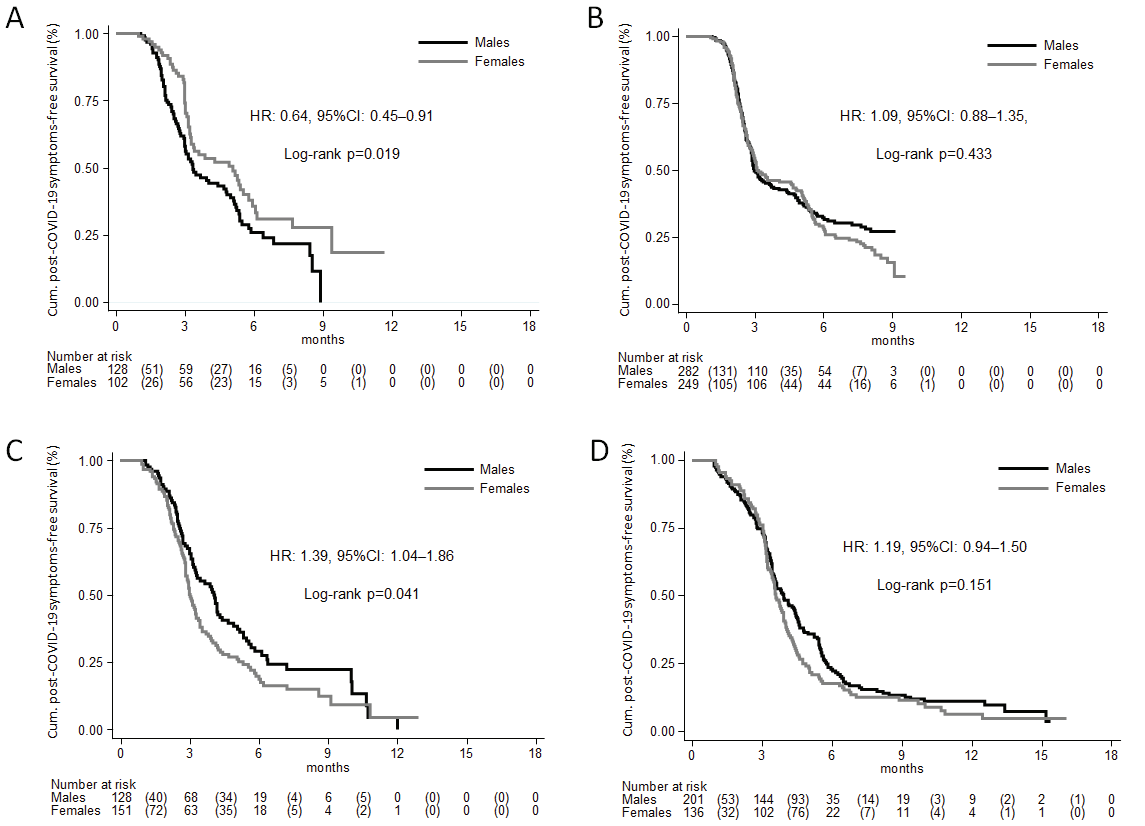

Figure 2. Kaplan-Meier estimates of cumulative reported frequency of post-COVID-19 symptoms, by sex, (**A**) in subjects 0–5 years of age, (**B**) 6–11 years of age, (**C**) 12–50 years of age, and (**D**) >50 years of age. The numbers in parentheses represent the number of symptoms that were reported within each time interval, by sex. The estimate of the hazard ratio (HR) was based on a Cox proportional hazards regression model, adjusted for age.

The forest plot in Figure 3 graphically summarizes the hazard ratios of females compared to males in each considered age class for each symptom category and overall for "Any Symptom".

The interaction between sex and age was statistically significant overall for Any Symptom ($p = 0.024$), and in particular for respiratory ($p = 0.010$), dermatological ($p = 0.008$), and gastrointestinal ($p = 0.030$) symptoms, sleep problems ($p = 0.006$), and the loss of appetite or weight loss ($p = 0.040$). Similarly, the analysis of hazard ratios for each symptom category and age range (Figure 3) suggested that the risk was higher for males in the 0–5 year age class for almost all categories, but this was not statistically significant; and for females in the 12–50 year class for almost all categories, and the association was statistically significant for cardiovascular, neurological, and gastrointestinal symptoms, and sleep. Lower but statistically significant HRs were also found for women >50 years of age in all categories except for musculoskeletal, sensory, and fatigue.

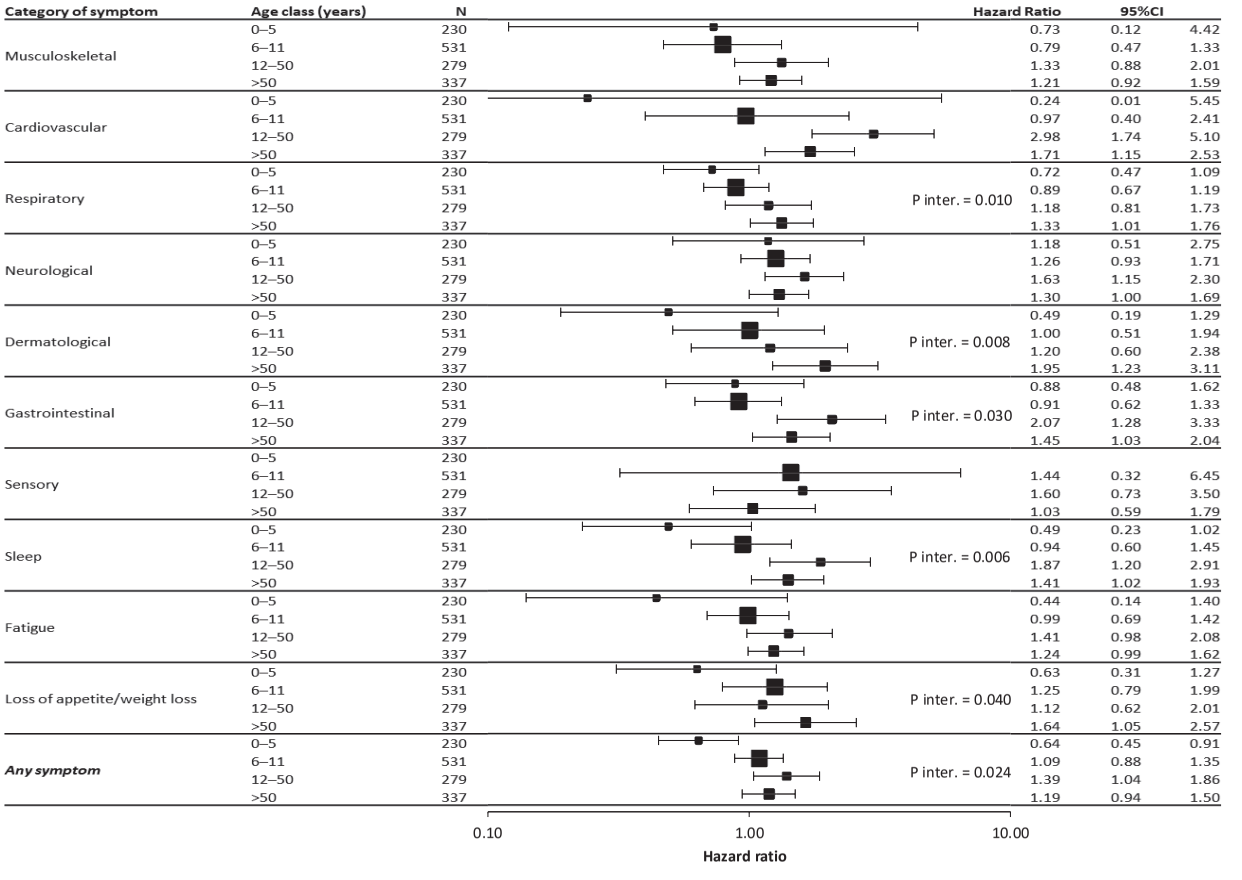

Figure 3. Forest plot of the risk estimate (hazard ratio, HR) of post-COVID-19 symptoms for females vs. males in each symptom category, stratified by age group. HRs are adjusted for age. Abbreviations: *p*-inter., *p*-value for the sex × age interaction term in the Cox model. Notes: HR for sensory symptoms in the 0–5 year group was not estimable because of a lack of events; data are from all of the surveys collected.

Finally, adopting the STEPP graphical technique (Figure 4), differences between the two sexes by age class appear to confirm our findings: the 3-months cumulative incidence rate of any post-COVID-19 symptoms is higher for females vs. men in the of 12–50 years of age range, while it seems lower below 6/7 years of age (interaction *p*-value based on cumulative incidence estimates = 0.012).

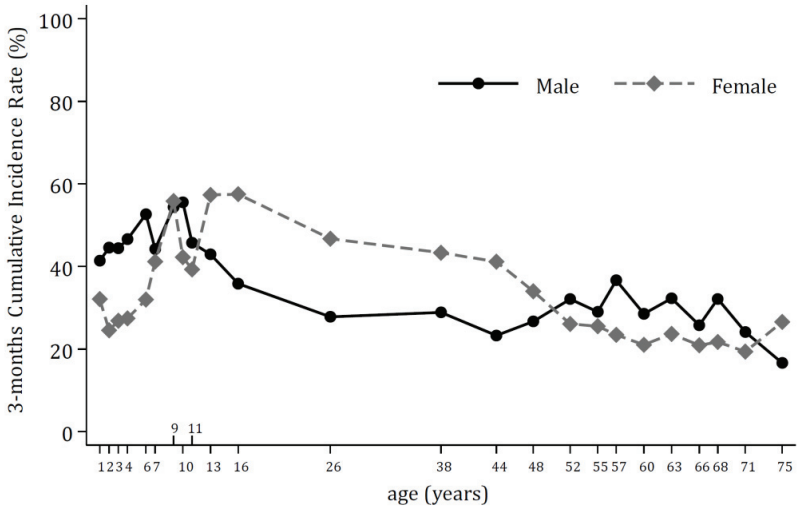

Figure 4. Subpopulation Treatment Effect Pattern Plot (STEPP) of the 3-month cumulative incidence rate of the reported symptoms in males (solid black line) and females (dotted grey line). The plot was drawn adopting the sliding window pattern, including $n = 100$ subjects in each subpopulation and $n = 75$ subjects in common among consecutive subpopulations, implementing 2500 permutations of the covariate age.

Sensitivity Analyses

The analysis considering hospitalized adults and pediatric outpatients separately (Figures S3–S5) confirmed the higher risk estimates for females in the 12–17 and 18–50 age ranges as compared to males and to other age classes. With regard to the further sensitivity analysis, it was carried out by excluding the data obtained from the first survey in pediatric patients (Figures S6 and S7), and despite the expected wider confidence intervals, the direction of the estimates in the age groups considered was almost superimposable.

4. Discussion

This paper reported the analysis of two follow-up cohorts of 452 adults and 925 children with a laboratory confirmed diagnosis of SARS-CoV-2 infection. This work was conducted in the framework of an international initiative led by ISARIC, and used the ISARIC Global COVID-19 follow-up protocol for adults and children. Numerous publications have been produced [6,18] mainly aimed at determining the frequencies of the reporting of PASC and characterizing the factors associated with their occurrence. Some of these studies have investigated the influence of sex or age separately, and we only know of one study [37] which described a higher prevalence of PASC in women <50 years of age among adult patients discharged from the hospital. Therefore, to better understand the factors influencing the magnitude or direction of the relationship between virus exposure and the onset of PASC, our study aimed to examine two potential moderators, sex and age, identified on the basis of previous research which, however, highlighted conflicting results [6,18].

To the best of our knowledge, this is the first study focused on assessing how the risk of PASC is modulated by the interaction between sex and age in children and adults.

While our overall analysis did not detect any effect considering these two factors separately, the analysis of the interaction suggests that women aged between 12 and 50 years of age exhibited a 40% higher risk than men for PASC; in particular, the risk was three times higher for cardiovascular symptoms, and twice as high for gastrointestinal and sleep problems. Sex differences in PASC have been attributed to biological (i.e., hormones and immune responses), and sociocultural (i.e., sanitary-related behaviors, psychological stress, and inactivity) aspects [18,38]. In particular, the higher prevalence of PASC in women between 12–17 and 18–50 years is an important and supporting clue of the role of sex hormones, also considering that the mean age of natural menopause is 51 years [33]. This hypothesis is plausible; however, considering that hormone levels vary considerably within such a wide age range, it does require further investigation, which was not feasible in our study due to the lack of adequate sample size in this age range.

Another finding emerging from our analysis is that among pediatric subjects, the risk for PASC appeared to be higher for males compared to females in the 0–5-year age range, although no significant associations were found in the analysis of individual symptom categories, which was also due to the high variability of risk estimates. We could not find studies exploring these aspects in the literature, and it is difficult to give a plausible explanation for this finding. It should also be taken into account that for children in this age class, responses were provided by parents, therefore the results should be interpreted with caution.

This study has some strengths, which include the use of standardized ISARIC Long-term Follow-up Study CRFs, the enrolment of both adults and children combined in the analysis of data, and a relatively large sample size of people attending multiple clinical centers.

Some limitations of the study must also be considered. Firstly, we did not include a control arm, and prevalence data may have been overestimated, as shown in the meta-analysis by Behnood et al. [6]; similarly, the combined effect between sex and age observed in this work may be due to factors other than COVID-19. Secondly, as our results were obtained with subgroup analyses without adjustments for multiple comparisons, which exhibit known limitations [39], they should be considered with caution and verified in ad hoc studies. Thirdly, this study is based on self-reported symptoms, and data may be biased due to psychological and sociocultural factors. Fourthly, the risk of potential bias should be pointed out. Selection bias may be present: for instance, individuals with symptoms may be more likely to respond to the survey. Notably, among potentially eligible subjects, only 42% of adults and 56% of children participated. The remaining individuals could not be contacted, did not give their informed consent, or were judged to be unable to take part in the survey. Furthermore, the proportion of responders decreased in subsequent follow-up surveys, which, however, was expected due to the voluntary nature of survey completion and web-based self-administration [40]. Again, it is possible that dropouts may be more frequent among individuals without important symptoms, or in those whose symptoms improved.

5. Conclusions

This study adds to the evidence on the importance of sex as a risk factor for PASC, but only in specific age ranges. In particular, the elevated risk found in women 12–50 years old emphasizes the need to further investigate the role of sex hormones on inflammatory/immune and autoimmune processes in greater depth. Future research on PASC should be considered from the perspective of sex and age. Taking these differences into account in the diagnosis, the prevention and treatment of COVID are critical steps towards precision medicine.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12082924/s1: Table S1. Categorization of post-COVID-19 symptoms; Table S2. Number of subjects interviewed and the timing of the interviews in adults and children; Table S3. Frequency of post-COVID-19 symptoms in categories, by sex and age classes;

Table S4: Vaccination status in adults and children analysed, at the time of the last survey; Figure S1: Kaplan-Meier estimates of cumulative reported frequency of post-COVID-19 symptoms in the whole cohort, by sex. Figure S2: Kaplan-Meier estimates of cumulative reported frequency of post-COVID-19 symptoms in the whole cohort, by age class; Figure S3: Kaplan-Meier estimates of cumulative reported frequency of post-COVID-19 symptoms, by sex; Figure S4: Forest plot of the risk estimate (hazard ratio, HR) of post-COVID-19 symptoms for females vs. males in each symptom category, stratified by age group, in pediatric outpatients.; Figure S5: Forest plot of the risk estimate (hazard ratio, HR) of post-COVID-19 symptoms for females vs. males in each symptom category, stratified by age group, in hospitalized adults; Figure S6: Forest plot of the risk estimate (hazard ratio, HR) of post-COVID-19 symptoms for females vs. males in each symptom category, stratified by age group, excluding data from the 1st survey of pediatric outpatients; Figure S7: Forest plot of the risk estimate (hazard ratio, HR) of post-COVID-19 symptoms for females vs. males in each symptom category, stratified by age group, in pediatric outpatients, excluding data from the 1st survey.

Author Contributions: Conceptualization and methodology, M.P., C.C. and S.E.; software, M.P.; validation, M.P.; formal analysis, M.P.; investigation, M.P. and C.C.; data acquisition, M.P., L.P., M.D., G.A., V.F. and University Hospital of Parma LONG-COVID Research Team; data curation, M.P. and C.M.P.; writing—original draft preparation, M.P.; writing—review and editing, M.P., C.C. and S.E.; supervision, C.C. and S.E.; project administration, M.P. and C.C. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Area Vasta Emilia Nord (AVEN) Ethics Committee (adult protocol code: 196/2021/OSS/AOUPR, approved on 13 April 2021; pediatric protocol code: 952/2021/OSS/AOUPR, approved on 30 November 2021).

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

Data Availability Statement: Data supporting the findings of this study are available from the corresponding author, M.P., upon reasonable request.

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

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Humoral and Cellular Response and Associated Variables Nine Months following BNT162b2 Vaccination in Healthcare Workers

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Abstract: In this study, we aimed to illustrate the trajectory of humoral and cellular immunity nine months after primary vaccination with the BNT162b2 mRNA vaccine among 189 healthcare workers (HCWs). Additionally, we endeavored to identify correlations between immunity parameters and a number of common variables and comorbidities. A total of 189 healthcare workers (HCWs), vaccinated against COVID-19, were finally included in the study. All of the subjects had received two doses of the BNT162b2 vaccine; had undergone antibody tests one, four and nine months post-vaccination; and had completed a medical questionnaire. Further samples taken at nine months were tested for cellular immunity. No participants had evidence of COVID-19 infection pre- or post-vaccination. An anti-S1 receptor binding domain (RBD) antibody assay was used to assess humoral response, and cellular immunity was estimated with an INF-γ release assay (IGRA). Statistical analysis was performed using STATA. We report a statistically significant antibody drop over time. Being above the age of 40 or a smoker reduces the rise of antibodies by 37% and 28%, respectively. More than half of the participants did not demonstrate T-cell activation at nine months. Female gender and antibody levels at four months predispose detection of cellular immunity at nine months post-immunization. This study furthers the qualitative, quantitative, and temporal understanding of the immune response to the BNT162b2 mRNA vaccine and the effect of correlated factors.

Keywords: antibodies; cellular immunity; COVID-19; humoral immunity; INF-γ release assay; mRNA vaccine

1. Introduction

The necessity to contain the COVID-19 pandemic impelled the emergency authorization of novel mRNA vaccines. The BNT162b2 mRNA vaccine has been administered to billions of people worldwide with a two-dose schedule proven to be 95% effective for preventing severe COVID-19 disease caused by wild-type virus and several mutations [1–5]. BNT162b2 has demonstrated a high efficacy rate even against variants of concern and has an acceptable safety profile [6]. Nevertheless, the decline of antibody levels post vaccination along with the increasing numbers of breakthrough infections among vaccinated individuals [7–9] has created uncertainty about the durability of protective immunity and has necessitated serial booster doses for the adult population.

The rise of specific antibodies against SARS-CoV-2 after natural infection or vaccination has been widely examined [10–14]. Evidence is scarce regarding the question as to whether these antibodies directly correlate with protection or constitute at least one of the protective immune mechanisms [15]. A large UK study (the SIREN study) has suggested that natural infection and induction of antibody response provides robust protection against asymptomatic and symptomatic reinfection [10]. Similarly, studies have demonstrated that available vaccines are able to elicit a significant humoral response in vaccinees with a peak antibody level measured one month after immunization [11,16–18]. Previous natural COVID-19 infection is associated with higher levels of humoral response in BNT162b2 mRNA vaccinated individuals, enabling hybrid immunity to promise long-term protection [19,20].

However, the rise of antibody titers per se is not necessarily associated with protection and the level above which we consider the antibodies to be protective is yet to be validated [21–24]. Conversely, the observation that antibody titers wane over time [21,25–33] has raised concerns regarding the level of residual protection and shifted the focus of scientific inquiry to other correlates of immunity to more accurately assess protection.

Vaccines are able to confer immunity by targeting not only the humoral but also the cellular branch of the immune system [34,35]. There is mounting evidence that T-cell response is elicited both in naturally infected patients and vaccinated individuals and can provide long-term protection [36–49]. Nevertheless, the trajectory of long-term antigen-specific T-cell response following mRNA vaccination remains incompletely investigated. Cellular assays are expensive and time-consuming and require experienced lab personnel to execute. Other methods that indirectly assess cellular response, such as interferon gamma release assays (IGRA), are emerging in the literature as both sensitive and accurate in assessing T-cell antigen-specific responses in cohorts of SARS-CoV-2 convalescent and vaccinated populations [50–54].

The most important risk factors for serious disease from SARS-CoV-2 are old age and the presence of comorbidities [27,29,30,55,56]. Male gender, smoking, and obesity are also well-established factors for worse outcomes [57–59]. According to the literature, the efficacy of the BNT162b2 vaccine against SARS-CoV-2 could be correlated with the above characteristics with a difference in elicited humoral responses [60–64].

This study aims to elucidate aspects of the humoral and cellular response to vaccination with the BNT162b2 vaccine, and to assess the magnitude and the longitude of antibody titers measured up to nine months post-vaccination.

2. Materials and Methods

2.1. Population and Study Design

This is a single-centered, prospective, longitudinal study conducted at 251 Air Force General Hospital, in Athens. Seven hundred and twenty-nine (729) healthcare workers (HCW) were vaccinated with two doses of the BNT162b2 mRNA vaccine 21 days apart during the period of 4 January 2021–19 February 2021, when a mass vaccination campaign was initiated in-house for hospital staff. The workers all had a measurement of total antibody titers one month after receiving the second dose as per a hospital offer to check

for immune response. Out of 729 individuals eligible for inclusion, 350 were selected after randomization to form our initial sample population. (Figure 1).

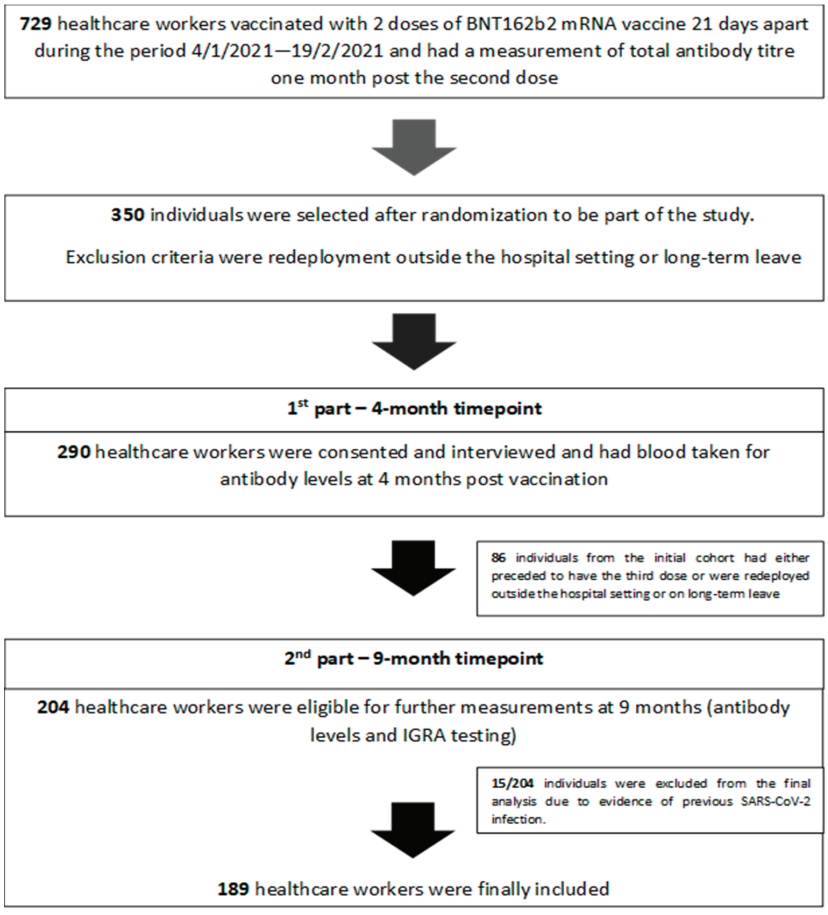

Figure 1. Study design.

2.2. Data Extraction

The sample population was contacted in person or by telephone by study investigators and written informed consent was obtained before enrollment. Out of 350 individuals randomly selected, 290 were consented and interviewed. Data were collected using questionnaires to investigate demographic features (age and gender), anthropometric data (weight and height), smoking habits, and past medical history/regular medications. Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters (kg/m^2). Morbidity recorded was classified into further subgroups including diabetes mellitus, lung disease (asthma, chronic obstructive pulmonary disease, pulmonary fibrosis), chronic renal failure, heart disease (coronary heart disease, myocardiopathy, heart failure), hypertension, dyslipidemia, immunosuppression (cancer or immunosuppressive treatment), autoimmunity.

2.3. Blood Collection Four and Nine Months Post Full Vaccination

Blood collection was scheduled four months after the second inoculation with a maximum delay of 10 days. A total of 290 blood samples were sent to the biochemistry lab

for centrifuge and quantification of post-vaccination antibody levels. Samples collected were kept in the refrigerator (between +2 °C and +8 °C) and were analyzed within seven days. At the nine-month timepoint, estimation of long-term antibody levels in addition to T-cell activation profile was attempted. Of 290, 204 individuals were eligible for the second phase of the study (Figure 1), and 204 paired blood samples were analyzed. In addition to biochemistry samples sent for antibody levels, another 5 mL of whole blood from each participant (collected in five lithium heparin tubes (1 mL each)) were sent to the lab for IGRA testing. Whole blood was harvested after 16–24 h of stimulation at 37 °C and then assessed for IFN-γ.

2.4. Laboratory Methods

2.4.1. Anti-SARS-CoV-2 Antibodies

Antibody levels were measured in serum samples using the ADVIA Centaur® SARS-CoV-2 IgG (sCOVG) (Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) assay, a quantitative chemiluminescence immunoassay that uses the receptor binding domain (RBD) of the spike protein 1 as capture antigen. All samples were processed according to the manufacturer's instructions using an automated platform (ADVIA Centaur® XP systems, Siemens (Siemens Healthineers, Erlangen, Germany)) and yielded results with 96.41% sensitivity and 99.9% specificity. A result of reactive or nonreactive was determined according to the index value established with the calibrators. A cut-off level of 1 U/mL determined a positive result.

2.4.2. IGRA

T-cell activation was evaluated using a COVI-FERON kit (SD Biosensor, Inc., Cheongju-si, Republic of Korea), an IGRA approved for use in in vitro diagnosis (IVD). The assay consists of five antigen tubes aiming to stimulate T-lymphocytes involved in cell-mediated immunity in heparinized whole blood. Nil tube estimates the background IFN-γ level of the sample. Original spike protein (OSP) antigen tube assesses the IFN-γ responses to SARS-CoV-2 spike protein (SP) antigen derived from the wild-type virus (Wuhan) and 20I/501Y.V1 (UK) variant. Variant spike protein (VSP) antigen tube assesses the IFN-γ responses to SARS-CoV-2 SP antigen derived from the 20H/501.V2 (South Africa) and 20I/501Y.V3 (Brazil) variants. A mitogen tube is used as positive control. NP Antigen tube is used to speculate IFN-γ responses to SARS-CoV-2 nucleocapsid protein (NP) antigen indicative of previous natural COVID-19 infection. Plasma from the stimulated samples was used for detection of INF-γ production using an enzyme-Linked immunosorbent assay (ELISA)-based platform. Specimens were processed as per the manufacturer's advice. Quantitative results (INF-γ concentration in IU/mL) were recorded and further analyzed. An elevated response was defined as a value greater than at least 0.3 IU/mL, implying detectable cellular immunity with 97% sensitivity and 94.2% specificity. Finally, results were appropriately modified to represent the index and >1 was considered positive.

2.5. Ongoing Disease Surveillance

A significant benefit of all the study subjects being members of the hospital staff was that it enabled in-house surveillance for disease incidence throughout the study via several modalities: biweekly nasopharyngeal testing in high exposure placements, prompt reporting of clinical signs and symptoms and immediate testing, and close tracking and tracing of index cases and high-risk exposures. As a result, before the final analysis, 15 participants were further excluded due to evidence of COVID-19 infection (Figure 1). This was evidenced either by positive PCR or by a positive result for previous natural infection in IGRA testing (NP index). A total of 189 samples remained eligible for analysis of humoral and cellular response in COVID-19 naïve individuals. At the time of the study, the delta variant had emerged and was responsible for the main burden of infections.

2.6. Statistical Analysis

Participants were divided into two age groups (≤40 and >40 years old), and three BMI subgroups (BMI: <25 kg/m^2 (Underweight/Normal), BMI: 25–29.9 kg/m^2 (Overweight), BMI: ≥30 kg/m^2 (Obese/Extremely Obese)). They were further classified according to their status of original SP and variant SP cellular immunity (no/yes). For descriptive analysis, continuous variables are presented as median with interquartile range (IQR) and categorical ones as absolute and relative frequencies. The antibody titers from the first, fourth, and ninth month are also presented as means with standard deviation, and the difference between the three measurements is accessed through a repeated-measures ANOVA test. Differences between the groups were compared with *t*-test or Mann–Whitney test for the continuous data and the chi-squared or Fisher exact test for the categorical variables, while the Shapiro–Wilk test was used to access the normality assumption of the data.

Mixed linear regression models were used to identify baseline characteristics associated with antibody titers. The natural logarithm (ln) of the antibody titers was used as the independent variable, and four multivariate models are presented for added robustness. Model 1 refers to the repeated antibody titer measurements for months 1 to 4, model 2 refers to months 4 to 9, model 3 refers to months 1 to 9, and model 4 also to months 1 to 9 including an interaction term of the variables with a significant univariate association (p-value < 0.05), that is, the age group and smoking status.

Logistic regression analysis was performed to detect factors that might be associated with original SP and variant SP cellular immunity. Antibody titers were evaluated both in their original and ln-transformed scale. Two multivariate models are presented, the first includes the antibody titers in their original scale, while the second represents their ln-transformed scale.

Statistical analysis was performed with STATA and a two-tailed p-value < 0.05 was considered significant.

3. Results

One hundred eighty-nine (189) HCWs were ultimately included in this analysis. The descriptive characteristics of the study group are summarized in Table 1. All participants were of Caucasian ethnicity, their median age was 43 years old (range: 36–50) and 48.7% (n = 92) of them were male. Additionally, 37.6% (n = 71) were younger than 40 years old, 39.2% (n = 74) and 14.8% (n = 28) were overweight and obese respectively, and 31.8% (n = 60) were current smokers. Moreover, 7.4% (n = 14) reported being hypertensive on medication, 5.8% (n = 11) reported having dyslipidemia, 9% (n = 17) endorsed autoimmunity (7 of them referred Hashimoto), and 2.1% (n = 4) claimed to have some degree of immunosuppression.

Mean antibody levels at one, four, and nine months after the second dose of BNT162b2mRNA were 153.49 U/mL, 32.38 U/mL, and 19.65 U/mL respectively. All participants had detectable antibodies (>1 U/mL) one and four months after their second dose but 7/204 (3.4%) dropped their antibody levels to less than 1 U/mL at nine months.

A 78.9% decline in median antibody levels was calculated between the first and fourth month, and a 39.31% decline between the fourth and ninth month, revealing a continued reduction, albeit at a slower pace. ANOVA test for repeated measurements was used and corroborated a significant time effect for the mean antibody level kinetics (p < 0.001). A post hoc pairwise comparison using the Bonferroni correction revealed a statistically significant drop in antibody levels between one and four months (p < 0.001), but the drop was not statistically significant between the timepoints of four and nine months (p = 0.458).

To determine whether vaccine-induced antibody responses depended on sex, age, BMI, or specific comorbidities, we investigated the induction in antibodies one, four, and nine months after the second vaccine dose concerning these variables (Table 2). We sought to examine which characteristics might be significantly associated with antibodies at their peak detection concentration one month after the second dose. Younger participants (<40 years old) had significantly higher antibody levels at one month (mean 172 (106–210),

$p = 0.003$), at four months (mean 32 (17–52), $p < 0.001$), and at nine months after a second dose (mean 5.66 (3.45–8.65), $p < 0.001$) than older participants. Moreover, the concentration of antibodies was increased in the non-smoker group at the first month (mean 159 (100–195), $p = 0.009$), at four months (mean 24 (13–49), $p < 0.001$), and at nine months after the second dose (mean 4.78 (2.84–8.22), $p < 0.001$) compared with smokers (Table 2). COVI-FERON ELISA assay demonstrated that, nine months after the second dose, the IFN-γ concentration against the original SARS-CoV-2 spike protein (OSP) was positive in 43.9% ($n = 83$) and the IFN-γ concentration against the variant SARS-CoV-2 spike protein (VSP) was positive in 27% ($n = 51$) (Table 1).

3.1. Mixed Linear Regression Analysis

To investigate the possible correlation of vaccine-induced antibody responses with age, sex, BMI, smoking habits, or specific comorbidities, we conducted univariate and multivariate mixed linear model analyses. Older age (>40) and smoking habit were steadily associated with lower antibody levels in measurements at all three of the time spans (one to four months ($p = 0.001$, $p = 0.03$), four to nine months ($p < 0.002$, $p = 0.007$), and one to nine months ($p = 0.002$, $p = 0.029$)) and these correlations were statistically significant (Figure 2, Table 3, models 1–3). BMI, sex, hypertension, dyslipidemia, immunosuppression, and autoimmune disease were not significantly associated to antibody levels in any case.

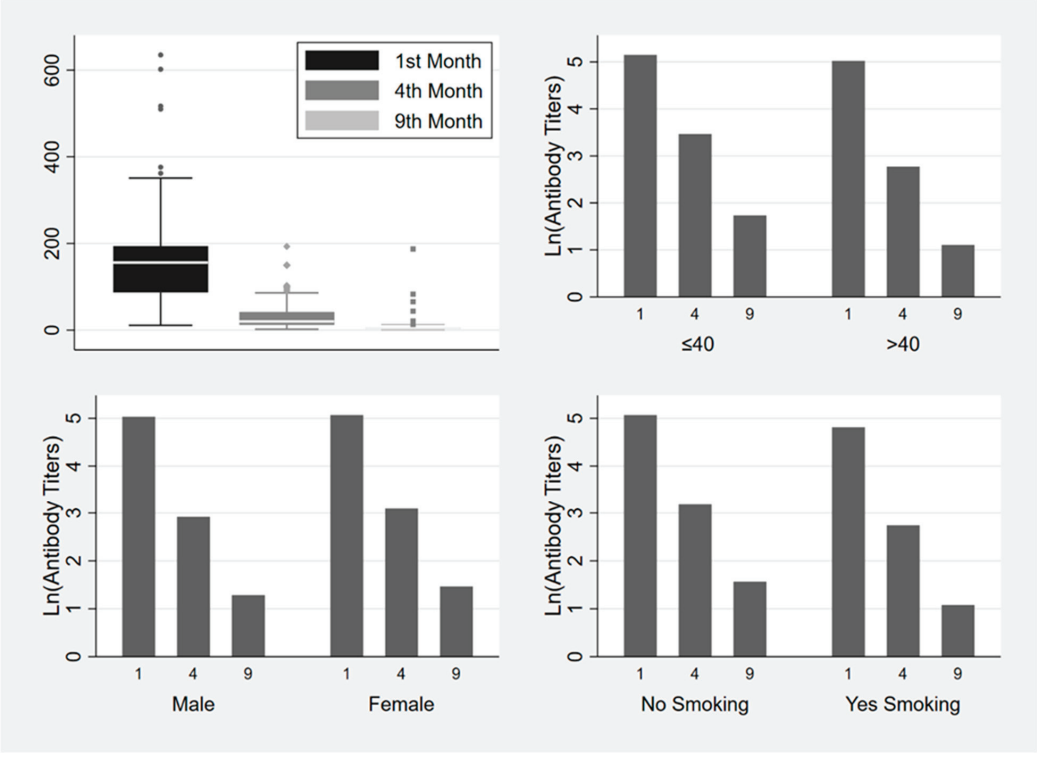

Figure 2. Box plot and bar plots (median values) of ln (antibody titers) variance one, four and nine months after the second dose of BNT162b2 vaccine and risk factors (age, gender, smoking status).

Table 1. Baseline demographics, antibody measurements and cellular immunity.

			Cellular Immunity Original Spike Protein			Cellular Immunity Variant Spike Protein		
	Initial Sample	Final Sample	Inactive	Active		Inactive	Active	
Characteristic	*n* = 290	*n* = 189	*n* = 106	*n* = 83	*p*-Value	*n* = 138	*n* = 51	*p*-Value
Age—median (IQR)	44.00 (36.00–51.00)	43.00 (36.00–50.00)	44.00 (36.00–51.00)	43.00 (35.00–50.00)	0.436	44.00 (36.00–50.00)	41.00 (35.00–51.00)	0.508
Age Category-no. (%)								
≤40	102 (35.17)	71 (37.57)	36 (33.96)	35 (42.17)	0.248	48 (34.78)	23 (45.10)	0.194
>40	188 (64.83)	118 (62.43)	70 (66.04)	48 (57.83)		90 (65.22)	28 (54.90)	
Gender								
Male	145 (50.00)	92 (48.68)	45 (42.45)	47 (56.63)	0.053	65 (47.10)	27 (52.94)	0.476
Female	145 (50.00)	97 (51.32)	61 (57.55)	36 (43.37)		73 (52.90)	24 (47.06)	
BMI-median (IQR)	25.35 (22.50–28.65)	25.35 (22.50–28.40)	25.12 (22.46–28.37)	25.86 (22.84–28.69)	0.492	25.20 (22.46–28.40)	25.88 (23.51–28.65)	0.762
BMI Category—no. (%)								
Underweight/Normal	134 (46.21)	87 (46.03)	52 (49.06)	35 (42.17)	0.599	66 (47.83)	21 (41.18)	0.713
Overweight	104 (35.86)	74 (39.15)	40 (37.74)	34 (40.96)		52 (37.68)	22 (43.14)	
Obese/Extreme Obese	52 (17.93)	28 (14.81)	14 (13.21)	14 (16.87)		20 (14.49)	8 (15.69)	
Smoking—no. (%)								
No	195 (67.24)	129 (68.25)	68 (64.15)	61 (73.49)	0.171	92 (66.67)	37 (72.55)	0.441
Yes	95 (32.76)	60 (31.75)	38 (35.85)	22 (26.51)		46 (33.33)	14 (27.45)	
Hypertension—no. (%)								
No	268 (92.41)	175 (92.59)	99 (93.40)	76 (91.57)	0.634	130 (94.20)	45 (88.24)	0.164
Yes	22 (7.59)	14 (7.41)	7 (6.60)	7 (8.43)		8 (5.80)	6 (11.76)	
Immunosuppression—no. (%)								
No	282 (97.24)	185 (97.88)	103 (97.17)	82 (98.80)	0.441	135 (97.83)	50 (98.04)	0.928
Yes	8 (2.76)	4 (2.12)	3 (2.83)	1 (1.20)		3 (2.17)	1 (1.96)	
Dyslipidemia—no. (%)								
No	265 (91.38)	178 (94.18)	100 (94.34)	78 (93.98)	0.916	130 (94.20)	48 (94.12)	0.982
Yes	25 (8.62)	11 (5.82)	6 (5.66)	5 (6.02)		8 (5.80)	3 (5.88)	
Autoimmune—no. (%)								
No	262 (90.34)	172 (91.01)	96 (90.57)	76 (91.57)	0.811	124 (89.86)	48 (94.12)	0.363
Yes	28 (9.66)	17 (8.99)	10 (9.43)	7 (8.43)		14 (10.14)	3 (5.88)	
Antibody Measurements								
1st month								
Median (IQR)	154.50 (69.00–189.00)	156.00 (77.00–194.00)	154.50 (61.00–188.00)	160.00 (100.00–201.00)	0.031	155.50 (73.00–191.00)	164.00 (114.00–195.00)	0.082
Mean (sd)	146.08 (100.16)	153.49 (97.24)	140.52 (91.15)	170.05 (102.68)		146.63 (94.35)	172.04 (103.33)	
4th month								
Median (IQR)	18.50 (11.00–38.00)	20.00 (12.00–41.00)	18.00 (11.00–34.00)	26.00 (13.00–53.00)	0.007	19.00 (11.00–39.00)	26.00 (13.00–60.00)	0.038
Mean (sd)	32.45 (37.52)	32.38 (32.22)	26.26 (25.33)	40.19 (38.07)		29.08 (28.71)	41.31 (39.18)	
9th month								
Median (IQR)	-	3.98 (2.46–6.95)	3.48 (2.38–5.99)	5.49 (2.80–8.82)	0.058	3.59 (2.31–6.40)	5.38 (2.87–8.44)	0.069
Mean (sd)		19.65 (109.17)	6.37 (18.36)	36.61 (162.40)		10.89 (56.09)	43.37 (188.17)	

Table 1. *Cont.*

Characteristic	Initial Sample	Final Sample	Cellular Immunity Original Spike Protein			Cellular Immunity Variant Spike Protein		
			Inactive	Active		Inactive	Active	
	n = 290	*n* = 189	*n* = 106	*n* = 83	*p*-Value	*n* = 138	*n* = 51	*p*-Value
INDEX OSP	-	0.72 (0.33–1.95)	0.36 (0.22–0.52)	2.14 (1.53–3.11)	<0.001	0.46 (0.26–1.03)	2.47 (1.74–4.75)	<0.001
INDEX VSP	-	0.43 (0.17–1.15)	0.19 (0.10–0.38)	1.24 (0.72–1.95)	<0.001	0.24 (0.12–0.47)	1.79 (1.41–2.88)	<0.001
INDEX Nucleocapsid Protein	-	0.04 (0.00–0.09)	0.05 (0.02–0.11)	0.00 (−0.04–0.07)	0.001	0.04 (0.01–0.09)	0.01 (−0.03–0.09)	0.715
Cellular immunity OSP—no. (%)								
No	-	106 (56.08)	106 (100.00)	0 (0.00)	<0.001	103 (74.64)	3 (5.88)	<0.001
Yes	-	83 (43.92)	0 (0.00)	83 (100.00)		35 (25.36)	48 (94.12)	
Cellular immunity VSP—no. (%)								
No	-	138 (73.02)	103 (97.17)	35 (42.17)	<0.001	138 (100.00)	0 (0.00)	<0.001
Yes	-	51 (26.98)	3 (2.83)	48 (57.83)		0 (0.00)	51 (100.00)	

Notes: *p*-values refer to Mann–Whitney test or independent-samples *t*-test for the continuous variables and Chi-squared or Fisher exact test for the categorical data. BMI in kg/m^2. Abbreviations: BMI = body mass index, IQR = interquartile range, no. = number, OSP = original spike protein, VSP = variant spike protein.

Table 2. Antibody values at one, four, and nine months after immunization and risk factors.

Characteristic	First Month—Median (IQR)	*p*-Value	Fourth Month—Median (IQR)	*p*-Value	Ninth Month—Median (IQR)	*p*-Value
Age Category						
≤40	172.00 (106.00–210.00)	0.003	32.00 (17.00–52.00)	<0.001	5.66 (3.45–8.65)	<0.001
>40	151.50 (67.00–184.00)		16.00 (10.00–33.00)		3.01 (1.92–6.30)	
Gender						
Male	153.50 (75.50–192.50)	0.425	18.50 (11.50–33.00)	0.151	3.60 (2.68–6.31)	0.515
Female	159.00 (84.00–198.00)		22.00 (12.00–52.00)		4.32 (2.34–8.25)	
BMI Category						
Underweight/Normal	156.00 (87.00–202.00)	0.877	22.00 (13.00–41.00)	0.372	4.32 (2.67–7.51)	0.086
Overweight	155.50 (69.00–191.00)		18.00 (11.00–42.00)		3.49 (2.26–6.89)	
Obese/Extreme Obese	161.00 (80.50–198.50)		24.00 (13.00–43.50)		3.59 (2.30–6.80)	
Smoking						
No	159.00 (100.00–195.00)	0.009	24.00 (13.00–49.00)	<0.001	4.78 (2.84–8.22)	<0.001
Yes	123.00 (58.00–184.00)		15.50 (10.00–29.50)		2.94 (1.61–5.54)	
Hypertension						
No	156.00 (77.00–194.00)	0.915	20.00 (12.00–42.00)	0.564	3.98 (2.53–7.14)	0.581
Yes	156.50 (61.00–212.00)		20.50 (7.00–35.00)		3.58 (1.37–6.95)	
Immunosuppression						
No	156.00 (77.00–194.00)	0.494	20.00 (12.00–41.00)	0.413	3.98 (2.46–6.95)	0.732
Yes	179.00 (126.00–196.50)		44.50 (13.50–109.50)		5.08 (2.36–10.79)	

Table 2. *Cont.*

Characteristic	First Month—Median (IQR)	*p*-Value	Fourth Month—Median (IQR)	*p*-Value	Ninth Month—Median (IQR)	*p*-Value
Dyslipidemia						
No	156.00 (74.00–194.00)	0.681	20.00 (12.00–41.00)	0.705	3.97 (2.53–6.95)	0.842
Yes	156.00 (94.00–224.00)		20.00 (10.00–69.00)		4.93 (2.00–7.51)	
Autoimmune						
No	156.00 (80.00–194.50)	0.488	21.00 (12.00–43.50)	0.172	4.16 (2.50–7.45)	0.245
Yes	154.00 (77.00–180.00)		18.00 (9.00–28.00)		3.02 (2.23–5.78)	

Note: *p*-values refer to Mann–Whitney test. Abbreviations: BMI = body mass index, IQR = interquartile range.

Table 3. Antibody kinetics dependent on time and the dependent variables (mixed linear regression).

	Univariate Mixed Linear Model	Multivariate Mixed Linear Model			
		(1)	(2)	(3)	(4)
	n = 567	*n* = 378	*n* = 378	*n* = 567	*n* = 567
	Months 1 to 9	Months 1 to 4	Months 4 to 9	Months 1 to 9	Months 1 to 9
Dependent Variable: Ln (Antibodies)	**β Estimate, 95% CI, *p*-Value**	**β Estimate, 95% CI, *p*-Value**	**β Estimate, 95% CI, *p*-Value**	**β Estimate, 95% CI, *p*-Value**	**β Estimate, 95% CI, *p*-Value**
Age > 40	−0.467 (−0.742–−0.193) p = 0.001	−0.454 (−0.717–−0.192) p = 0.001	−0.537 (−0.815–−0.258) p < 0.001	−0.466 (−0.765–−0.167) p = 0.002	-
Female gender	0.067 (−0.201–0.336) p = 0.622	0.132 (−0.127–0.391) p = 0.317	0.069 (−0.205–0.344) p = 0.620	0.071 (−0.224–0.365) p = 0.638	0.073 (−0.223–0.369) p = 0.628
BMI (ref < 25) Overweight	−0.062 (−0.354–0.229) p = 0.676	−0.014 (−0.296–0.268) p = 0.923	−0.004 (−0.303–0.296) p = 0.981	−0.010 (−0.332–0.311) p = 0.950	−0.009 (−0.331 –0.313) p = 0.958
Obese/Extreme Obese	0.141 (−0.259–0.542) p = 0.490	0.262 (−0.114–0.638) p = 0.171	0.397 (−0.002–0.796) p = 0.051	0.317 (−0.111–0.746) p = 0.146	0.313 (−0.119–0.745) p = 0.155
Smoking	−0.408 (−0.694–−0.122) p = 0.005	−0.287 (−0.545–−0.028) p = 0.030	−0.378 (−0.652–−0.103) p = 0.007	−0.328 (−0.623–−0.033) p = 0.029	-
Hypertension	−0.106 (−0.619–0.406) p = 0.684	0.046 (−0.421–0.513) p = 0.847	0.074 (−0.422–0.570) p = 0.769	0.066 (−0.467–0.598) p = 0.809	0.068 (−0.465–0.602) p = 0.802
Immunosuppression	0.232 (−0.700–1.164) p = 0.626	0.335 (−0.505–1.175) p = 0.435	0.283 (−0.608–1.174) p = 0.534	0.243 (−0.714–1.199) p = 0.619	0.242 (−0.715–1.200) p = 0.620
Dyslipidemia	0.102 (−0.471–0.675) p = 0.728	0.294 (−0.221–0.809) p = 0.263	0.320 (−0.227–0.866) p = 0.252	0.286 (−0.301–0.872) p = 0.340	0.284 (−0.304–0.872) p = 0.344
Autoimmune	−0.273 (−0.742–0.195) p = 0.253	−0.267 (−0.700–0.166) p = 0.226	−0.372 (−0.831–0.087) p = 0.112	−0.292 (−0.785–0.201) p = 0.246	−0.291 (−0.785–0.203) p = 0.248
Interaction Effect (ref: Age ≤ 40 and No Smoking)					
Age ≤ 40 and Smoking					−0.289 (−0.820 –0.241) p = 0.285
Age > 40 and No Smoking					−0.451 (−0.797 –−0.106) p = 0.010
Age > 40 and Smoking					−0.796 (−1.177 –−0.416) p < 0.001

Notes: (1) Multivariate analysis for months one to four, (2) multivariate analysis for months four to nine, (3) multivariate analysis for months one to nine, and (4) multivariate analysis for months one to nine including interaction term. Abbreviations: CI: confidence interval, BMI: body mass index.

Mixed linear model analysis showed that being above the age of 40 or being a smoker reduces the development of antibodies by 37% (β Estimate: −0.466, CI: −0.765–−0.167, *p*-value = 0.002) and 28% (β Estimate: −0.328, CI: −0.623–−0.033, *p*-value = 0.029) respectively, during the first nine months after vaccination (Table 3—model 3). The interaction effect between the two variables was also found to be significant. Specifically, it was revealed that being above the age of 40 and a smoker reduces the development of antibodies by 55% (β Estimate: −0.796, CI: −1.177–−0.416, *p*-value < 0.001) (Table 3—model 4).

3.2. Original SP Index

The univariate associations with the original SP index of cellular immunity were examined for all baseline demographic data, clinical characteristics, and antibody levels at one, four, and nine months on the original and ln-transformed scale. Female gender and antibody levels at one and four months on the original scale, and ln-transformed antibody levels for all months were found to have a significant univariate association as opposed to age, underlying conditions, and antibody levels at nine months (original scale) (Table 4, Part A). In the multivariable analysis, only in model 1, female gender (OR: 0.477; 95% CI: 0.238–0.956; $p = 0.037$) and antibody levels at four months (OR: 1.016; 95% CI: 1.002–1.031; $p = 0.028$) were found to be significantly associated with the presence of original SP index cellular immunity at nine months post-vaccination (Table 4, Part A). Results are similar if we account for the standardized or the normalized transformed scales of the antibody levels.

3.3. Variant SP Index

Antibody levels at four months in their original scale and antibody levels at four and nine months in their ln-transformed scale were found to have a significant univariate association with the VSP index of cellular immunity (Table 4, Part B). In the multivariate analysis for the VSP index, none of the examined parameters were found to have a significant association (Table 4, Part B).

Table 4. Predictors of positive cellular immunity (logistic regression).

Variable	Univariate Analysis	Multivariate Analysis	
		(1)	(2)
	n = 189	n = 189	n = 189
	OR, 95% CI, p-Value	OR, 95% CI, p-Value	OR, 95% CI, p-Value
Part A: Cellular immunity OSP			
Age > 40	0.705 (0.390–1.276) p = 0.248	0.931 (0.458–1.894) p = 0.844	0.939 (0.463–1.906) p = 0.862
Female gender	0.565 (0.316–1.010) p = 0.054	0.477 (0.238–0.956) p = 0.037	0.515 (0.260–1.021) p = 0.057
BMI (ref < 25) Overweight	1.263 (0.675–2.363) p = 0.465	0.972 (0.465–2.033) p = 0.940	1.065 (0.514–2.209) p = 0.866
Obese/Extreme Obese	1.486 (0.631–3.496) p = 0.365	0.889 (0.325–2.434) p = 0.819	1.073 (0.404–2.852) p = 0.888
Smoking	0.645 (0.344–1.210) p = 0.172	0.720 (0.362–1.432) p = 0.349	0.731 (0.367–1.458) p = 0.374
Hypertension	1.303 (0.438–3.872) p = 0.634	1.519 (0.459–5.027) p = 0.493	1.419 (0.428–4.706) p = 0.567
Immunosuppression	0.419 (0.043–4.100) p = 0.455	0.235 (0.018–3.004) p = 0.265	0.369 (0.034–4.011) p = 0.412
Dyslipidemia	1.068 (0.314–3.630) p = 0.916	1.070 (0.266–4.301) p = 0.924	1.204 (0.308–4.704) p = 0.790
Autoimmune	0.884 (0.322–2.432) p = 0.812	1.293 (0.418–3.995) p = 0.656	1.151 (0.372–3.557) p = 0.807
Antibodies 1st month	1.003 (1.000–1.006) p = 0.044	1.001 (0.997–1.005) p = 0.775	
Antibodies 4th month	1.015 (1.004–1.025) p = 0.005	1.016 (1.002–1.031) p = 0.028	
Antibodies 9th month	1.007 (0.994–1.021) p = 0.276	1.005 (0.997–1.013) p = 0.224	
Ln (Antibodies 1st month)	1.645 (1.073–2.523) p = 0.023		1.139 (0.594–2.187) p = 0.695
Ln (Antibodies 4th month)	1.601 (1.135–2.258) p = 0.007		1.309 (0.694–2.470) p = 0.406
Ln (Antibodies 9th month)	1.545 (1.125–2.124) p = 0.007		1.253 (0.866–1.812) p = 0.232
Part B: Cellular immunity VSP			
Age > 40	0.649 (0.338–1.248) p = 0.195	0.677 (0.311–1.474) p = 0.326	0.719 (0.328–1.574) p = 0.409
Female gender	0.791 (0.416–1.506) p = 0.476	0.828 (0.385–1.778) p = 0.628	0.848 (0.396–1.815) p = 0.671
BMI (ref < 25) Overweight	1.330 (0.660–2.677) p = 0.425	1.265 (0.561–2.851) p = 0.570	1.332 (0.593–2.992) p = 0.488
Obese/Extreme Obese	1.257 (0.483–3.269) p = 0.639	0.833 (0.261–2.658) p = 0.757	1.006 (0.330–3.074) p = 0.991
Smoking	0.757 (0.372–1.539) p = 0.441	0.893 (0.413–1.931) p = 0.773	0.904 (0.416–1.963) p = 0.799
Hypertension	2.167 (0.713–6.584) p = 0.173	3.518 (1.077–12.102) p = 0.054	3.162 (0.920–10.860) p = 0.067
Immunosuppression	0.900 (0.091–8.855) p = 0.928	0.730 (0.053–10.020) p = 0.814	0.966 (0.078–11.892) p = 0.978
Dyslipidemia	1.016 (0.259–3.987) p = 0.982	1.086 (0.240–4.924) p = 0.915	1.158 (0.260–5.165) p = 0.847
Autoimmune	0.554 (0.152–2.012) p = 0.369	0.660 (0.157–2.768) p = 0.570	0.609 (0.145–2.546) p = 0.496
Antibodies 1st month	1.003 (0.999–1.006) p = 0.118	1.001 (0.997–1.005) p = 0.766	
Antibodies 4th month	1.011 (1.001–1.020) p = 0.026	1.010 (0.997–1.023) p = 0.123	
Antibodies 9th month	1.003 (0.999–1.006) p = 0.153	1.003 (0.999–1.006) p = 0.116	
Ln (Antibodies 1st month)	1.607 (0.982–2.631) p = 0.059		1.231 (0.580–2.614) p = 0.589
Ln (Antibodies 4th month)	1.534 (1.053–2.235) p = 0.026		1.113 (0.562–2.204) p = 0.759
Ln (Antibodies 9th month)	1.455 (1.076–1.968) p = 0.015		1.294 (0.909–1.843) p = 0.152

Notes: (1) Multivariate analysis with antibodies as continuous variables. (2) Multivariate analysis with antibodies as logarithmic variables. Abbreviations: OR: odds ratios, CI: confidence interval, BMI: Body mass index.

4. Discussion

In our study, anti-RBD antibody levels are observed to drop over nine months following vaccination, yet remain detectable in most cases; findings that are in agreement with other studies [31,65–69]. In our cohort of 189 HCWs, antibody decay was estimated to be 78.9% between one and four months after the second dose, and a further 39.3% between four and nine months (Figure 2). Similar antibody trajectories derive from other studies where the anti-S-RBD antibody fall rate six months after the initial vaccination scheme is estimated to range between 60–90% [65,69].

Several studies provide reassuring evidence that robust and long-lasting activation of spike-specific T-cells takes place and can outweigh humoral response as the main indicator of vaccine effectiveness [70–72]. Malipiero et al. have demonstrated that IGRA can be used as an accurate laboratory method to estimate cellular immune response to BNT162b2 mRNA vaccine in both immunocompetent and immunocompromised patients where the humoral response is undetectable [52]. Tychala et al. found a positive correlation of antibody levels with INF-γ-based cellular response five months post BNT162b2 vaccination [73].

In our study, IGRA estimation of cellular immunity nine months post vaccination shows an active cellular response to original SP in 83 participants (44%) and variant SP in 51 (26%), meaning that more than half of the participating individuals lose their highly desired cellular response by nine months (Table 1). Interestingly, we report a positive association of original SP cellular immunity with female sex and antibody levels at four months that was well-supported through multivariate regression analysis (Table 1). These findings have not been previously reported. Similar correlations are not found for cellular immunity as a response to variant SP.

A clear negative correlation is noted between increasing age and antibody titers at one, four, and nine months post vaccination, which is in line with other studies [21,31,69,74–79]. This correlation is persistent in all three measurements and is validated with logistic regression analysis and a mixed linear model. Other studies also corroborate an age-related impairment of binding and neutralizing antibodies after vaccination [80–82], while cellular immunity does not appear to be affected by age in our study or elsewhere [83–85]. We also report that being a smoker weakens antibody development by 37%, whereas being above the age of 40 and a smoker reduces the development of the antibodies by 55%. Likewise, Nomura et al. found that age and smoking habit determine antibody response three months post receipt of second vaccine [79], and the VASCO study describes a rapid decrease in antibody levels post BNT162b2 mRNA vaccine among smokers [83].

Conflicting data in the literature demonstrate either an increased capacity for females to mount humoral immune responses compared with males [62,85–89], or do not associate gender with antibody response [56,69,90–92]. In our study, we did not find an association between the female gender and antibody levels post full immunization, but we report a correlation between the female gender and the presence of activated anti-S T-cells at nine months post-immunization [31,65,93,94].

Studies have shown that mRNA COVID-19 vaccines had similar efficacy among obese and non-obese individuals [1,16,89], while others report a decreased antibody response to the first dose of BNT162b2 COVID-19 vaccine in the obesity or pre-obesity group [62,95]. In the present study, BMI is not found to significantly impair antibody or cellular response. Watanabe et al. have correlated central obesity (defined as higher waist circumference) as a factor negatively affecting the post-vaccination development of antibodies [56]. Alternatively, several studies suggest that obese individuals develop surprisingly higher neutralizing antibodies compared with their recipients with normal weight [31,96].

Regarding the effects of comorbidity, findings from several studies indicate blunted post-vaccination humoral response in people with diabetes [17,56], hypertension [56,89], dyslipidaemia [56], immunosuppression [97], autoimmune diseases and heart disease [66,93,98]. In our study, no association is found between humoral or cellular immunity and underlying medical conditions, possibly because of the small number of participants with comorbidities in this cohort. It should be acknowledged that this is a limitation of our study.

This is a single-centered study, yet the size of the sample is relatively high compared with others published so far. Another limitation of the study is that there is no baseline sample collected before vaccination and COVID-19 negative status was determined by infection surveillance. Moreover, the study population in this report is relatively young and healthy without a significant burden of comorbidities. Therefore, the generalizability of our results may not be taken for granted. Demographic and anthropometric data in this study were obtained via a standardized structured self-reporting questionnaire, introducing the possibility of recall bias. Additionally, cell-mediated immunogenicity was only measured at nine months post-vaccination, as our country's kit for cellular immunity was not commercially available earlier. The company we sourced it from lists the date of registration in their database as 24 May 2021. In our study, vaccinees with previous COVID-19 have been excluded, thus our measurements of humoral and cellular immunity reflect the duration of vaccine-induced immunity in COVID-19 naive population. The limitations of this study prevent us from drawing definitive conclusions but do add to existing and future scientific data.

5. Conclusions

The long-term effectiveness of the primary vaccination scheme of mRNA vaccines is unknown and results from large-scale trials are warranted to indicate the optimal vaccination strategy against SARS-CoV-2 and the necessity of serial booster doses. This study provides insights into the evolution, duration, and associated factors of vaccine-induced immunity—both humoral and cellular—long after the completion of the two-dose BNT162b2 vaccine schedule.

Author Contributions: N.S. and F.S. conceived the study; F.S., N.S. and M.-C.G. designed the study; N.S., F.S., M.-C.G., E.R., Z.V., E.S., N.L., D.G. and A.Z. collected samples and questionnaires from participants; E.T., P.V., V.G. and E.L. performed IgG assay; P.G. performed IGRA testing; D.K., M.-C.G. and A.P. analyzed the data. N.S., M.-C.G. and D.K. drafted the manuscript; L.P. revised the manuscript; D.H. supervised the study. All authors have read and agreed to the published version of the manuscript.

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Predictive Attributes for Developing Long COVID—A Study Using Machine Learning and Real-World Data from Primary Care Physicians in Germany

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Abstract: (1) In the present study, we used data comprising patient medical histories from a panel of primary care practices in Germany to predict post-COVID-19 conditions in patients after COVID-19 diagnosis and to evaluate the relevant factors associated with these conditions using machine learning methods. (2) Methods: Data retrieved from the IQVIA™ Disease Analyzer database were used. Patients with at least one COVID-19 diagnosis between January 2020 and July 2022 were selected for inclusion in the study. Age, sex, and the complete history of diagnoses and prescription data before COVID-19 infection at the respective primary care practice were extracted for each patient. A gradient boosting classifier (LGBM) was deployed. The prepared design matrix was randomly divided into train (80%) and test data (20%). After optimizing the hyperparameters of the LGBM classifier by maximizing the F2 score, model performance was evaluated using several test metrics. We calculated SHAP values to evaluate the importance of the individual features, but more importantly, to evaluate the direction of influence of each feature in our dataset, i.e., whether it is positively or negatively associated with a diagnosis of long COVID. (3) Results: In both the train and test data sets, the model showed a high recall (sensitivity) of 81% and 72% and a high specificity of 80% and 80%; this was offset, however, by a moderate precision of 8% and 7% and an F2-score of 0.28 and 0.25. The most common predictive features identified using SHAP included COVID-19 variant, physician practice, age, distinct number of diagnoses and therapies, sick days ratio, sex, vaccination rate, somatoform disorders, migraine, back pain, asthma, malaise and fatigue, as well as cough preparations. (4) Conclusions: The present exploratory study describes an initial investigation of the prediction of potential features increasing the risk of developing long COVID after COVID-19 infection by using the patient history from electronic medical records before COVID-19 infection in primary care practices in Germany using machine learning. Notably, we identified several predictive features for the development of long COVID in patient demographics and their medical histories.

Keywords: COVID-19; long COVID; machine learning; gradient boosting classifier

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1. Introduction

COVID-19 is the global pandemic of the 21st century. As of 21 February 2023, there have been approximately 757 million confirmed cases of COVID-19 worldwide, including 6.9 million deaths [1]. In many COVID-19 patients, symptoms persist for at least several months. In a systemic review and meta-analysis of 50 studies, the prevalence of long COVID-19 symptoms 28 days to 12 months after COVID-19 infection was 54% in hospitalized individuals and 34% in non-hospitalized individuals [2]. The proportion of individuals affected by long COVID-19 symptoms has decreased since 2021 due to the emergence of milder COVID-19 variants [3].

An increasing number of decisions in medical applications are being made on the basis of machine learning (ML) algorithms. In view of this, COVID-19 research is also

focusing on the development of machine learning algorithms to optimize the prediction of COVID-19 [4] and estimate COVID-19 vaccination side effects [5] or the risk of death as a result of COVID-19 for patients in hospital intensive care units (ICU) [6]. When it comes to long COVID, a study successfully predicted long COVID conditions mainly based on sociodemographic variables and symptom severity during acute COVID-19 infection using a case–control design [7]. In addition, one study was conducted to identify potential long COVID patients using gradient boosting models that had been trained on patients treated in a specialized long COVID clinic [8] and applied to patient cohort data from a US COVID-19 database. Nevertheless, the authors themselves state that their study does not represent all population strata, especially because it does not include people who are not insured and people who are unable to afford medical treatment in the US. The disadvantages associated with their data also apply to electronic medical records (EMR) such as in the database used in the present study. For example, these records only document patient visits to general practitioners (GPs) and do not document patient visits to different specialty practices or hospitals. Additionally, the data are skewed towards patients who visit their general practitioners regularly. Nevertheless, the advantage of having defined trajectories for a cross-section of the population allows the model to include chronic and acute diseases, sick leave days, treatments, and other information. Finally, GPs are the primary point of contact for patients suffering from long COVID.

In the present exploratory study, we used data comprising patient medical histories from a panel of primary care practices in Germany to predict long COVID symptoms in patients after COVID-19 diagnosis and to evaluate the relevant factors associated with these symptoms using ML methods. To our knowledge, this is one of the first studies using electronic medical records to identify potential features predictive for the development of long COVID.

2. Materials and Methods

2.1. Data Set

The data used in the present study were retrieved from the IQVIA^{TM} Disease Analyzer database, which contains information from approximately 3% of primary care practices in Germany, and includes demographics, diagnoses, and prescription data, in an anonymized format, retrieved from the computer systems of cooperating practices. Previous research has shown that the panel is representative of primary care practices in Germany [9].

2.2. Study Population

Patients with at least one COVID-19 diagnosis (ICD-10: U07.1 or U08.9) between January 2020 and July 2022 were selected for inclusion in this study. Of these patients, a subpopulation was then formed comprising patients with one recorded certain diagnosis of long COVID (ICD-10: U09.9). Data on age, sex, and the complete history of diagnoses and prescription data at the respective primary care practice were extracted for each patient before their first COVID-19 infection. A categorical variable representing each primary care practice ID was also added.

We applied several filters when selecting patients for analysis. First, the distances between all patients' first COVID-19 diagnoses and the long COVID diagnoses were calculated. The 75% quartile of the distribution (86 days) was considered the minimum distance to the last available timepoint in the database. All patients who received their first COVID-19 diagnosis less than 86 days prior to the last available timepoint of the database were therefore excluded from further analysis. In addition, patients with less than 30 days between their first recorded COVID-19 diagnosis and the long COVID diagnosis were excluded from further analysis. Patient history was analyzed prior to the first COVID-19 diagnosis to exclude COVID-19-related diagnoses or medication as predictors. Furthermore, patients with a documented long COVID diagnosis but no prior documented COVID-19 infection were excluded from the dataset, as the date of the first COVID-19 infection is necessary to determine the cutoff for the patient's history.

Finally, 272,588 patients were available for the ML models, 5440 of whom had a long COVID diagnosis.

2.3. Feature Preparation

Data cleansing and preprocessing were conducted using SAS (version 9.4, SAS Institute, Cary, NC, USA). Each diagnosis was classified into third-level ICD-10 categories based on the classification of the Federal Institute for Drugs and Medical Devices [10]. Similarly, prescriptions were classified into third-level ATC categories based on the anatomical chemical classification (ATC) of the European Pharmaceutical Market Research Association (EphMRA) [11]. After this, the number of diagnoses and prescriptions within the respective ICD-10 or ATC category across the entire patient history were counted to assess patients' general utilization of the health care system. To reduce the number of features for training, the 50 most frequent ICD-10 and ATC categories were selected within the present patient population.

We added the number of COVID-19 diagnoses per patient as another feature. Distinct diagnoses were assumed if the time between two diagnoses was more than four weeks. While for the other features we only looked at the patient's history prior to the first COVID-19 infection, for this feature we looked at additional COVID-19 diagnoses after the first COVID-19 diagnosis but before a potential long COVID diagnosis.

Further features were again based on the history available for each patient. We included the time span between the first and the last record of a patient (visibility days). Patients with visibility of under 100 days were excluded. The median visibility among the remaining patients was 5.9 years (10% quantile: 1.3 years, 90% quantile: 17.5 years). Explicitly including visibility as a feature allows the classifier to account for different lengths of patient histories in its decision. In addition, the number of sick leave days was calculated based on the medical history. Similarly, the number of recorded hospital referrals was calculated. These newly created variables were normalized by relating them to the length of the respective patient visibility.

Using data from the Robert Koch Institute (RKI), the corresponding relative probabilities of each virus strain (wild type, Alpha, Beta, Delta, various Omicron subtypes) were assigned to the first COVID-19 diagnosis of a patient [12]. The current vaccination rates of the population were assigned in a similar fashion based on the date of the first COVID-19 infection of a patient to estimate the probability of vaccination-related immunity [13]. Two vaccination rates were used representing the basic immunization rate (two shots administered) and the first booster (third shot administered). This modeling using external data was necessary because only a small portion of COVID-19 vaccinations is reported in our initial data, as the vaccination campaign in Germany was distributed across fixed and mobile vaccination centers and vaccinations were not administered solely by GPs.

The data were entered into a design matrix, with each row representing one patient and each column representing one variable as described above. The target variable was a binary vector considering a long COVID diagnosis (=1) or no long COVID diagnosis (=0) after COVID-19 diagnosis. All further processing was conducted in Python (v. 3.9.15) using sklearn (v. 1.1.3). Categorical variables were one-hot encoded. Where values were missing in the categorical variables, the redundant column representing the missing value was dropped from the data set (i.e., sex, <0.1%). In the case of count variables, missing values were imputed with zeros. The prepared design matrix was randomly divided into two data sets: the train data (80%) and the test data (20%). Missing values in the age variable (<0.1%) were imputed with the median age derived from the train data.

2.4. Training

In this study, we deployed the light gradient boosting machine (LGBM), a performant gradient boosting algorithm based on decision trees [14]. It was used because algorithms of this kind are widely used to identify potential features and disease outcomes [8], and are supposed to perform better than, e.g., neural networks in tabular data [15]. In addition,

the LGBM algorithm used here is a well-established classifier which is used in a variety of classification approaches [5,16,17]. It is equally performant to other boosting classifiers and, therefore, is a good choice for the classification of long COVID in primary care practices [18].

An LGBM binary classifier was trained using the Python lightGBM (v. 3.3.3 [14]) package. Several hyperparameters were optimized using a grid search with 5-fold cross-validation within the train data set (Table S1). Hyperparameters were optimized to maximize the F2 score of the model. The F2 score is a weighted harmonic mean of precision and recall, whereby recall is weighted double relative to precision [19]. A higher weighting of recall was applied in order to acknowledge potential false-negative labels in the train data so as to correct for patient hopping and diagnoses at other practices in particular.

Model performance was evaluated on the test data set illustrating a contingency matrix, precision, recall, specificity, F2 score, ROC-AUC, and accuracy metrics.

2.5. Feature Importance

Shapley Additive Explanation (SHAP) values were calculated (v. 0.41.0 [20]) to evaluate the contribution of the individual features to the model's performance. SHAP is a generic game theoretic approach allowing for the interpretation of features for any machine learning model [20]. Contrary to many other approaches, SHAP allows the direction of the effects of features onto the target variable to be interpreted. SHAP takes into consideration the contribution of each feature in conjunction with all possible combinations of other features in the model, and therefore returns an integrated view of feature importance.

The one-hot encoded variable describing the practice identifiers comprised many columns in the design matrix, as several thousand practices were included. Therefore, the SHAP values were summarized across practices within each row (patient) of the design matrix to estimate the overall effect of the category "practice," rather than the contribution of each single practice [21].

3. Results

3.1. Model Performance

Across the entire train data set, the model showed an accuracy of 80%, a precision of 8%, a recall (sensitivity) of 81%, a specificity of 80%, an ROC-AUC of 0.9, and an F2 score of 0.28. On the test data set, the model showed an accuracy of 80%, a precision of 7%, a recall of 72%, a specificity of 80%, an ROC-AUC of 0.84, and an F2 score of 0.25. Note that the data set classes were imbalanced. Accuracy and ROC-AUC are therefore not particularly suitable as criteria for model effectiveness, but are reported nevertheless for the convenience of the reader. The contingency matrices for train and test data sets are illustrated in Figure 1. A total of 81% and 72% of long COVID patients were identified correctly by the model from the train and test data sets, while 80% and 80% of patients, respectively, without a long COVID diagnosis were identified correctly by the model in the train and test data sets. All further inferences will be made based on the test data set only.

3.2. Feature Importance

SHAP was used to estimate feature importance. Figure 2 illustrates the 20 most important features and the relative impact of a variable expression for the development of long COVID in our data. Feature values are displayed in either red or blue. When higher feature values (red) are associated with positive SHAP values (positive range on the *x*-axis), and lower feature values (blue) are associated with negative SHAP values (negative range on the *x*-axis), the variable expression is positively associated with the development of long COVID. By contrast, if higher feature values distribute to the negative range and lower feature values distribute to the positive range, the feature is negatively associated with the development of long COVID.

For the top 19 features (there are actually 20 features, but we are excluding the summarized categorical feature "practice") identified in our SHAP analysis, we also illustrated the SHAP values as a function of the variable expression (Figure 3).

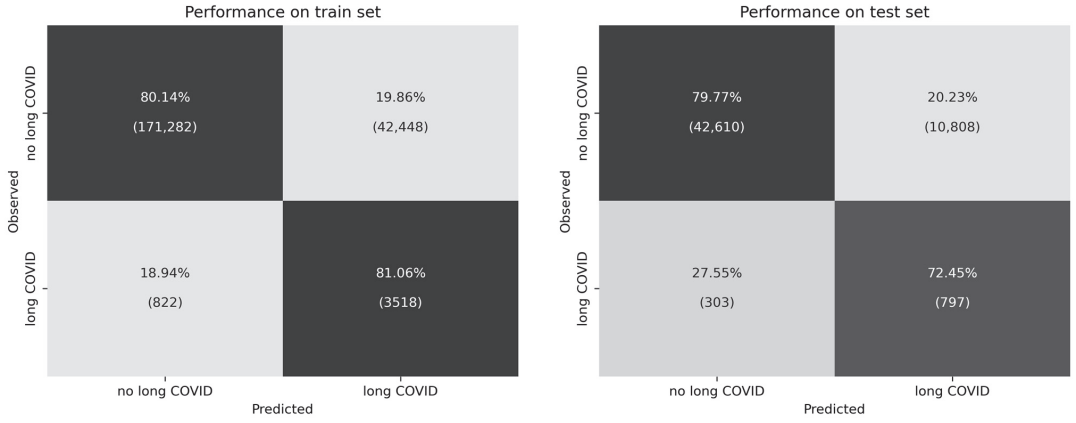

Figure 1. Contingency matrices of model performance on the train and test data sets. Left: train data set. Right: test data set. The *y*-axes represent the "true" observed data labels, i.e., no long COVID diagnosis, or long COVID diagnosis. The *x*-axes represent the data labels predicted by the model. True negatives (correctly identified patients without long COVID diagnoses) are illustrated in the upper left. True positives (correctly identified long COVID patients) are illustrated in the lower right. Each cell contains percentages relating to the total proportion of patients with or without long COVID diagnoses labeled in the data (i.e., row-wise). Brackets contain the total amount of patients in each cell.

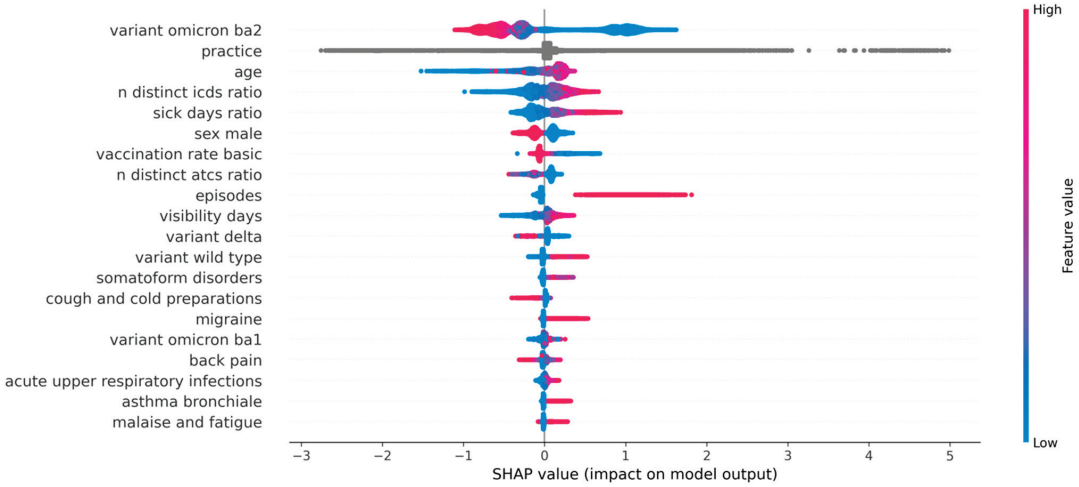

Figure 2. Feature importance as estimated via SHAP. Only the 20 most important features are displayed in descending order (top to bottom). SHAP values are illustrated on the *x*-axis. Higher feature values (red) represent data points with higher variable expression. Lower feature values (blue) represent data points with lower variable expression. Gray values represent the influence of the categorical practice IDs. When higher feature values (red) are distributed to the positive range of the *x*-axis, and lower feature values (blue) are distributed to the negative range of the *x*-axis, the variable expression is positively associated with the development of long COVID. By contrast, if higher feature values distribute to the negative range and lower feature values distribute to the positive range, the feature is negatively associated with the development of long COVID.

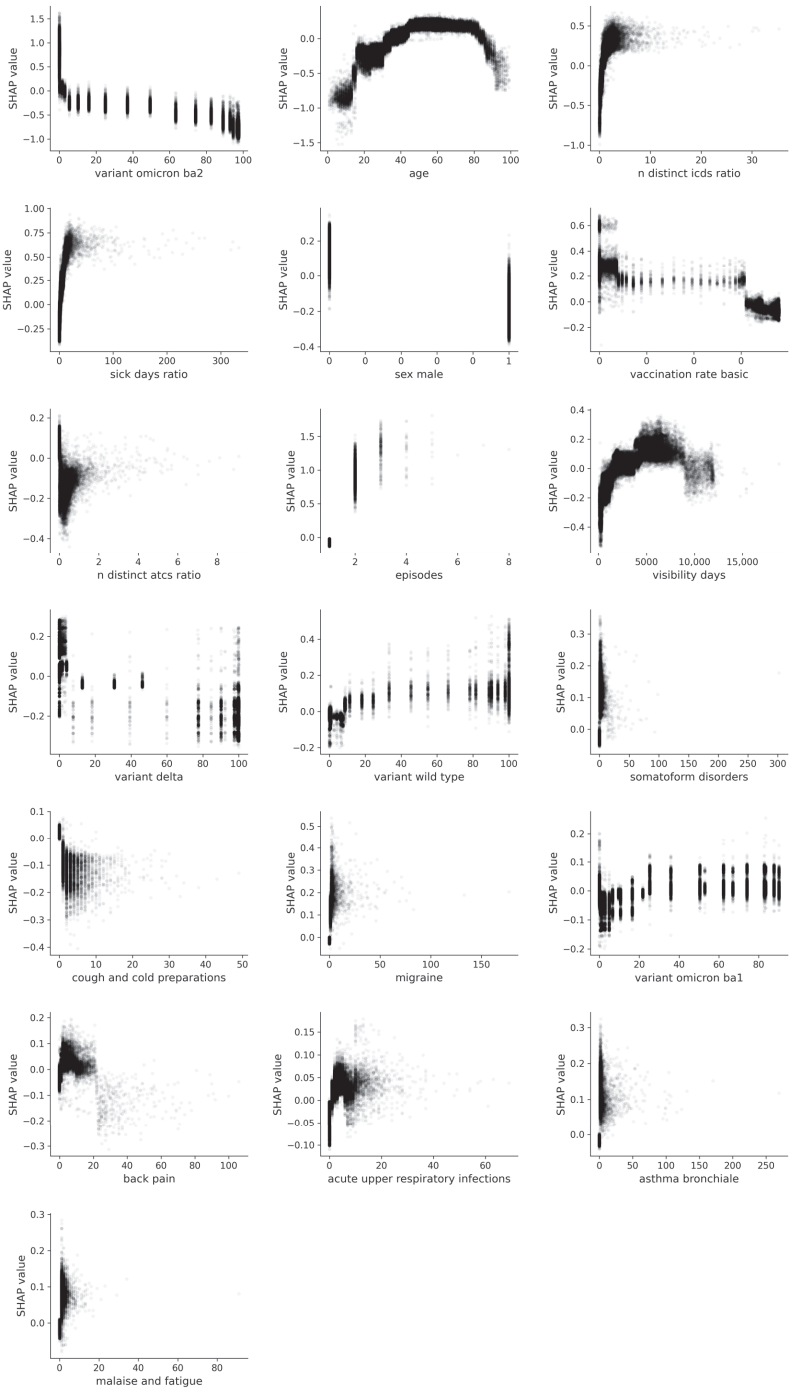

Figure 3. Dependency of long COVID on feature expression. The 19 most important features (when the categorical variable "practice" is excluded) according to the SHAP analysis (Figure 2) are displayed from top left to bottom right. For each feature, the corresponding SHAP value (*y*-axis) is related to the respective variable expression (*x*-axis).

3.2.1. SARS-CoV-2 Variants

The most important feature in our analysis was variant Omicron-BA2, indicating that patients with a COVID infection at a time with a higher proportion of variant Omicron-BA2 had a lower probability of developing long COVID (Figures 2 and 3). Conversely, this highlights that patients with COVID infection at a time when the proportion of variant Omicron-BA2 was lower (and, in turn, the probability of other variants was higher) were more likely to develop long COVID. While the influence of the Delta variant is similar (Figures 2 and 3), it is less strong, as highlighted by its lower feature importance (order on the y-axis in Figure 2). Furthermore, the relative proportion of the wild type variant of SARS-CoV-2 showed a positive association with long COVID, with higher probability of being infected with the wild type strain pointing towards an increased risk of developing long COVID (Figures 2 and 3).

This is more clearly reflected in Figure 3, which depicts the dependence of SHAP-values on feature expression. Here, the SHAP values are shown as a function of the variable expression, i.e., the proportion of the respective strain in all sequenced samples for a given point in time. The SHAP value for the wild type variant was higher when the proportion for the wild type in the population was highest, indicating a higher probability of long COVID when the probability of being infected with the wild type strain (on first COVID diagnosis) was higher. However, the opposite effect can be observed for the Omicron-BA2 variant. The highest SHAP values are found where the proportions of the variant were lowest (Figures 2 and 3). A mixture of the two is shown for the Delta variant, with a tendency to show lower SHAP values at higher proportions of the variant (Figures 2 and 3). For the Omicron-BA1 variant, the effect is rather similar to that of the wild type variant, with higher proportions of Omicron-BA1 at the time of infection associated with higher probability of long COVID. Note that the stepwise representation of the proportions of the variants in Figure 3 results from the weekly data used from the RKI tables. In these tables, the proportions of the strains can change rapidly between successive weeks.

3.2.2. Sociodemographic and Practice Effects, and General Diagnosis and Medication Counts

To also control for the effect of the individual physician on long COVID diagnosis, the sum of SHAP values of all practice IDs was consolidated, resulting in practice being the second most important feature (Figure 3). The third most important feature was patient age. Age had a strong impact on the model prediction, with low age values leading to negative SHAP values, whereas high age values led to higher SHAP values and, therefore, a higher probability of long COVID. When looking at the feature expressions, the SHAP values were distributed as an inverted U (Figure 3). Higher SHAP values—indicative of a higher probability of long COVID—were associated with an age of between 30 and 80 years. Higher and lower age showed negative SHAP values, with very low values before an age of 15 and after an age of 80.

The ratio of distinct ICD-10 classes and the sick day ratio, as the fourth and fifth most important features, show a similar distribution of SHAP values in Figure 2. For both, the SHAP value increased with higher feature expressions, indicating a positive association between the development of long COVID and having multiple different diagnoses before COVID-19 infection, as well as having more sick days before COVID-19 infection (both relative to the observation period of a particular patient). Furthermore, SHAP values for the number of COVID episodes showed a high positive impact on the model. The number of episodes is a proxy for the number of COVID infections (cf. Section 2). For this purpose, distinct diagnoses were counted no earlier than 4 weeks after the previous COVID diagnosis. This includes patients with either a long-lasting infection or recurring infections. The analysis of the dependence plots in Figure 3 demonstrates that as few as two episodes already lead to higher SHAP values and, therefore, a higher probability of long COVID, with each additional episode increasing the risk. Longer visibility of a patient within our database also contributed to higher SHAP values.

Male sex reduced the probability of developing long COVID, as this feature showed an inverted pattern of SHAP values around the *x*-axis (Figure 2). This is also illustrated in Figure 3, as the "1" depicts male sex and is therefore associated with a lower SHAP value than patients with female or unknown ("0") sex. The risk of long COVID was also reduced if patients had most likely received the basic vaccination (Figure 2), which is defined as the first two vaccination shots. This can also be seen in the dependence plots (Figure 3), where the increasing rate of full vaccination across Germany is related to lower SHAP values. In addition, the distinct number of different ATC classes in patient history was slightly negatively associated with a higher risk of developing long COVID (Figure 2). The dependence analysis here did not provide a clear picture (Figure 3). Higher SHAP values were slightly associated with a lower number of distinct ATC classes. However, with a very low number of distinct ATC classes (i.e., 0), both low and high SHAP values can be discerned.

3.2.3. ICD-10 Classes

Within the 20 most important features, features describing ICD-10 classes were ranked lowest (Figure 2). The feature expression of each ICD-10 class stands for the number of the respective diagnosis in the patient history before the first recorded COVID-19 infection. Within the ICD-10 classes, somatoform disorders (ICD-10: F45) were the most important feature (Figure 2). The SHAP values suggest that patients diagnosed with somatoform disorders had a higher risk of developing long COVID (Figure 2). SHAP values for back pain (ICD-10: M54) showed high feature values on the positive part of the *x*-axis and on the negative part of the *x*-axis, making the interpretation less clear in Figures 2 and 3. For migraine (ICD-10: G43), asthma (ICD-10: J45), and malaise and fatigue (ICD-10: R53), positive feature values also tended towards positive SHAP values, indicating a higher probability of long COVID (Figure 2). In the dependence analysis (Figure 3), back pain and acute upper respiratory infections (ICD-10: J06) had similar SHAP value distributions, with lower SHAP values connected to a low number of diagnosis codes. SHAP values increased generally with an increasing number of the respective diagnosis codes in the respective patient history. Somatoform disorders, malaise and fatigue, asthma, and migraine all had a broad variety of SHAP values associated with already low numbers of diagnosis codes, accumulating around 0.

To better determine the effects of finding particular diagnoses (and medications) in a patient history on the probability of developing long COVID, we also dichotomized each ICD and ATC code into patients with either a zero or non-zero amount of a particular diagnosis code or medication code in their histories. We then averaged the SHAP values of all patients in each group (zero and non-zero, respectively). Figure 4 illustrates the mean SHAP values for each of the most predictive diagnoses and medication codes, averaged for patients with and without the code, respectively. Figure 4 clearly illustrates that the effects point mainly in the positive direction, i.e., where a patient history includes a particular diagnosis, the SHAP values tend to be positive, while otherwise, they tend to be negative. This circumvents the limitations of the dependence plots (Figure 3), where it is difficult to infer the exact density of the SHAP values in particular regions. For most ICD-10 codes in Figure 3, there is a point mass of data points visually hidden with a negative SHAP value at a feature expression of zero.

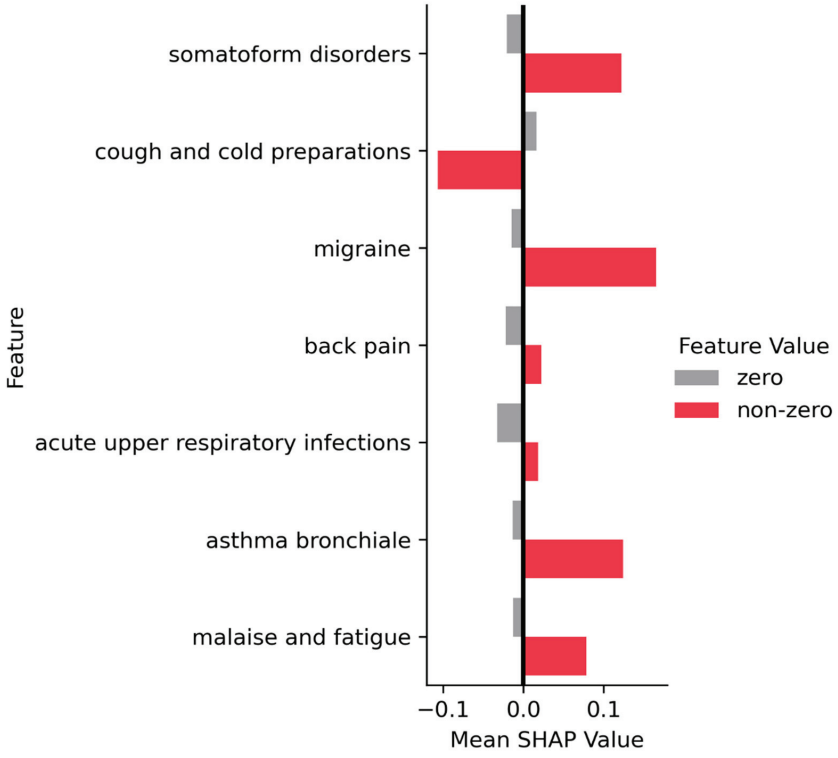

Figure 4. Average SHAP values for diagnosis and medication codes, averaged within two respective patient groups with one or more occurrences of the code (*non-zero*) or with no occurrence (*zero*). Top to bottom order reflects feature importance. *X*-axis illustrates mean SHAP value for the respective subgroup. Note that the absolute length of the bars does not directly indicate the importance of the feature.

3.2.4. ATC Classes

Only one ATC class was predictive enough to be included as one of the 20 most important features for the model. The ATC class R05C (cough-related products including antihistamines and bronchodilators) shows a negative impact on the model. Patients receiving products in this ATC class are less likely to develop long COVID (Figures 2–4). The higher number of prescriptions of this class is associated with decreasing SHAP values, and therefore a lower probability of long COVID.

4. Discussion

In this retrospective, exploratory study including more than 270,000 patients with COVID-19, a good prediction of long COVID was achieved using an LGBM classifier. This is, to the best of our knowledge, the first investigation on the prediction of potential features increasing the risk for developing Long-COVID after COVID-19 infection in primary care practices in Germany. Additionally, particularly novel is the use of electronic medical record data for the prediction of long COVID, having been performed only a few times, such as in [8]. Further, this is the first study that focuses on the first point of medical contact of patients.

The first finding of our study is the good performance of the LGBM classifier. In the train dataset, 81% of long COVID patients and 80% of non-long COVID patients were correctly identified by the model. In the test dataset, the proportions were 72% and 80%, respectively. Aktar et al. also successfully attempted to predict clinical outcomes in COVID-

19 patients based on different peripheral blood values, using several ML models to identify blood parameters that can predict the risk of serious illness among COVID-19 patients [22]. Furthermore, Sudre et al. predicted long COVID conditions based on symptoms during the first week of illness and sociodemographic factors [7] using a matched case–control design and achieved good model performance. Because the data set we used is unbalanced with respect to diagnoses (long COVID vs. no long COVID), and because it does not comprise case–control matched groups, direct comparison of model performance to many other studies is difficult. However, there was no marked drop in model goodness between train and test data, suggesting good generalizability of our model.

The second finding of our study is the identification of a number of important features. Patients who were diagnosed with COVID-19 at a time with a higher proportion of the Omicron-BA2 variant had a lower risk of developing long COVID, whereas a higher proportion of the wild type variant of SARS-CoV-2 was positively associated with the risk of developing long COVID. This finding is partly in line with other published research. Du et al. performed a systematic review and meta-analysis including a total of 51 studies with 33,573 patients to evaluate the characteristics of long COVID caused by different SARS-CoV-2 variants. While authors suggested that there was no significant difference between different variants in terms of long COVID incidence, symptoms of long COVID differed strongly depending on SARS-CoV-2 variant. For example, $\geq$1 general symptoms and fatigue occurred most commonly in patients infected with the Alpha variant, followed by patients with the wild type strain, and less often among patients with the Omicron variant [23].

The second most important feature was the practice in which a patient was treated. The high importance of this variable is interesting, but not surprising, as it captures different diagnostic styles in medical practices. Especially with such new diagnostic codes and for such a heterogeneous clinical picture as long COVID, for which guidelines and information change rapidly, individual doctors can come to very different assessments as to whether or not a patient suffers from long COVID. Furthermore, some of the practices might also (begin to) treat long COVID with a focus, while other practices might not approach a diagnosis.

In our study, age 30–80 and female sex were associated with a higher risk of long COVID. Interestingly, in another study based on the same database but using logistic regression to analyze associations between different variables and long COVID, age 45–60 was associated with a 2.1 times higher risk of long COVID compared with age group 18–30; female sex was associated with a 1.2 times higher risk of developing long COVID. However, further variables such as asthma and somatoform disorders were also positively associated with long COVID [24]. Although the association between sex and long COVID is still insufficiently understood, several other studies also reported that the prevalence of long COVID was higher in women than in men [7,25–29].

Somatoform disorders which were associated with a higher risk of long COVID in our study can be characterized by symptoms such as back pain, headache, fatigue, dizziness, and shortness of breath without an adequate medical explanation. COVID-19 patients who have a coexisting somatoform disorder may harbor a belief that these symptoms are due to long COVID [30]. Another study from Poland which assessed factors associated with prolonged symptoms in non-hospitalized patients with COVID-19 demonstrated that female sex, asthma, history of myocardial infarction, and severity of symptoms in the acute phase of COVID-19 were the predictors of long COVID [31]. Two of these predictors (female sex, asthma) were found among the top 20 features in our study. Another study also used symptom severity in an early phase of COVID infection to successfully predict long COVID in a case–control designed analysis [7]. In addition to symptom severity and symptom quantity, female sex, age, and asthma were also predictive for long COVID in their study, nicely converging on our findings.

In our study, we observed a positive association between multimorbidity (multiple different diagnoses or a higher number of sick days before COVID-19 infection) with subsequent long COVID. Wilk et al. analyzed data from different European countries and

found that multimorbid individuals had an increased risk of experiencing symptoms of long COVID, identifying a slightly increased relative risk of 1.12 for such individuals [32]. However, multimorbidity is known to impact COVID-19 severity and mortality as well as the risk of long COVID [27,33]. On the other hand, polypharmacy was negatively associated with the risk of long COVID in our study.

A further finding of our study is that a higher likelihood of COVID-19 vaccination was negatively associated with the risk of long COVID. Although we used a proxy for vaccination (cf. Methods), this finding is not surprising and was already reported in a systematic review by Notarte et al. [34]. Based on case–control and cohort studies included in this review, the authors suggested that vaccination before SARS-CoV-2 infection could reduce the risk of subsequent long COVID [34]. This finding is quite prevalent, such as, for example, in a recent meta-analysis [29].

Further variables included in the top 20 features identified by our model such as migraine, malaise and fatigue, or back pain include symptoms which can also occur as symptoms of long COVID (headache, back pain, fatigue). These symptoms may worsen after COVID-19 infection and, thus, transition to long COVID. In general, many physical and also mental disorders have been shown to increase risk for long COVID [27,28].

Prescription of cough medication and polypharmacy are two further variables that are included in our top 20 features. Considering the effect of polypharmacy, one could speculate, for example, that it counteracts the negative effect of multimorbidity when therapies have been claimed. However, making a statement is difficult, and an investigation of the time course and composition of the therapies would be necessary to understand the effect. The only single drug class that appears to be predictive and simultaneously protective was cough medication. Patients receiving cough medications might be more prone to the hazard of bronchial diseases. Therefore, those patients might have additional medications for the treatment of bronchial diseases mitigating acute COVID-19 symptoms, which are in turn predictive for long COVID [7]. Further research is needed here as well. All other individual drug groups did not make it onto the list of highly predictive features, unlike the individual diagnostic codes, some of which were represented.

The strengths of this study are the inclusion of more than 270,000 patients, the use of data from clinical practice, and the use of ML methodology. However, the study is also subject to several limitations, which should be acknowledged at this point. First, all diagnoses relied on ICD-10 codes pre-COVID-19 infection only, and no data were available on symptoms of long COVID. Second, long COVID may sometimes have been diagnosed in specialized practices (e.g., pneumology) or hospitals, and some of the related data may have gone undocumented in the Disease Analyzer database, potentially leading to an underestimation of the prevalence of this condition. The prevalence of long COVID observed in our study was much lower than that in published investigations, probably due to the rare use of the ICD-10 codes U09.9 in the first year after the beginning of the pandemic and also due to our exclusion criteria. Third, no medications used for COVID-19 therapy were analyzed, as these are usually given in hospitals and are only administered for severe courses of COVID-19. Fourth, viral variants were not determined individually for patients, but rather assigned based on the predominant variant at the time the patient was first diagnosed with COVID-19. Since we did not have any information about the genome sequence of the virus, the estimation via the time of infection and the inclusion of the epidemiological situation was the most obvious way to estimate the strain. However, an interpretation of the virus strains should be approached with caution and confirmed with actual sequencing studies. Fifth, we trained the model to achieve a high recall, and have compromised on a lower level of precision, therefore allowing for a high proportion of false positives to achieve a very low proportion of false negatives. The focus could have been set differently to achieve a better accuracy of the model. Since the main reason for us was to identify predictive features, we deliberately set a high recognition rate of long COVID patients in our model to correct for the underrepresentation of this diagnosis at the GP. Finally, limitations come with the analysis of real-world data, which are temporally

unstructured and full of missing values compared with studies designed in a matched case–control fashion. Insights, however, highly converged on other studies using matched study designs and different data sources.

5. Conclusions

The present study describes an initial investigation of the prediction of potential features increasing the risk of developing long COVID after COVID-19 infection in primary care practices in Germany using machine learning on the patient history before COVID-19 infection retrieved from electronic medical record data. Importantly, we identified several predictive features for the development of long COVID in patient demographics and their medical histories.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jcm12103511/s1, Table S1: Hyperparameters were optimized in grid search. Asterisks indicate the hyperparameters of the optimal model, which were used for further analysis. Learning rate was set to 0.05. The remaining hyperparameters were set to their default values.

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Informed Consent Statement: The database used includes only anonymized data in compliance with the regulations set forth in the applicable data protection laws. German law allows the use of anonymous electronic medical records for research purposes under certain conditions. According to this legislation, it is not necessary to obtain informed consent from patients or approval from a medical ethics committee for this type of observational study that contains no directly identifiable data. Because patients were only queried as aggregates and no protected health information was available for queries, no Institutional Review Board approval was required for the use of this database or the completion of this study.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflict of interest.

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Characteristics of Chemosensory Perception in Long COVID and COVID Reinfection

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Abstract: Emerging data suggest an increasing prevalence of persistent symptoms in individuals affected by coronavirus disease-19 (COVID-19). The objective of this study was to determine the relative frequency of altered taste and smell in COVID reinfection (multiple COVID positive tests) and long COVID (one COVID positive test). We sent an electronic survey to patients in the Indiana University Health COVID registry with positive COVID test results, querying if they were experiencing symptoms consistent with long COVID including altered chemosensory perceptions. Among the 225 respondents, a greater long COVID burden and COVID reinfection was observed in women. Joint pain was reported as the most common symptom experienced by 18% of individuals in the long COVID cohort. In the COVID reinfection cohort >20% of individuals reported headache, joint pain, and cough. Taste perception worse than pre-COVID was reported by 29% and 42% of individuals in the long COVID and COVID reinfection cohorts, respectively. Smell perception worse than pre-COVID was reported by 37% and 46% of individuals in long COVID and COVID reinfection cohorts, respectively. Further, Chi-square test suggested significant association between pre-COVID severity of taste/smell perception and headache in both cohorts. Our findings highlight the prevalence of persistent chemosensory dysfunction for two years and longer in long COVID and COVID reinfection.

Keywords: long COVID; reinfection; taste dysfunction; smell perception

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1. Introduction

Globally as of April 2023, there have been 762,201,169 confirmed cases of coronavirus disease-2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus-2 (CoV-2) [1]. While mass vaccination has lessened the acute illness and most individuals with COVID return to their baseline state of health, a proportion of individuals exhibit persistent symptoms for extended period [2–4]. Variably referred to as post COVID, post-acute sequelae of SARS-CoV-2 infections (PASC) or long COVID, the condition is defined as symptoms that occur in individuals with a history of probable or confirmed CoV-2 infection that begins within three months of the onset of COVID and lasts at least 2 months and cannot be explained by an alternate diagnosis [5]. Amongst the myriad of symptoms that have been reported in confirmed and suspected cases of long COVID, the most frequent are fatigue, cognitive dysfunction, depression, chemosensory dysfunction, shortness of breath and cough [5–7]. Furthermore, age, sex, pre-infection comorbidities (diabetes, asthma) and severity of acute CoV-2 infection (symptomatic/asymptomatic, hospitalization) are confounding factors that could contribute to the development and/or persistence of heterogeneous post-COVID-19 conditions [2,8,9].

Intense disturbances of taste, smell, and chemesthesis were widely reported in acute COVID-19 infection with a prevalence of over 30% in most studies [10–13]. These chemosensory dysfunctions have been shown to be persistent for several months after primary infection [3,11,14–16]. Further, some individuals developed qualitative taste disturbances such as phantosomia or phantosmia months after primary CoV-2 infection [17,18]. In addition, the symptoms may fluctuate and relapse over time. To our knowledge, few studies have evaluated the prevalence of chemosensory dysfunction in patients with repeat COVID infections. In this study, we report a detailed analysis of population-based, self-reported survey data from hospitalized and non-hospitalized individuals with a history of a CoV-2 positive test. Our objectives were to assess the frequency of altered taste and smell in individuals with single and repeat CoV-2 infections and correlate them with common post-COVID neurological symptoms.

2. Materials and Methods

2.1. Study Design

An electronic survey was sent to individuals aged 18 years and older, who had previously agreed to be notified about COVID-19 studies registered at IU School of Medicine's COVID-19 Research Registry. The questionnaire was designed in consultation with the Indiana Clinical and Translational Sciences Clinical Research Core and included demographics, COVID-19 test results and questions on commonly reported long COVID symptoms. Only individuals who self-identified as testing positive for COVID completed the survey. The survey was divided into two parts: the first part included 9 items on sociodemographic characteristics and the second part consisted of 30 items that measure the following eight dimensions: general health (GH: perception of overall personal health); physical activities (PA: limitations in performing everyday work and other daily activities); emotional/mental health (E/MH: MH, feeling depressed or anxious); specific general health symptoms previously reported in long COVID (GH-LC, headache, muscle pain, chest pain, joint pain, cough); social function (SF: effect on social activities); chemosensory perception (CP: effects on taste and smell sensations); and oral health (OH: oral health). For positive COVID tests, the responders reported the date of initial positive testing and the number of times they tested positive subsequently. Specific questions on physical/emotional health and limitation in activities recorded binary responses as yes/no. Response to symptom specific items were recorded on a five-point scale for long COVID symptoms (all the time/always, most of the time/often, some of the time/sometimes, a little of the time/rarely and none of the time/never). Response to taste- and smell-specific questions were recorded on a four-point scale (same as before, worse than before, better than before and total loss). Study data were collected and managed using REDCap (Research Electronic Data Capture) tools hosted at Indiana clinical and translational sciences institute [19,20].

2.2. Statistical Analysis

Data from surveys completed between August and September 2022 were analyzed in this study. Descriptive statistics were used to express categorical data (frequency and percentage) of long COVID symptoms and changes in chemosensory perceptions. Age was summarized using means and standard deviations. Chi-square test was used to study the association between the parameters of duration, number reporting specific symptoms, and the intensity of the symptoms. Data are provided as mean $\pm$ SD or as Chi-square score and p value.

3. Results

3.1. Participant Characteristics

The survey was distributed to 13,561 volunteers. Two hundred and twenty-five responders self-reported the date and the number of times they had COVID-19 positive test results. Of the 225 responders, 57 were males and 157 were females, and the rest chose not to identify themselves. The mean age was 45.8 years (range: 19–84 years). One hundred

and ninety-four identified as white, 18 as African American, 7 as Asian, 3 as Hispanic and rest chose not to report their race.

One hundred and seventy-two individuals reported a single COVID-19 positive test. The duration since testing positive ranged between 6 and 906 days with a median of 222 days (Table 1A). Only data from individuals testing positive at least 60 days prior to the date of responding to the survey were selected for analysis (Table 1B). This included a total of 127 respondents and constituted the long COVID cohort. There were more females (92) than males (29) in this cohort. This is consistent with the reports of higher preponderance of females being diagnosed with long COVID [21,22]. Eight individuals reported hospitalization due to COVID in this cohort. Amongst these individuals, the duration of persistent symptoms was less than two months in two individuals, one individual each experienced taste perception worse than pre COVID for 4 months and 28 months, respectively, and four did not experience any change post COVID. We excluded all individuals with a history of hospitalization due to COVID in further analyses to minimize confounding factors.

Table 1. (A): Participant characteristics of the long COVID and COVID reinfection cohorts. (B): Frequency distribution.

(A)

	Long COVID (N = 127)	COVID reinfection (N = 47)	*p* value
Age	46.7 ± 17.8 yrs	45.8 ± 13.9 yrs	0.37
Sex	29 M:92 F *	11 M:36 F	
Mean duration	352.4 ± 250 days	488 ± 228.5 days	0.0007
Vaccination: % N (V + booster)	2% (1 + 0)	6% (1 + 0)	0.54
	19% (1 + 1)	15% (1 + 1)	
	57% (1 + 2)	55% (1 + 2)	
	13% (1 + 3)	13% (1 + 3)	
	6% (1 + 4)	11% (1 + 4)	
	2% (1 + 5)	0% (1 + 5)	

(B)

	Number of individuals	
Duration (days)	Long COVID	COVID reinfection
60–119	25	4
120–179	9	1
180–239	31	5
240–299	13	6
300–359	7	1
360–419	2	0
420–479	0	0
480–539	0	4
540–599	8	4
600–659	9	8
660–719	10	9
720–779	4	3
780–839	2	0
840–899	6	2
900–959	1	4
Total	127	47
History of hospitalization	6	4

* no response was provided in six surveys.

Fifty-two individuals self-reported testing positive for COVID-19 two times or more. Of these, 78.8% (41) tested positive twice, 17.3% (9) tested positive three times, and two individuals (3.8%) reported four positive COVID-19 tests (Table 1A). The duration since the first positive COVID-19 test ranged between 21 and 875 days with a median of 556 days. Responses from five individuals with two positive COVID-19 tests and duration of symptoms less than 60 days were not included for further analysis. The remaining cohort, with

47 (11 males and 36 females) individuals with the minimum duration of 101 days or three months, constituted the COVID reinfection cohort (Table 1B). Four individuals reported a history of hospitalization due to COVID in this cohort and data from these individuals were excluded in further analysis to minimize confounding factors.

All individuals in this study were vaccinated, with 57% in the long COVID cohort and 55% in the COVID reinfection cohort receiving two boosters. In the COVID reinfection cohort, 11% received four boosters and in the long COVID cohort, 2% received five boosters (Table 1).

3.2. Incidence of Long COVID Symptomatology

Common symptoms that comprise the post-COVID conditions include tiredness or fatigue that interferes with daily life activities that get worse after physical or mental effort (also known as "post-exertional malaise"), joint pain, muscle pain, respiratory symptoms such as cough and chest pain, neurological symptoms such as headache, depression, anxiety and changes in taste or smell [2,3,5,23]. In our long COVID cohort, joint pain was reported as the symptom experienced most often or always by 19% of individuals, and sometimes by 16%. The symptoms of headache, cough, and muscle pain were reported to be experienced sometimes by 16–21% and often by 7–9% of individuals (Figure 1A). Further, joint pain, muscle pain, and headache were experienced at least sometimes for longer than one year in 10% of individuals with long COVID.

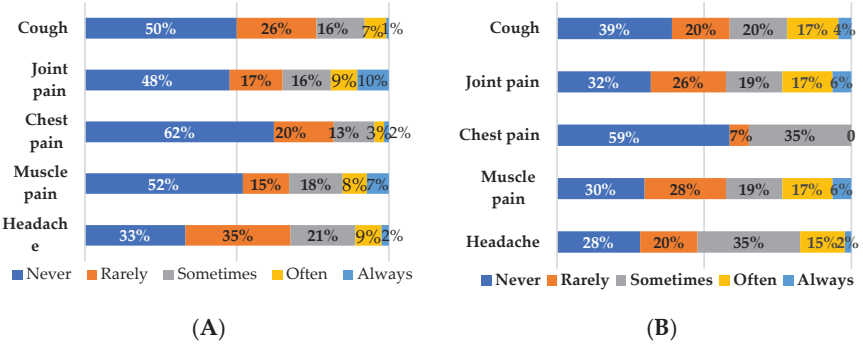

Figure 1. Proportion of individuals reporting the indicated symptom in the (**A**) long COVID and (**B**) COVID reinfection cohorts.

In the COVID reinfection cohort the symptoms of joint pain, muscle pain, cough, and headache were reported to be experienced often or always by 23%, 23%, 21% and 17% of individuals, respectively. The frequency of symptoms that was experienced sometimes was higher for chest pain and headache (35%), followed by cough (20%), joint pain (19%), and muscle pain (19%) (Figure 1B). In this cohort, headache, joint pain, cough and muscle pain were reported to be present often and always by 8%, 11%, 22% and 15% for two years. However, it is relevant to note here that since all individuals were responding to a survey question on the experience of these symptoms post-positive COVID-19 test, it is not known whether they are reporting symptoms being experienced after the first infection that persisted and/or increased, or symptoms that began after subsequent re-infections.

3.3. Chemosensory Symptoms in Long COVID and COVID Reinfection

3.3.1. Changes in Taste Perception

In our long COVID cohort, 29% (35/121) reported worse taste perception (Figure 2A) with duration ranging between two and thirty months. Equivalent number of individuals reported experiencing worse taste perception for >60 days and <12 months and for periods >12 months. Further, one individual experienced total loss and three reported better taste perception than pre-COVID. The Chi-square test for the groups of no change in taste

perception and taste perception worse than pre COVID across the durations of 2–6 months, >6–12 months, >12–24 months and >24 months was 13.72, $p < 0.003$ (Figure 2B, Table 2A).

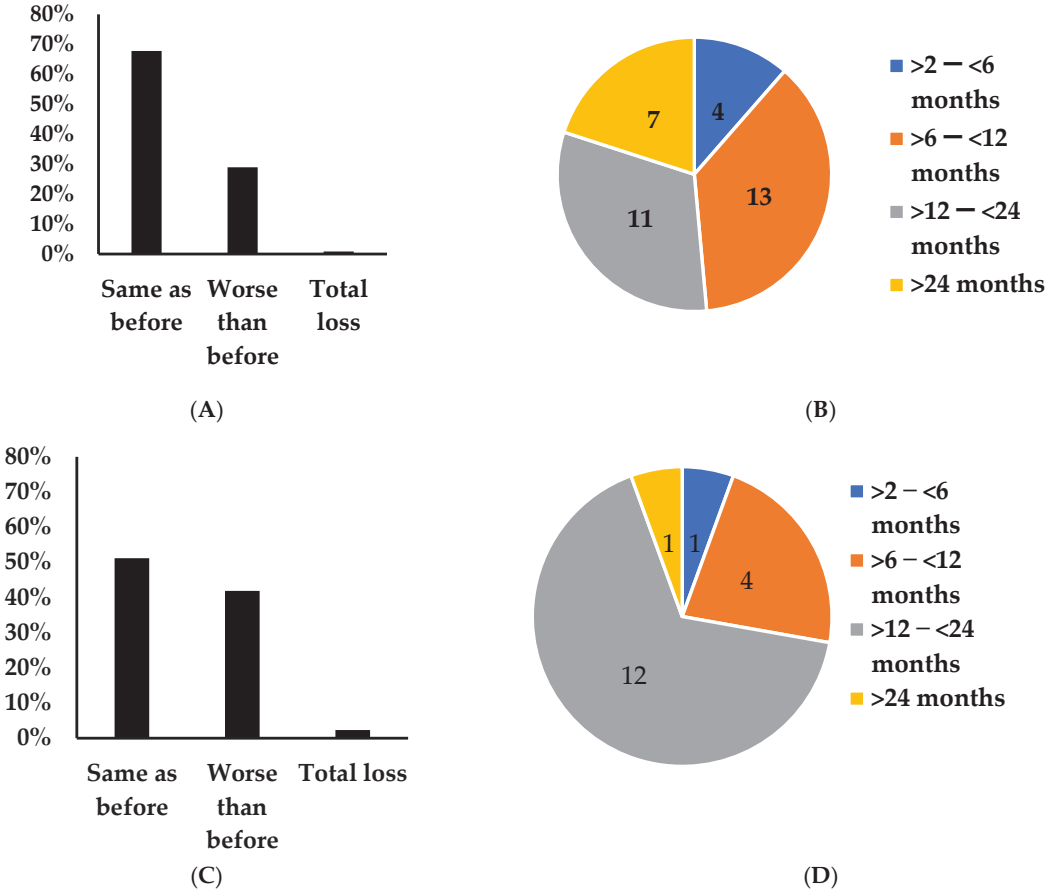

Figure 2. Characteristics of changes in taste perception. Distribution of altered taste among individuals in the long COVID (**A**) and COVID reinfection (**C**) cohorts. Distribution of duration of taste perception worse than pre COVID in the long COVID (**B**) and COVID reinfection (**D**) cohorts.

In the COVID reinfection cohort, 42% (18/43) reported worse taste perception than pre COVID with duration ranging between 79 and 875 days (Figure 2C). Further, two individuals reported total loss and one reported experiencing better taste perception than pre COVID. Of the 18 individuals with taste perception worse than pre COVID, one reported being infected by SARS-CoV-2 four times, three individuals were infected thrice, and 13 were infected twice by the COVID-19 virus. The Chi-square test for the groups of no change in taste perception and taste perception worse than pre COVID across the durations of 2–6 months, >6 to 12 months, >12–24 months and >24 months was 3.2, $p < 0.36$ (Figure 2D, Table 2A).

Table 2. (A) Association between duration and taste perception in long COVID and COVID reinfection. (B) Association between duration and smell perception in long COVID and COVID reinfection.

(A)				
Taste	Long COVID		COVID Reinfection	
Duration	No Changes (82)	Worse [@] (35)	No changes (23)	Worse [@] (18)
2 to 6 months	27 (23%)	4 (3%)	3 (7%)	1 (2%)
>6 to 12 months	36 (31%)	13 (11%)	8 (20%)	4 (10%)
>12 to 24 months	16 (14%)	11 (9%)	9 (22%)	12 (29%)
>24 months	3 (3%)	7 (6%)	3 (7%)	1 (2%)
Chi-square	13.72		3.2	
p value	0.003		0.36	
(B)				
Smell	Long COVID		COVID reinfection	
	No changes (71)	Worse [@] (45)	No changes (22)	Worse [@] (19)
2 to 6 months	25 (22%)	6 (5%)	4 (7%)	3 (7%)
>6 to 12 months	30 (26%)	18 (16%)	3 (10%)	3 (7%)
>12 to 24 months	13 (11%)	14 (12%)	11 (27%)	13 (32%)
>24 months	3 (3%)	7 (6%)	4 (10%)	0
Chi-square	11.1		4.1	
p value	0.012		0.25	

[@] = worse than pre COVID. The absolute number for the indicated response is given with the percentage with respect to the total number in the cohort in parenthesis.

3.3.2. Changes in Smell Perception

In the long COVID cohort, 37% (45/121) reported worse smell perception worse than pre COVID for durations between two and thirty months (Figure 3A). Further, one individual experienced total loss and three reported better smell perception than pre COVID. With respect to the frequency and duration, the Chi-square test for the groups of no change in smell sensation and perception of smell worse than pre COVID across the durations of 2–6 months, >6 to12 months, >12–24 months and >24 months was 11.1, $p < 0.012$ (Figure 3B, Table 2B).

In the COVID reinfection cohort, 46% (19/43) reported worse smell perception than pre COVID for durations ranging between 79 days and 875 days (Figure 3C). Further, two individuals reported better smell perception than pre COVID. The Chi-square test for the groups of no change in smell and smell perception worse than pre COVID across the durations of 2–6 months, >6–12 months, >12–24 months and >24 months was 4.1, $p < 0.25$ (Figure 3D, Table 2B).

3.3.3. Correlation between Vaccination and Smell/Taste Perception in Long COVID and COVID Reinfection

In both the long COVID and the COVID reinfection cohorts, a higher percentage of individuals that received two boosters experienced worse taste (14% and 22%, respectively) or smell perception (19% and 22%, respectively) (Figure 4A,B). Interestingly, the number of individuals reporting chemosensory dysfunction decreased precipitously with increasing number of boosters, with 4% and 2% experiencing altered smell in the long COVID and COVID reinfection cohorts, respectively. However, this is attributed to the higher number of individuals ($\geq$55%) (Table 1) receiving two boosters, as opposed to 20% receiving three or four boosters in our study cohort. The association between the number of boosters and the duration of worse taste/smell perception was not significant with Chi-square values of 12.8 and 9 respectively, $p < 0.3$.

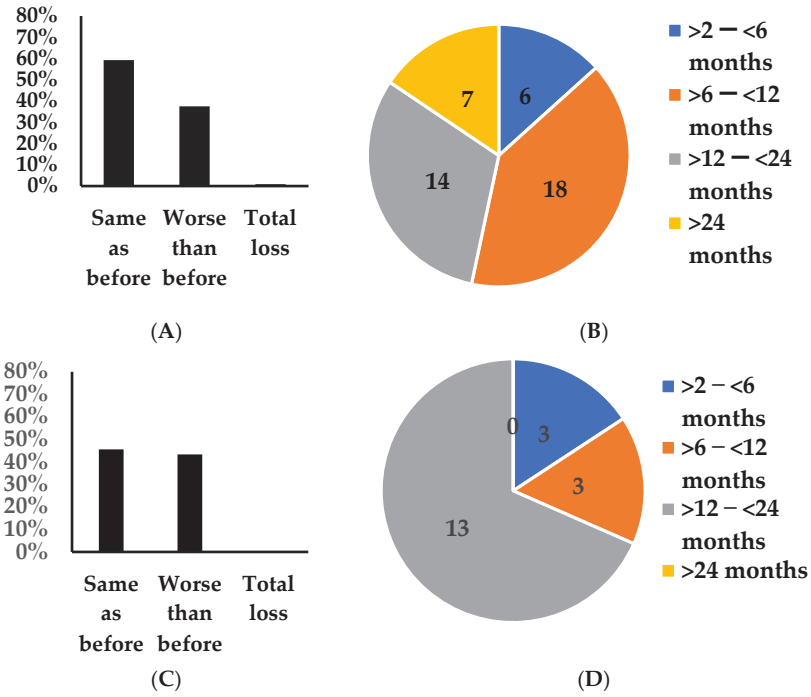

Figure 3. Characteristics of changes in smell perception. (**A**) Distribution of altered smell among individuals in the long COVID (**A**) and COVID reinfection (**C**) cohorts. Distribution of duration of taste perception worse than pre COVID in the long COVID (**B**) and COVID reinfection (**D**) cohorts.

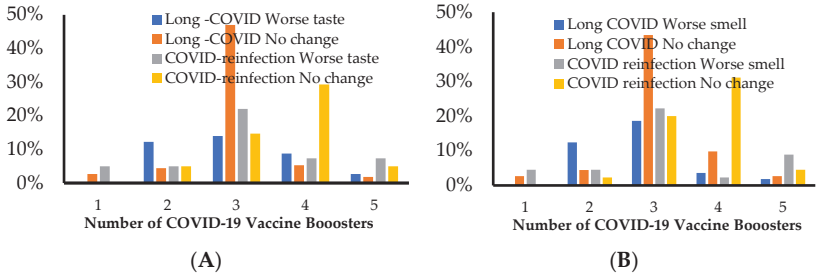

Figure 4. Correlation of vaccinations with taste and smell changes in long COVID and COVID reinfection. Shows distribution of number of individuals (as percent of the total cohort) experiencing altered taste (**A**) or smell (**B**) with respect to the number of COVID-19 vaccine boosters received.

3.3.4. Correlation between Taste and Smell Perception and with Other Neurological Symptoms

In both the long COVID and COVID reinfection cohorts, a greater number of individuals reported experiencing both taste and smell perception worse than pre COVID than either taste or smell dysfunction alone as is evident by the r value of 0.7 (Figure 5A–D). The Chi-square test statistic for comparison of long COVID and COVID reinfection groups for persistent taste dysfunction post COVID across the three discrete time periods of less than one year, one to two years, and over two years was 7.5, $p < 0.024$. The Chi-square test statistic for the comparison of long COVID and COVID reinfection groups for persistent smell dysfunction across the three discrete time periods of less than one year, one to two years, and over two years was 8.7, $p < 0.045$ (Table 3).

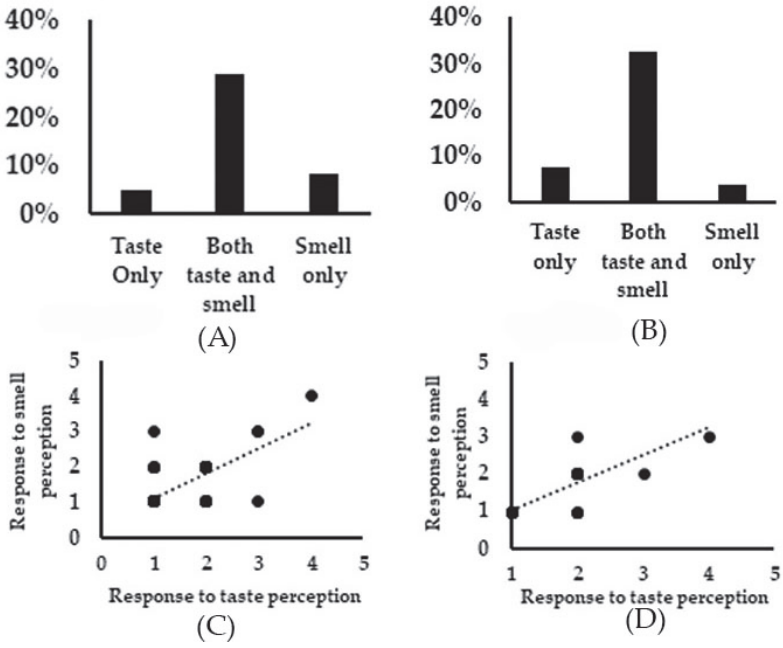

Figure 5. Correlation of taste and smell changes. (**A**) A higher percentage of individuals reported both taste and smell perception worse than pre COVID in both the long COVID (**A**) and COVID reinfection (**B**) cohorts. (**C**) Pearson correlation (r) between taste and smell worse than pre COVID in long COVID (**C**) and COVID reinfection (**D**) cohorts.

Table 3. Comparison of dysgeusia and dysosmia across the specified duration between long COVID and COVID reinfection cohorts.

Worse than Pre COVID		<12 Months	12–24 Months	>24 Months	Chi-Square	*p*-Value
Taste	Long COVID (35)	17 (49%)	11 (31%)	7 (20%)	6.23	0.043
	COVID reinfection (18)	5 (28%)	12 (67%)	1 (6%)		
Smell	Long COVID (45)	24 (53%)	14 (31%)	7 (16%)	17.56	0.0002
	COVID reinfection (19)	6 (32%)	13 (68%)	0		

In the long COVID cohort, the Chi-square test statistic for the relation between pre COVID severity of taste perception (no change, worse, total loss) and headache (never and rarely; sometimes and often and always) was 17.2, $p < 0.0002$. The Chi-square value for similar comparison in the COVID reinfection cohort was 9.3, $p < 0.01$. The Chi-square test statistic for the relation between pre-COVID severity of taste perception and joint pain 28.4, $p < 0.7 \times 10^{-7}$ and 15.5, $p < 4.40 \times 10^{-4}$ in the long COVID and COVID reinfection cohorts, respectively. With respect to smell perception, in the long COVID cohort, the Chi-square test statistic for relation between pre-COVID severity and headache was 18.7, $p < 4.40 \times 10^{-4}$. The Chi-square test value for severity of changes in smell perception and joint pain with respect to pre-COVID levels was 30.9, $p < 2.20 \times 10^{-6}$ and 15.5, $p < 0.0004$ in the long COVID and COVID reinfection cohorts, respectively (Table 4).

Table 4. Association between long COVID or COVID reinfection with headache or joint pain.

		Change from Pre COVID			Chi-Square	*p*-Value
Long COVID	Headache	78 (69%) (Never and rarely)	27 (20%) Sometimes	17 (10%) Often and always	17.16	0.0002
	Taste	81 (66%) (No change)	41 (33%) Worse	1 (1%) Total loss		
COVID reinfection	Headache	23 (50%)	18 (39%)	11 (24%)	9.25	0.0098
	Taste	27 (59%)	23 (50%)	1 (2%)		
Long COVID	Joint pain	82 (65%)	20 (15%)	24(19%)	28.36	0.0000007
	Taste	81 (66%)	41 (33%)	1 (1%)		
COVID reinfection	Joint pain	29 (63%)	10 (22%)	13 (28%)	15.5	0.00044
	Taste	27 (59%)	23 (50%)	1 (2%)		
Long COVID	Headache	78 (69%)	27 (20%)	17 (10%)	18.71	0.000087
	Smell	78 (63%)	45 (34%)	1 (1%)		
COVID reinfection	Headache	23 (50%)	18 (39%)	11 (24%)	10.04	0.0066
	Smell	25 (54%)	19 (41%)	0		
Long COVID	Joint pain	82 (65%)	20 (15%)	24 (19%)	30.86	0.0000002
	Smell	78 (63%)	45 (34%)	1 (1%)		
COVID reinfection	Joint pain	29 (63%)	10 (22%)	13 (28%)	15.5	0.00044
	Smell	25 (54%)	19 (41%)	0		

4. Discussion

In this single survey study, we assessed non-hospitalized individuals with long COVID (one positive COVID test) and with COVID reinfection (two or more positive COVID tests) for persistent symptoms and chemosensory dysfunction. Our data show that 29% and 42% reported worse taste and 37% and 46% reported worse smell perception than pre COVID in the long COVID and COVID reinfection cohorts, respectively. This is consistent with the previous reports of persistent dysgeusia and anosmia in long COVID studies [6,24].

Viral infections such as influenza have been associated with altered smell and taste [25–27]. Typically, in respiratory infections including the previous coronavirus infections, smell disturbances occur due to localized impediment to airflow conduction by excessive mucus and/or to the swelling of the respiratory mucosa [28]. In contrast, in a study comparing gustatory functions in patients affected by COVID-19 and/or the common cold, it was observed that the sweet and bitter taste scores were significantly worse in COVID-19 patients without nasal congestion or discharge [29]. This suggests that the taste disturbances reported by COVID patients may reflect actual impairment of gustatory abilities, rather than olfactory dysfunction. Other unique features of the altered taste and smell in the COVID-19 pandemic are their higher incidence, persistence, and occurrence of specific taste dysfunction without smell loss [11,15,30,31]. Consistently, we observed that in the long COVID cohort, while 29% reported both taste and smell dysfunction post COVID, 5% of individuals reported gustatory dysfunction alone. In the COVID reinfection cohort 8% reported persistent worse taste perception post COVID but no change in smell perception.

A recent observational study showed that repeat CoV-2 infections increases risk for cardiac, pulmonary, or neurological problems [32]. We observed that in our long COVID and COVID reinfection cohorts, at least 20% reported persistent (often/always) experience of at least two common long COVID symptoms. To our knowledge, this is the first study reporting on the altered taste and smell perception amongst individuals with COVID re-infection. We observed that while >30% experienced persistent dysgeusia, 18% reported persistent dysosmia in the long COVID cohort. However, in the COVID reinfection group, an equivalent number of individuals reported experiencing persistent dysgeusia and olfactory dysfunction for longer than one year.

The available literature suggests that the severity of smell and taste alterations is reduced in CoV-2-infected individuals during the periods of Omicron variant dominance [33,34]. However, in our study cohort, the experience of altered (worse than pre

COVID) taste and smell perception does not seem to be related to the frequency of infection or the number of boosters received. This could be attributed to the difference in the global prevalence of different variants and that the positive COVID-19 test in our cohort was not-restricted to one variant. Interestingly, Notarte et al., in a systematic review, show that there is a low level of evidence to suggest that vaccination before SARS-CoV-2 infection could reduce the risk of developing subsequent long COVID [35].

The interest in elucidating the pathogenesis of altered taste and smell has escalated since COVID-19, with several hypothesis projected including viral infection-induced changes in tongue biofilm, local inflammatory responses, and neurological disturbances [30,36]. Interestingly, we observed that in both the long COVID and COVID reinfection cohorts, the association between persistent taste/smell dysfunction and headache was significant, thus suggesting a role for neurological mechanism.

Limitations: While our study provides significant new knowledge with respect to COVID reinfection and chemosensory dysfunction, multiple limitations are recognized. 1) Our data does not specify the time period between the COVID-19 positive tests in the reinfection cohort, and hence could potentially include repeat positive tests in relation to the first infection. However, since we analyzed only data from responders reporting symptoms that lasted longer than two months, and >60% of individuals experienced symptoms for longer than one year and 10% for longer than two years, it is likely that the responses are symptoms that persisted after first positive COVID-19 test in the COVID re-infection cohort. A second limitation is that the retrospective questions about pre-COVID health status may be biased by the current health status. While we report age, sex, and race distribution, we did not perform symptom analyses by subgroup. Further, while the relative prevalence of altered chemosensation in our study is concomitant with previous studies [10,16,17], comorbidities such as diabetes that existed pre COVID or newly developed post COVID could have potentially contributed to the altered chemosensation in both long COVID and COVID reinfection cohorts [2,22]. The objectives of our study were to examine the relative frequency of altered taste and smell associated with COVID reinfection compared with that of a single infection. The results do not represent an assessment of severity of a second infection versus that of a first infection.

5. Conclusions

In summary, our data are consistent with the observations that the long COVID characterized by persistent multi-organ symptoms affects a proportion of COVID-19 infected individuals despite vaccination. Repeat infections are more likely associated with altered chemosensory perception for extended periods. Since chemosensory dysfunction has been strongly associated with neurological pathologies, depression, and inadequate quality of life, protective measures to prevent reinfection with SARS-CoV-2 are warranted. Future longitudinal follow-up studies or in-depth electronic health data mining studies are needed to better elucidate these relationships.

Author Contributions: Conceptualization: M.S. and T.P.T.; methodology, M.J. and G.E.; software, T.P.T.; validation, M.S., T.P.T. and G.E.; formal analysis, G.E.; investigation, M.S. and T.P.T.; resources, M.S.; data curation, M.J., M.S. and G.E.; writing—original draft preparation, M.S. and M.J.; writing—review and editing, M.S., T.P.T. and G.E.; supervision, M.S.; project administration, M.S.; funding acquisition, M.S. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of Indiana University–Purdue University at Indianapolis (protocol code 15239, approved 25 May 2022) for the electronic survey study.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study as part of the survey in accordance with the IRB approval.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to individual privacy and ethical reasons.

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Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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Neurological Manifestations and Complications of the Central Nervous System as Risk Factors and Predictors of Mortality in Patients Hospitalized with COVID-19: A Cohort Study

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Abstract: The aim of this study was to analyze the risk factors and predictors of mortality in a retrospective cohort of patients with coronavirus disease (COVID-19) who presented central nervous system (CNS) manifestations and complications when admitted to hospital. Patients hospitalized from 2020 to 2022 were selected. Demographic variables; history of neurological, cardiological and pulmonary manifestations; comorbidities; prognostic severity scales; and laboratory tests were included. Univariate and adjusted analyses were performed to determine risk factors and predictors of mortality. A forest plot diagram was used to show the strength of the associated risk factors. The cohort included 991 patients; at admission, 463 patients presented CNS damage and of these, 96 hospitalized patients presented de novo CNS manifestations and complications. We estimate a general mortality of 43.7% (433/991) and 77.1% (74/96), for hospitalized patients with de novo CNS manifestations and complications, respectively. The following were identified as risks for the development of hospital CNS manifestations and complications when in hospital: an age of $\geq$64 years, a history of neurological disease, de novo deep vein thrombosis, D-dimer $\geq$ 1000 ng/dL, a SOFA $\geq$ 5, and a CORADS 6. In a multivariable analysis, the mortality predictors were an age of $\geq$64 years, a SOFA $\geq$ 5, D-dimer $\geq$ 1000 ng/mL and hospital CNS manifestations and complications when admitted to hospital. Old age, being hospitalized in critical condition, and having CNS manifestations and complications in hospital are predictors of mortality in hospitalized patients with COVID-19.

Keywords: central nervous system; COVID-19; risk factors; predictors of mortality

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1. Introduction

At the end of 2019, an emerging viral infectious pathology called COVID-19 emerged, caused by the severe acute respiratory syndrome virus 2 (SARS-CoV-2) [1]. Globally, as of 15 January 2023, a total of 671,314,125 confirmed cases of COVID-19 and 6,730,278 deaths have been reported, with a recovery rate of about 96%. In Mexico, as of that same date, a total of 7,309,154 cases and 331,510 deaths have been reported, with a recovery rate of 89% (ranking 19th) (https://www.worldometers.info/coronavirus/, accesed on 16 October 2022) [2].

It has been reported that up to 80% of patients hospitalized with COVID-19 present some neurological manifestation; moreover, manifestations and complications specifically

of the CNS (mainly strokes) have been observed as a predictive factor for mortality [3]. Battaglini et al. [4] in particular cases, reported a mortality of up to 92% (stroke and ischemic-hypoxic brain damage).

SARS-CoV-2 has been shown to enter directly through the olfactory nerves where the axons connect to different regions of the CNS [5]. In hematological dissemination, the virus reaches the brain and infects the endothelial cells of the blood–brain barrier (BBB) and of the cerebrospinal fluid in the choroid plexus. The three mechanisms for dissemination through the BBB are: (1) the Trojan horse pattern, where the virus uses leukocytes and myeloid cells to enter the CNS; (2) paracellular migration, where the virus crosses the BBB by destroying the Trojan horse complex; and (3) in the vascular endothelium of the BBB, where SARS-CoV-2 binds to the human receptor for angiotensin-converting enzyme 2 (ACE-2). In addition to the mechanisms of invasion of the CNS, there are other neuropathogenic mechanisms such as hypoxic brain injury and immunological injury mediated by a cytokine storm. The latter leads to rupture of the BBB, manifesting in elevations in interleukin 6 (IL-6), D-dimer, C-reactive protein (CRP), and lymphopenia inflammatory markers [3,6,7].

The main hospital neurological manifestations of the CNS in patients with COVID-19 that have been reported are delirium, hallucinations, drowsiness, stupor, dysarthria, aphasia, hemiparesis, hemiplegia, anisocoria, and seizures, and complications such as ischemic stroke, hemorrhagic stroke, subarachnoid hemorrhage, encephalitis, epilepsy NOVO, ataxia, and others have been reported [4,8–10]. A series of studies have been published establishing associations between neurological manifestations and complications of the CNS and mortality [1,4,8]. In our study, we had the opportunity to perform an adjusted odds ratio analysis to determine predictors of mortality in our cohort of COVID-19 patients during hospital evolution, which allowed us to emphasize the role of neurological manifestations and complications of the CNS de novo. Therefore, the objective of this study was to analyze the risk factors and predictors of mortality in a retrospective cohort of patients with COVID-19 who presented CNS manifestations and complications when admitted to hospital.

2. Materials and Methods

2.1. Participants and Procedures

This study was performed on a retrospective cohort and it was carried out in a medical and surgical specialty hospital in western Mexico. Of 1977 hospitalized patients (April 2020 to March 2022) with a confirmed diagnosis of COVID-19 by PCR, 991 complete medical records were reviewed. Of the 463 patients who presented with CNS neurological damage (any neurological signs or symptoms) during admission and/or during hospitalization, 96 hospitalized patients with de novo (recently presented or newly diagnosed) CNS manifestations and complications (neurological cohort) were selected for further analysis (Figure 1). The study cohort includes patients $\geq$18 years of age and of both genders.

The study variables included were demographic data, comorbidities, previous history of neurological manifestations, cardiopulmonary manifestations, history of pulmonary manifestations, and the time of onset of general and respiratory symptoms.

On admission, the patients were assessed in the emergency department and/or in the hospital area through the protocol established in the hospital that includes a SOFA ≥ 5 (Sepsis Related Organ Failure Assessment), a NEWS2 (National Early Warning Score), a CORADS 6, and also, severe acute respiratory distress syndrome (ARDS) ($PaO_2/FiO_2 \leq 100$ mmHg) and laboratory test results showing D-dimer $\geq$ 1000 ng/mL, troponin, B-type natriuretic peptide (BNP), thrombocytopenia $\leq$ 150 cells ($10^3/\mu L$) and lymphopenia $\leq$ 900 cells ($10^3/\mu L$) (including cerebrospinal fluid). Assessment was supported with images such as computed tomography and/or magnetic resonance images with and without gadolinium of the skull and/or electroencephalograms.

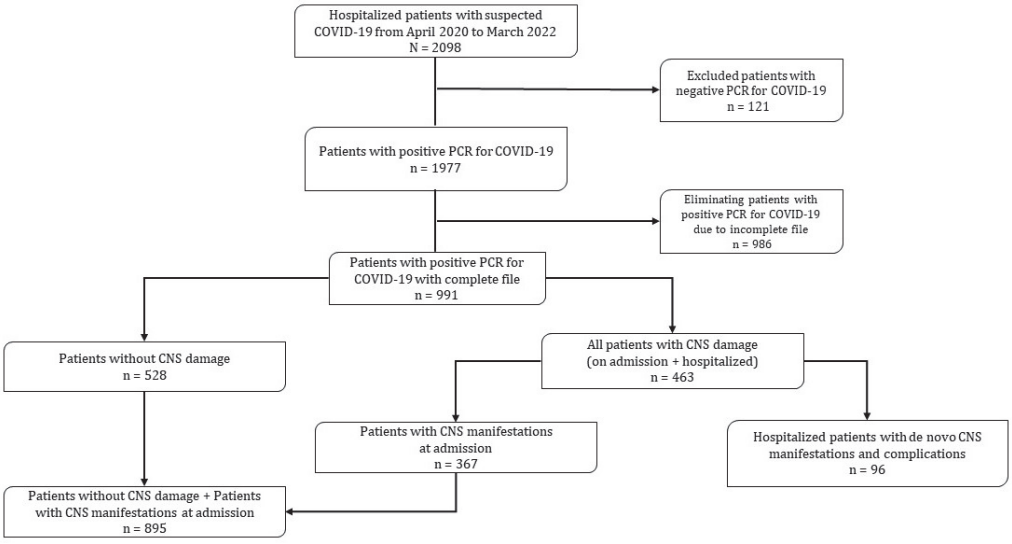

Figure 1. Flow diagram. CNS, central nervous system.

A neurological diagnosis of patients admitted to hospital (with CNS manifestations and complications of CNS, neurological cohort) was determined by neurology and neurosurgery specialists.

The following were considered neurological manifestations of the CNS:

- At admission: headache and vomiting.
- At hospital: delirium, hallucinations, altered state of consciousness (drowsiness and stupor), language disorders (dysarthria and aphasia), motor deficits (hemiparesis, hemiplegia and quadriparesis), anisocoria, and seizures.

Likewise, the following neurological complications of the CNS were considered:

- At hospital: ischemic stroke (left and right middle cerebral artery; right and left carotid artery), hemorrhagic stroke, subarachnoid hemorrhage, encephalitis, de novo epilepsy, and ataxia (uncoordinated movements).

The following were considered neurological manifestations of the PNS: myalgias, anosmia, and dysgeusia (disturbances of taste and smell) at admission; and cranial neuropathy, Guillain Barré syndrome and myopathy at hospitalization. Other extraneurological complications were also analyzed, such as hematological and cardiovascular complications (pulmonary thromboembolism, disseminated intravenous coagulation, deep venous thrombosis, de novo congestive heart failure and acute myocardial infarction, among others).

Patients were monitored from admission to discharge or outcome (improvement or death).

2.2. Statistical Analysis

Quantitative variables were presented through medians and interquartile ranges (IQR) and nominal variables in percentage. To compare the differences between the variables (including risk factors and mortality predictors), a Mann–Whitney U test, an X2 test, or a Fisher's exact test were used. Univariate analysis was performed between the variables included in the cohort of patients with and without de novo CNS neurological manifestations and/or complications Univariate analyses and multivariate logistic regression analyses were performed to estimate adjusted odds ratios and determine risk factors and predictors of mortality, respectively. A p-value of <0.05 (two tails) was considered statistically significant. An adjusted odds ratio model was designed with the variables that were statistically significant in the univariate analysis of mortality. Additionally, a forest plot diagram was

used to show the risk factors associated with the presentation of neurological manifestations and complications in the CNS observed in hospitalized patients with COVID-19. SPSS V25® (IBM, Armonk, NY, USA) and RevMan version 5.3 software were used for statistical analyses.

3. Results

Of the 991 complete records reviewed, we found 614 male patients (62.0%) and 377 females (38.0%) with a median age of 62 years (IQR 50–71). Of these, a total of 96 hospitalized patients with de novo CNS manifestations and complications were found; 57 patients (59.4%) were men and 39 (40.6%) were women, with an age range of 19–91 years of age and a median age of 70 (IQR 62–77).

Table 1 describes the frequency of presentation of the neurological study variables (CNS and PNS manifestations and complications) during admission and the evolution of the hospitalized patients included in the COVID-19 cohort.

Table 1. Frequency of neurological manifestations and complications at admission and while in hospital in the cohort of COVID-19 patients.

Study Variables	All Patients (n = 991)	Without Neurological Complications in the CNS n = 895 (90.3%)	Neurological Complications in the CNS n = 96 (9.7%)
Neurological manifestations in the central nervous system (CNS) on admission			
Headache, n (%)	392 (39.6)	350 (39.1)	42 (43.8)
Vomiting, n (%)	31/641 (4.87)	25/553 (4.5)	6/88 (6.8)
Neurological manifestations in the central nervous system (CNS) while in hospital			
Delirium, n (%)	63 (6.4)	-	63 (65.6)
Hallucinations, n (%)	4 (0.4)	-	4 (4.2)
Drowsiness, n (%)	32/990 (3.2)	-	32 (33.3)
Stupor, n (%)	21 (2.1)	-	21 (21.9)
Dysarthria, n (%)	5 (0.5)	-	5 (5.2)
Aphasia, n (%)	5 (0.5)	-	5 (5.2)
Hemiparesis, n (%)	9 (0.9)	-	9 (9.4)
Hemiplegia, n (%)	4 (0.4)	-	4 (4.2)
Quadriparesis, n (%)	3 (0.3)		3 (3.1)
Anisocoria, n (%)	4 (0.4)	-	4 (4.2)
Seizures, n (%)	9/990 (0.9)	-	9 (9.4)
Hospital neurological complications in the central nervous system (CNS) while in hospital			
Stroke, n (%)	19 (1.9)	-	19 (19.8)
Ischemic stroke, n (%)	14 (1.4)	-	14 (14.6)
Ischemic in the left middle cerebral artery, n (%)	7 (0.7)	-	7 (7.3)
Ischemic in right middle cerebral artery, n (%)	5 (0.5)	-	5 (5.2)
Left carotid ischemic stroke, n (%)	1 (0.1)	-	1 (1.0)
Right carotid ischemic stroke, n (%)	1 (0.1)	-	1 (1.0)
Hemorrhagic stroke, n (%)	5 (0.5)	-	5 (5.2)
Subarachnoid hemorrhage, n (%)	4 (0.4)	-	4 (4.2)
Encephalitis, n (%)	3 (0.3)	-	3 (3.1)
Epilepsy NOVO, n (%)	9 (0.9)	-	9 (9.4)
Ataxia, n (%)	7 (0.7)	-	7 (7.3)
Neurological manifestations in the peripheral nervous system (PNS) on admission			
Myalgias, n (%)	185 (18.7)	168 (18.8)	17 (17.7)
Anosmia, n (%)	82 (8.3)	75 (8.4)	7 (7.3)
Dysgeusia, n (%)	94 (9.5)	86 (9.6)	8 (8.3)
Hospital neurological complications in the peripheral nervous system (PNS) while in hospital			
Cranial neuropathy, n (%)	7 (0.7)	7 (7.3)	-
Guillain Barré syndrome de novo, n (%)	1 (0.10)	1 (0.11)	-
Myopathy, n (%)	7 (0.7)	5 (0.6)	2 (2.1)

At admission, headache was observed as the main neurological manifestation, and de novo CNS manifestations observed in hospitalized patients were delirium, drowsiness, and stupor. Among the de novo CNS complications, stroke and ischemic stroke were

most frequently observed. The most frequent neurological manifestations in the peripheral nervous system (PNS) on admission were myalgia, anosmia, and dysgeusia. The main neurological complications were cranial neuropathy, de novo Guillain Barré syndrome and myopathy. Twenty patients had concomitant manifestations or complications of the central and peripheral nervous system (stroke and dysgeusia, delirium and dysgeusia) (Table 1).

At admission, among the comorbidities presented by the patients that made up the study cohort, we found the following in descending order of frequency: arterial hypertension (549 patients, 55.4%), diabetes mellitus (412, 41.6%), obesity (396/814, 48.6%), smoking (221/982, 25.5%), chronic kidney disease (100, 10.1%), history of any pulmonary disease (76, 7.7%), previous coronary artery disease (55, 5.5%), chronic obstructive pulmonary disease (COPD) (50, 5.0%), malignancy (30, 3.0%), and any neurological disease (30, 3.0%). The following comorbidities were present in less than 3.0% of patients: history of heart failure, steroid use, asthma, history of previous stroke, chronic liver disease, kidney transplant, previous peripheral vascular event, history of epilepsy, infection by human immunodeficiency virus (HIV), and multiple sclerosis (Table 2).

Table 2. Demographic and laboratory characteristics at admission in COVID-19 patients with hospital CNS manifestations and complications while in hospital.

Demographic Characteristics and Risk Factors	All Patients n = 991	Without De Novo CNS Disorders n = 895 (90.3%)	With De Novo CNS Disorders n = 96 (9.7%)	OR	CI 95%	*p*
Age years, median (IQR) (range)	62 (50–71)	60 (49–70), (20–95)	70 (62–76.8), (19–91)			0.000
≥64 years, n (%)	453 (45.7)	389 (43.5)	64 (66.7)	2.60	1.67–4.06	0.000
Male gender, n (%)	614 (62.0)	557 (62.2)	57 (59.4)			0.583
Unvaccinated patients, n (%)	752/771 (97.5)	679/695 (97.7)	73/76 (96.1)			0.422
Comorbidities						
Hypertension, n (%)	549 (55.4)	482 (53.9)	67 (69.8)	1.98	1.26–3.12	0.003
Diabetes mellitus, n (%)	412 (41.6)	360 (40.2)	52 (54.2)	1.76	1.15–2.68	0.008
Obesity, n (%)	396/814 (48.6)	366/735 (49.8)	30/79 (38.0)			-
Smoking, n (%)	221/982 (22.5)	195/886 (22.0)	26/96 (27.1)			0.258
Chronic kidney disease, n (%)	100 (10.1)	86 (9.6)	14 (14.6)			0.124
Any pulmonary history, n (%)	76 (7.7)	65 (7.3)	11 (11.5)			0.142
Chronic obstructive pulmonary disease, n (%)	50 (5.0)	41 (4.6)	9 (9.4)	2.15	1.01–4.58	0.041
Asthma, n (%)	26 (2.6)	24 (2.7)	2 (2.1)			1.000
History of coronary disease, n (%)	55 (5.5)	51 (5.7)	4 (4.2)			0.813
History of heart failure, n (%)	28 (2.8)	21 (2.3)	7 (7.3)	3.27	1.35–7.91	0.005
History of peripheral vascular event, n (%)	15 (1.5)	12 (1.3)	3 (3.1)			0.171
Malignancy, n (%)	30 (3.0)	25 (2.8)	5 (5.2)			0.202
Any neurological history, n (%)	30 (3.0)	20 (2.2)	10 (10.4)	5.09	2.31–11.22	0.000
History of cerebrovascular disease, n (%)	22 (2.2)	16 (1.8)	6 (6.3)	3.66	1.40–9.59	0. 005
History of epilepsy, n (%)	6 (0.6)	3 (0.3)	3 (3.1)	9.59	1.91–48.20	0.014
Multiple sclerosis, n (%)	2 (0.2)	1 (0.1)	1 (1.0)			0.184
Chronic steroid use, n (%)	23/842 (2.7)	19/754 (2.5)	4/88 (4.5)			0.289
Chronic liver disease, n (%)	11 (1.1)	7 (0.8)	4 (4.2)	5.52	1.58–19.20	0.016
Kidney transplant, (%)	17 (1.7)	17 (1.9)	-			-
HIV infection, n (%)	5 (0.5)	4 (0.4)	1 (1.0)			0.400
Clinical and laboratory findings						
Headache	392 (39.6)	350 (39.1)	42 (43.8)			0.377
Vomiting	31/641 (4.8)	25/553 (4.5)	6/88 (6.8)			0.351
Lymphopenia ≤ 0.900 cells ($10^3/\mu L$), n (%)	386/978 (39.5)	335/883 (37.9)	51/95 (53.7)	1.90	1.24–2.90	0.003
Platelets ≤ 150 cells ($10^3/\mu L$), n (%)	137/982 (14.0)	109/887 (12.3)	28/95 (29.5)	2.98	1.84–4.84	0.000
D-dimer ≥ 1000 ng/mL, n (%)	361/912 (39.6)	303/828 (36.6)	58/84 (69.0)	3.87	2.38–6.27	0.000
Troponin ng/mL, median (IQR)	12.4 (2.9–36.7)	11.4 (2.6–32.4)	27.5 (11.3–27.5)			0.000
BNP pg/mL, median (IQR)	635 (195–2304)	561 (176–1940)	1858 (410–6216)			0.000

COVID-19: coronavirus disease 2019. CNS: central nervous system. IQR: interquartile range. HIV: human immunodeficiency virus. BNP: B-type natriuretic peptide. Ng/mL: nanograms/milliliters. Pg/mL: picograms/milliliters.

3.1. Risk Factors Associated with Hospital CNS Manifestations and Complications in Patients with COVID-19

In the univariate analysis, we found the following as statistically significant risk factors: age ≥ 64 years (OR 2.60, CI 1.67–4.06, $p = 0.000$), arterial hypertension (OR 1.98, CI 1.26–3.12, $p = 0.003$), diabetes mellitus (OR 1.76, CI 1.15–2.68, $p = 0.008$), chronic liver disease (OR 5.52, CI 1.58–19.20, $p = 0.016$), history of any neurological disease (OR 5.09, CI 2.31–11.22, $p = 0.000$), history of stroke (OR 3.66, CI 1.40–9.59, $p = 0.005$), history of heart failure (OR

3.27, CI 1.35–7.91, $p = 0.005$), history of epilepsy (OR 9.59, CI 1.91–48.20, $p = 0.014$), and history of COPD (OR 2.15, CI 1.01–4.58, $p = 0.041$) (Table 2).

Among the laboratory findings, the following risk factors were found: lymphopenia ($\leq$0.900 10^3 cells/μL) (OR 1.90, IC 1.24–2.90, $p = 0.003$), platelets ($\leq$150 10^3 cells/μL) (OR 2.98, IC 1.84–4.84, $p = 0.000$), D-dimer ($\geq$1000 ng/mL) (OR 3.87, CI 2.38–6.27, $p = 0.000$), troponin ng/mL, median 27.5 (IQR 11.3–27.5, $p = 0.000$), and B-type natriuretic peptide pg/mL, median 1858 (IQR 410–6216, $p = 0.000$) (Table 2).

Table 3 presents a description and univariate analysis of the evolution of the patients during admission and their hospital stay and the outcomes.

Table 3. Evolution (admission–inpatient stay–outcomes) in COVID-19 patients with CNS manifestations and complications while in hospital.

Covariates	All Patients n = 991	Without De Novo CNS Disorders n = 895 (90.3%)	With De Novo CNS Disorders n = 96 (9.7%)	OR	CI 95%	*p*
Onset of symptoms at hospital admission (days) median (IQR) (range)	7 (5–11)	7 (5–11) (1–41)	7 (4–10.3) (1–53)			0.172
Severity of illness on admission associated with neurological complications						
CORADS 6, n (%)	141/803 (17.6)	111/725 (15.3)	30/78 (38.5)	3.46	2.10–5.69	0.000
Severe ARDS, n (%) on admission	301/902 (33.4)	256/810 (31.6)	45/92 (48.9)	2.07	1.34–3.20	0.001
CURB-65, median (IQR)	1 (0–2)	1 (0–2)	2 (1–3)			0.000
qSOFA, median (IQR)	1 (1–1)	1 (1–1)	2 (1–3)			0.000
SOFA $\geq$5, n (%)	191/702 (27.2)	149/635 (23.5)	42/67 (62.7)	5.48	3.23–9.29	0.000
NEWS2 score $\geq$8, n (%)	313/874 (35.8)	267/793 (33.7)	46/81 (56.8)	2.59	1.63–4.12	0.000
Days from hospital admission to discharge (days) median (IQR)	10 (6–17)	10 (3–17) (1–94)	11 (7–18) (1–75)			0.324
Extraneurological complications associated with complications in the central nervous system (CNS) in patients with COVID-19						
Cardiovascular manifestations de novo, n (%)	43 (4.3)	31 (3.5)	12 (12.5)	3.98	1.97–8.04	0.000
Congestive heart failure de novo, n (%)	20 (2.0)	13 (1.5)	7 (7.3)	5.34	2.08–13.72	0.000
Pulmonary thromboembolism de novo, n (%)	8 (0.8)	5 (0.6)	3 (3.1)	5.74	1.35–24.41	0.034
Deep venous thrombosis de novo, n (%)	11 (1.1)	5 (0.6)	6 (6.3)	11.87	3.55–39.65	0.000
Outcomes						
Invasive mechanical ventilation (IMV), n (%)	310/989 (31.3)	265/893 (29.7)	45/96 (46.9)	2.09	1.36–3.20	0.001
Admission to intensive care unit (ICU), n (%)	146/889 (16.4)	136/801 (17.0)	10/88 (11.4)			0.177
Septic shock, n (%)	277 (28.0)	230 (25.7)	47 (49.0)	2.77	1.80–4.25	0.000
Death, n (%)	433 (43.7)	359 (40.1)	74 (77.1)	5.02	3.06–8.23	0.000

CNS: central nervous system. SOFA: Sepsis Related Organ Failure Assessment. ARDS: acute respiratory distress syndrome. qSOFA: Quick Sepsis Related Organ Failure Assessment. CURB-65: pneumonia prognostic scale, C: confusion, U: urea, R: respiratory rate, B: blood pressure, 65: age > 65 years.

Admission. A median of 7 days (IQR 5–11 days) from COVID symptom onset to hospital admission was calculated for the 991 patients.

In assessing the severity of the disease on admission associated with neurological manifestations and complications in the CNS through study protocols, we found the following prognostic scales to be statistically significant in these patients: CORADS 6 (OR 3.46, CI 2.10–5.69, $p = 0.000$), severe ARDS (OR 2.07, CI 1.34–3.20, $p = 0.001$), SOFA $\geq$ 5 (OR 5.48, CI 3.23–9.29, $p = 0.000$), and NEWS2 score $\geq$ 8 (OR 2.59, CI 1.63–4.12, $p = 0.004$).

The range in duration of the hospital stay for all patients was 1–53 days, with a median of 10 days (IQR 6–17). The neurological cohort presented various extraneurological complications, among the most important were de novo cardiovascular manifestations

(OR 3.98, CI 1.97–8.04, $p = 0.000$), de novo congestive heart failure (OR 5.34, CI 2.08–13.72, $p = 0.000$), and de novo pulmonary thromboembolism (OR 5.74, CI 1.35–24.41, $p = 0.034$). Among the hematological complications, de novo deep vein thrombosis was found to be significant (OR 11.87, CI 3.55–39.65, $p = 0.000$).

Outcomes. In the patients included in the study cohort, there were 433/991 deaths (43.7%). Of the 96 hospitalized patients in the neurological cohort with de novo CNS manifestations and complications, 74 people (77.1%) had a fatal outcome (OR 5.02, CI 3.06–8.23, $p = 0.000$); 73 of these deaths occurred within 29 days. For patients in the neurological cohort, the risks of requiring invasive mechanical ventilation (OR 2.09, CI 1.36–3.20, $p = 0.001$) and of presenting septic shock (OR 2.77, CI 1.80–4.25, $p = 0.000$) were statistically significant (Table 3).

Figure 2 presents a diagram of the most relevant results of the univariate analysis of the risk factors associated with CNS manifestations and complications in patients hospitalized with COVID-19. The result of the test for heterogeneity was calculated as $I^2 = 48\%$. The test for overall effect was OR 3.08, 95% CI 2.53–3.75, Z = 1, $p < 0.00001$.

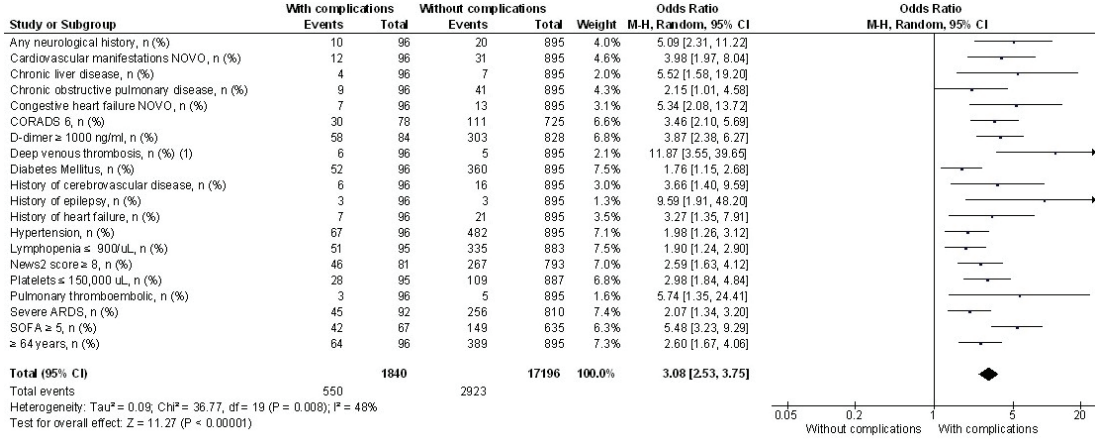

Figure 2. Forest plot of risk factors associated with CNS manifestations and complications in patients hospitalized with COVID-19.

3.2. Mortality Predictors Associated with Hospital CNS Manifestations and Complications in Patients with COVID-19

In the univariate analysis, age, male sex and the following comorbidities were found to be risk factors for mortality: arterial hypertension, diabetes mellitus, chronic kidney disease, any pulmonary history, COPD, and history of heart failure. Additionally, de novo CNS manifestations and complications present during hospitalization were a risk factor of mortality (OR 5.02, IC 3.06–8.23, $p = 0.000$), with delirium, drowsiness, stupor, stroke (OR 7.10, IC 2.06–24.52, $p = 0.001$), and ischemic stroke (OR 4.82, CI 1.34–17.39, $p = 0.012$) the most significant.

Laboratory findings that were consistent with an increased risk of mortality were lymphopenia $\leq 0.900\ 10^3$ cells/μL, platelets $\leq 150\ 10^3$ cells/μL and D-dimer ≥ 1000 ng/mL. In the severity scales, CORADS 6, Severe ARDS, SOFA ≥ 5 and NEWS2 ≥ 8 were also observed as risk factors of mortality for patients with COVID-19.

A multivariate logistic regression analysis was designed selecting the following variables: age of 64 years, sex, diabetes mellitus, arterial hypertension, any pulmonary history, general neurologic (CNS + PNS) manifestations and complications, hospital CNS manifestations, and complications, D-dimer ≥ 1000 ng/mL, lymphopenia $\leq 0.900\ 10^3$ cells/μL, and a SOFA ≥ 5.

When adjusting the ORs, the following were observed as predictors of mortality: age ≥ 64 years (OR 2.87, CI 2.02–4.08, $p = 0.000$), SOFA ≥ 5 (OR 4.52, CI 2.93–6.99, $p = 0.000$),

D-dimer ≥ 1000 ng/mL (OR 2.25, CI 1.57–3.23, $p = 0.000$). and hospital CNS manifestations and complications (OR 3.05, CI 1.39–6.72, $p = 0.006$) (Table 4).

Table 4. Mortality predictors in COVID-19 patients with de novo CNS manifestations and complications while in hospital.

Demographic Characteristics and Risk Factors	All Patients n = 991	No Deaths n = 558/991 (56.3%)	Deaths n = 433/991 (43.7%)	CI 95%	*p*	aOR (95%CI)	*p*
≥64 years, n (%)	453 (45.7)	184 (33.0)	269 (62.1)	3.33 (2.57–4.33)	0.000	2.87 (2.02–4.08)	0.000
Male gender, n (%)	614 (62.0)	328 (58.8)	286 (66.1)	1.36 (1.05–1.77)	0.019		
Hypertension, n (%)	549 (55.4)	269 (48.2)	280 (64.7)	1.97 (1.52–2.54)	0.000		
Diabetes mellitus, n (%)	412 (41.6)	205 (36.7)	207 (47.8)	1.58 (1.22–2.04)	0.000		
Chronic kidney disease, n (%)	100 (10.1)	43 (7.7)	57 (13.2)	1.82 (1.20–2.76)	0.005		
Any pulmonary history, n (%)	76 (7.7)	28 (5.0)	48 (11.1)	2.36 (1.45–3.83)	0.000		
COPD, n (%)	50 (5.0)	11 (2.0)	39 (9.0)	4.92 (2.49–9.73)	0.000		
History of heart failure, n (%)	28 (2.8)	9 (1.6)	19 (4.4)	2.80 (1.25–6.25)	0.009		
General neurological (CNS + PNS) manifestations and complications, n (%)	202 (20.3)	97 (17.4)	105 (24.2)	1.52 (1.12–2.08)	0.008		
De novo CNS manifestations and complications while in hospital, n (%)	96 (9.7)	22 (3.9)	74 (17.1)	5.0 (3.06–8.23)	0.000	3.05 (1.39–6.72)	0.006
CNS manifestations							
Delirium, (%)	63 (6.4)	15 (2.7)	48 (11.1)	4.51 (2.49–8.18)	0.000		
Altered consciousness, n (%)	50 (5.0)	6 (1.1)	44 (10.2)	10.41 (4.39–24.66)	0.000		
Drowsiness, n (%)	32/990 (3.2)	7 (1.3)	25/432 (5.8)	4.84 (2.07–11.29)	0.000		
Stupor, n (%)	21 (2.1)	3 (0.5)	18 (4.2)	8.02 (2.35–27.42)	0.000		
Seizures, n (%)	9 (0.9)	4 (0.7)	5 (1.2)		0.515		
Hemiplegia, n (%)	4 (0.4)	1 (0.2)	3 (0.7)		0.324		
Dysarthria, n (%)	5 (0.5)	1 (0.2)	4 (0.9)		0.174		
Aphasia, n (%)	5 (0.5)	0 (0.0)	5 (1.2)		-		
Anisocoria, n (%)	4 (0.4)	0 (0.0)	4 (0.9)		-		
Hallucinations, n (%)	4 (0.4)	1 (0.2)	3 (0.7)		0.324		
CNS syndrome or complications							
Stroke, n (%)	19 (1.9)	3 (0.5)	16 (3.7)	7.09 (2.06–24.52)	0.001		
Ischemic stroke, n (%)	14 (1.4)	3 (0.5)	11 (2.5)	4.82 (1.34–17.39)	0.012		
Ischemic in the left middle cerebral artery, n (%)	7 (0.7)	2 (0.4)	5 (1.2)		0.250		
Ischemic in right middle cerebral artery, n (%)	5 (0.5)	1 (0.2)	4 (0.9)		0.174		
Left carotid ischemic stroke, n (%)	1 (0.1)	0 (0.0)	1 (0.2)		-		
Right carotid ischemic stroke, n (%)	1 (0.1)	0 (0.0)	1 (0.2)		-		
Hemorrhagic stroke, n (%)	5 (0.5)	1 (0.2)	4 (0.9)		0.174		
Subarachnoid hemorrhage	4 (0.4)	0 (0.0)	4 (0.9)		-		

Table 4. *Cont.*

Demographic Characteristics and Risk Factors	All Patients n = 991	No Deaths n = 558/991 (56.3%)	Deaths n = 433/991 (43.7%)	CI 95%	*p*	aOR (95%CI)	*p*
Epilepsy de novo, n (%)	9 (0.9)	4 (0.7)	5 (1.2)		0.515		
Ataxia, n (%)	7 (0.7)	1 (0.2)	6 (1.4)		-		
Encephalitis, n (%)	3 (0.3)	2 (0.4)	1 (0.2)		1.000		
PNS manifestations and complications, n (%)	126 (12.7)	80 (14.3)	46 (10.6)		0.082		
Myalgias, n (%)	185 (18.7)	109 (19.5)	76 (17.6)		0.427		
Dysgeusia, n (%)	94 (9.5)	60 (10.8)	34 (7.9)		0.122		
Anosmia, n (%)	82 (8.3)	58 (10.4)	24 (5.5)		-		
Myopathy, n (%)	4 (0.7)	4 (0.7)	3 (0.7)		1.000		
Guillain Barré syndrome de novo, n (%)	1 (0.1)	1 (0.2)	0 (0.0)		-		
Extraneurological complications associated with complications in patients hospitalized with COVID-19							
Cardiovascular manifestations de novo, n (%)	43 (4.3)	10 (1.8)	33 (7.6)	4.52 (2.20–9.28)	0.000		
Laboratory findings							
Lymphopenia $\leq$ 0.900 ($10^3/\mu L$), n (%)	386/978 (39.5)	189/553 (34.2)	197/425 (46.4)	1.66 (1.28–2.16)	0.000		
Platelets $\leq$ 150 ($10^3/\mu L$), n (%)	137/982 (14.0)	53/553 (9.6)	84/429 (19.6)	2.30 (1.59–3.33)	0.000		
D-dimer $\geq$ 1000 ng/mL, n (%)	361/912 (39.6)	141/522 (27.0)	220/390 (56.4)	3.49 (2.65–4.62)	0.000	2.25 (1.57–3.23)	0.000
CORADS 6, n (%)	141/803 (17.6)	60/461 (13.0)	81/342 (23.7)	2.07 (1.44–2.99)	0.000		
Severe ARDS, n (%) on admission	301/902 (33.4)	59/473 (12.5)	242/429 (56.4)	9.08 (6.51–12.67)	0.000		
SOFA $\geq$ 5, n (%)	191/702 (27.2)	39/349 (11.2)	152/353 (43.1)	6.01 (4.05–8.91)	0.000	4.52 (2.93–6.99)	0.000
NEWS2 score $\geq$ 8, n (%)	313/874 (35.8)	120/469 (25.6)	193/405 (47.7)	2.65 (1.99–3.52)	0.000		
Outcomes							
IMV, n (%)	310/989 (31.3)	32 (5.7)	278/431 (64.5)	29.87 (19.87–44.90)	0.000		
Intensive care unit (ICU), n (%)	146/889 (16.4)	51/469 (10.9)	95/420 (22.6)	2.39 (1.66–3.47)	0.000		

CNS: central nervous system. PNS: peripheral nervous system. COPD: chronic obstructive pulmonary disease. ARDS: acute respiratory distress syndrome. SOFA: sepsis related organ failure assessment. ng/mL: nanograms/milliliters. IMV: invasive mechanical ventilation.

4. Discussion

The mortality of patients without de novo CNS neurological manifestations and complications was 40.1% (359/895), and in patients with de novo CNS neurological manifestations and complications, mortality was higher, 77.1% (74/96). The above shows the role of a compromised neurological system in the outcome of patients hospitalized with COVID-19 and expresses the mortality predictors found in this study, such as older age, de novo CNS manifestations and complications, D-dimer, and SOFA $\geq$ 5, in analytical terms.

Among the extrapulmonary manifestations and complications observed in patients with COVID-19, neurological manifestations of the central nervous system had a great impact due to their risk of high mortality [3,4,11,12].

Risk factors and predictors of mortality were analyzed in a cohort of patients with COVID-19 who presented with de novo hospital neurological manifestations and complications, using adjusted statistical analysis models. A forest plot diagram was also used to show the strength of the associated risk factors.

Given the low mortality in patients with PNS manifestations and complications (10.6%), we considered analyzing patients with de novo CNS manifestations and complications separately. Through the analysis strategy, we were able to identify the risks and

predictors of mortality in the neurological cohort, whose manifestations and complications were severe and that resulted in a poor prognosis.

The most frequent neurological complication associated with mortality in our study was stroke (OR 7.09, CI 2.06–24.52, $p = 0.001$)—particularly, ischemic stroke. Hingorani et al., (2022) reported that this cerebrovascular event is associated with older age, comorbidities and critical illness, with mortality being five times higher than in patients with stroke not infected by COVID-19. In addition to the fact that acute ischemic stroke tends to be more severe, its predominant etiology is associated with large vessel occlusion and cardioembolic events.

Among extraneurological complications, de novo cardiovascular complications (mainly, myocardial infarction, heart failure, pulmonary thromboembolism, and cardiac arrhythmias) were observed as a risk factor associated with mortality in the patients included in our study.

The study cohort was represented by a greater number of men than women, with a median age of 62 years. This distribution of patients was similar to that reported in other studies [8,12–14]. The cohort presenting with neurological CNS symptoms while in hospital also had a greater number of men than women, but with a higher mean age in this study (70 years).

The chronic degenerative diseases that occurred most frequently in the general study cohort were, as in other reviewed reports, arterial hypertension, diabetes mellitus, COPD, history of heart failure, and history of any neurological disease, mainly cerebrovascular diseases [14–16].

The following were classified as risk factors in hospitalized patients with de novo CNS manifestations and complications: age $\geq$ 64 years, arterial hypertension, diabetes mellitus, history of any neurological disease, history of stroke, history of heart failure, history of epilepsy, and a history of COPD. These have also been reported in other studies as risk factors [7,8,14].

Lymphopenia, thrombocytopenia, and elevated levels of D-dimer, troponin, and B-type natriuretic peptide were among the risk factors in the hospital neurological cohort among the laboratory findings, as reported in previously reviewed studies [4,7,14,16].

Additionally, in the univariate analysis, the following severity scale results, assessed during the admission of the patients and commonly used in our hospital for severity assessment in patients with COVID-19, were found as risk factors: a CORADS score of 6, severe ARDS, SOFA $\geq$ 5, and a NEWS2 score $\geq$ 8. In a study on mortality by Na et al. [17], they reported the use and factors associated with mortality of older adult patients with COVID-19 with a univariate analysis and Cox regression through the SOFA, CURB-65 score, and MEWS (modified early warning score) severity scales. The study was, however, applied to overall mortality from COVID-19. Flores-Silva et al. [18] reported the statistically significant effect of the CALL score and NEWS2 score assessed on admission in patients with COVID-19 analyzed through a semiquantitative score (low, medium and high risk) in a univariate analysis of the presentation of neurological signs and symptoms. These data indicate the usefulness of these scales in the management of COVID-19.

It has been reported that myocardial ischemia may predispose patients to further stroke damage as an extraneurological complication associated with CNS complications in patients with COVID-19 [3]. It has also been reported that the risk of presenting arterial and venous thrombosis is increased, favoring the occurrence of an ischemic stroke, even without a history of vascular disease. This occurrence is also associated with elevated levels of D-dimer and troponin [19].

The general cohort of patients included in our study presented a mortality of 43.7%, well above different rates reported worldwide; for example, in the Republic of Korea, the mortality of an elderly cohort of patients in hospitals was estimated at 25.5% [17]. However, patients with neurological manifestations and/or complications of the CNS while in hospital registered worse outcomes in our study among patients with COVID-19 (mortality of 77.1%).

The mortality rate in patients with COVID-19 due to respiratory complications ranges in different countries from 13 to 73%, since mortality varies from one country to another due to the age of patients and the level of access to treatment [1].

It is difficult to compare hospital mortality because the cohorts in the published studies refer to different stages of the COVID-19 pandemic in relation to time, different places, and different levels of access to vaccination. In addition, the different hospital conditions due to installed capacity, the different number of beds, the availability of human and material resources, etc., together with the great sociodemographic inequalities in the population and between the different hospitals, further complicates the comparison of said mortality. In a second-level hospital in Mexico City, a general hospital mortality from COVID-19 of 68.3% was observed in a retrospective cohort. The authors explain that this high mortality is partly justified by the low socioeconomic conditions of their custom population and the great inequities in hospital resources in the area of said hospital [20].

It must be considered, on the one hand, that our hospital regularly admits patients with severe complications referred from second-level hospitals and from the suburban and rural areas of Western Mexico. On the other hand, the low levels of prior vaccination in admitted patients should also be noted. Including a forest plot diagram allowed us to evaluate the findings of greatest interest and improve the precision of estimating risks. The diamond in the diagram is clearly to the right of the reference line, with a narrow confidence interval that allows one to determine the precision of the risk estimate and that the association studied was not due to chance [21].

Another one of the main strengths of the present study was the inclusion of just over 280 variables during the analysis of admission, hospitalization, and mortality.

Through a multivariate logistic regression analysis, it was found that an age of $\geq$64 years, hospital de novo CNS manifestations and complications while in hospital, D-dimer $\geq$ 1000 ng/mL and a SOFA $\geq$ 5 were predictors of mortality. In the reviewed literature, the works that present regression models to calculate adjusted odds ratios present results of general hospital mortality from COVID-19 and of patients with neurological manifestations and complications jointly from both the PNS and the CNS and other approaches with different designs [8,17].

Of the demographic variables, advanced age has been reported in different studies as a risk factor and/or predictor of mortality for COVID-19 patients and COVID-19 cohorts presenting with neurological damage, as determined in the present work [3,8,17,22].

The fact that the neurological evaluation was carried out by neurologists and neurosurgeons may contribute to reducing misclassifications of the data on neurological manifestations and complications, in addition to reducing the difficulties in discriminating the effects of aging in our patient cohort, which included elderly patients who have chronic degenerative diseases.

Battaglini et al. [4], specify that the patients who presented with more severe COVID-19 had greater CNS involvement (particularly patients with stroke) and higher D-dimer levels in their study on neurological manifestations in patients with SARS-CoV-2 infection.

In Na et al.'s [17] study on mortality predictors, they specify the utility of the SOFA score, where the highest score was associated with the highest mortality rate in older adult patients with severe COVID-19, which was also found to be statistically significant in the present study.

The SOFA score has been shown to be an important tool in the evaluation of critically ill patients, since it includes clinical, physiological and laboratory parameters that assess pulmonary, hematological, neurological, renal, hepatic, and cardiovascular functions. However, additional study is required to examine its scope in the patients included in this study.

In a review article [3], when analyzing the neurological manifestations and complications of the CNS of patients with COVID-19, the authors explained that stroke has been systematically associated with more serious and fatal outcomes, estimating that hospital mortality could be 5-times higher in COVID-19 patients with de novo CNS neurological

complications. Furthermore, they reported that the pathophysiology of these neurological events can be explained by virally mediated hypercoagulability, cytokine storm, cardiac effects and/or cerebrovascular arteriopathy. Additionally, in a study by Battaglini et al. [4], the authors concluded that patients who suffered a stroke have a worse prognosis.

5. Limitations

In the multivariate logistic regression analysis on the predictors of mortality, the variables of invasive mechanical ventilation and an admission to the ICU were excluded due to the indications of the triage protocol for the admission and hospitalization of patients with COVID-19 at the critical point of hospital saturation. In addition, many of the same patients rejected intubation for cultural reasons.

Other limitations of the study included the retrospective and longitudinal nature of this work, in addition to the health emergency which restricted the availability of data for excluded patients.

The present study considers patients from a one hospital, so it was limited in relation to the human and material resources available during the COVID-19 pandemic. This also limited the number of subjects that could be included in the cohort; however, the fact that this study only considers one hospital allowed us to better systematize and unify the data collected.

The authors of this article, like all health professionals around the world, had to face this serious emerging disease without knowing its natural history, diagnosis, prognosis. and treatment. We present this publication to allow the reader to refer to data on our specific patient population, including data on hospital mortality from COVID-19, among other characteristics.

6. Conclusions

Old age, being admitted in a critical condition and presenting comorbidities of chronic degenerative diseases, as well as having hospital manifestations and complications of the CNS, are determining factors in the prediction of mortality in patients hospitalized with COVID-19 as observed in the present study.

It is important to regularly update specific strategies for patients with COVID-19 who have risk factors for developing neurological complications mainly of the CNS to understand the pathophysiology of these complications and to detect cerebrovascular alterations earlier, thus avoiding a poor prognosis.

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Informed Consent Statement: As it was a retrospective cohort study, it was not necessary to obtain the consent of the included patients, only the medical records were reviewed. However, the confidentiality of the data was maintained.

Data Availability Statement: Not applicable.

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Conflicts of Interest: The authors of this research study declare that they have no conflict of interest.

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