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Original Article

Internal Medicine Section

Relationship between Disease Severity, Immune Response, and Viral Clearance in Unvaccinated Patients with COVID-19: A Cross-sectional Study

TUSHAR RAMESH SAHASRABUDHE[1], HARSHMEET SINGH GUJRAL[2], NIRMALA ANANTHI MUTHUKARUPPAN[3], MAHAVIR SATISHCHAND BAGRECHA[4], MADHU SUDAN BARTHWAL[5], SHAHZAD MIRZA[6]

ABSTRACT

Introduction: The clinical manifestations of Coronavirus Disease-2019 (COVID-19) range from asymptomatic cases to severe respiratory failure. It is unclear whether disease severity is determined by an excess viral load or a dysregulated immune response. It is also unclear whether the severe immune response is successful in rapid viral clearance.

Aim: To understand the relationship between immune response, viral clearance, and the severity of illness in unvaccinated patients suffering from COVID-19.

Materials and Methods: This was a cross-sectional observational study of 65 unvaccinated patients suffering from Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) confirmed COVID-19, conducted at a dedicated COVID-19 centre, Dr. D.Y. Patil Hospital, Pimpri, Pune, Maharashtra, India. from September 2020 to April 2021. The subjects were enrolled between day 10 and day 14 of the onset of symptoms and from two distinct clinical groups. Group A consisted of 34 mild cases, and Group B consisted of 31 severe cases, classified according to the national guidelines issued by the Indian Council of Medical Research (ICMR). The clinical progress of the illness and laboratory test records were carefully reviewed. A repeat throat swab for Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) RT-PCR, blood levels of COVID-19 specific antibodies Immunoglobin G (IgG) and IgM, C-reactive protein (CRP), and D-dimer levels were measured on day 14 of the illness. The data were analysed using MedCalc and Epi Info software. Chi-square and Fisher's-Exact tests were used to assess variables such as viral detection, antibody response, and inflammatory markers.

Results: In Group A, 2/34 (5.88%) patients tested RT-PCR negative {Cycle threshold (Ct) value cut-off above 35} compared to 5/31 (16.13%) in Group B (p=0.0829). In Groups A and B, respectively, the mean antibody titers were 35390.47 and 36426.11 (p=0.7469), the mean CRP values were 4.91 (p<0.001) and 31.01 mg/dL, and the mean D-dimer levels were 895 and 2896 ng/mL (p<0.001).

Conclusion: Both mild and severe COVID-19 cases had a significant antibody response which, however, did not help in viral clearance. Most patients remained RT-PCR positive on day 14 regardless of the disease severity.

Keywords: Antibody, Coranavirus disease-2019, D-dimer, Markers

INTRODUCTION

The pandemic of coronavirus disease 2019 or COVID-19, caused by Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), has led to millions of infections and deaths worldwide. A striking feature of COVID-19 is the wide variance in clinical severity among infected people, ranging from asymptomatic cases to severe respiratory failure and multi-organ dysfunction [1]. It has been a matter of debate whether disease severity is driven by the viral load or a dysregulated immune response. It is also unclear if the antibody response helps in viral clearance or reduction in infectiousness of the case, as conflicting results were obtained in some observational studies [2,3]. Symptomatic COVID-19 patients develop clinical manifestations within a mean duration of 14 days (ranging from 2 to 24 days) and can exhibit a range of symptoms, including fever, dry cough, malaise, anosmia, dyspnea, myalgia, headache, diarrhoea, etc., [4].

Various predictors of disease severity include older age, body mass index, various co-morbidities notably diabetes, chronic kidney disease, malignancies, hypertension, Acquired Immunodeficiency Syndrome (AIDS), and immunosuppressant therapy [5]. Vaccination status also significantly reduces disease severity [6,7]. The role of sex, genetics, and socioeconomic factors and their impact on the pathogenic mechanisms and disease outcome is incompletely understood [8-11]. Hypoxia has been used as a major parameter for clinical disease severity classification and is also a predictor of the outcome [12]. During the first wave of COVID-19, it was observed in the hospital that mild cases generally remained throat swab positive for a longer duration of time, sometimes even up to six weeks, whereas severe cases showed relatively earlier viral clearance. During the initial days of the pandemic, ICMR prescribed quarantine of COVID-19 patients for two weeks or until a negative repeat throat swab, whichever was later [13].

The hypothesis of dead virus being detected by RT-PCR after two weeks was popular too. Confirmation would then require either viral cultures or documentation that there is no difference in viral clearance regardless of the immune response. Another debatable issue was whether the severity of the disease is due to the viral load or due to the dysregulated excessive inflammatory immune response and whether such a response served any benefit in clearing the virus earlier. Such data would guide in reducing the unnecessary quarantine period, use of hospital resources, and manpower. The initial trend, however, was to quarantine people until and unless they turned throat swab negative, which was later changed to 14 days for all patients, and repeat RT-PCR was thought to be unnecessary. Consequently, the quarantine period was reduced to one week [13].

The present study thus, aimed to test the hypothesis that there might be an inverse relationship between immune response and severity of illness with viral clearance in patients suffering from COVID-19.

The secondary objective was to see if an exaggerated inflammatory immune response helped in early viral clearance, and the secondary objective was to see whether the inflammatory response correlated with protective immune response.

MATERIALS AND METHODS

This was a cross-sectional observational study conducted at a dedicated COVID-19 hospital, Dr. DY Patil Hospital in Pimpri, Pune, Maharashtra, India. The study was approved by the Institutional Ethics Committee before the commencement of enrollments (DYPV/EC/591/2020). The enrollment period was from September 2020 to April 2021, and enrollments were stopped after the vaccination drive started as it was an important confounding factor for the study.

Sample size calculation: According to the variables to be tested, the sample size was calculated to be 58, with 29 in each group, assuming a moderate effect size with a confidence interval of 95%. The Type 1 error was kept at 0.05, and the Type 2 error was kept at 2. The power of the study was 80%. The software used was G*power version 3.1.9.7 [14,15].

Inclusion and Exclusion criteria: All adult patients admitted to the dedicated COVID-19 hospital with a confirmed diagnosis of COVID-19 were screened as potential participants of the study. None of these patients had received even a single dose of any COVID-19 vaccine, and the study was conducted during the period before the emergence of the delta and omicron variants. A confirmed case of COVID-19 was defined as a patient whose nasopharyngeal swab was positive for SARS-CoV-2 by RT-PCR (Quantitative real-time reverse transcription polymerase chain reaction). Patients with known co-morbidities dampening adequate immune response, such as uncontrolled diabetes, Acquired Immunodeficiency Syndrome (AIDS), transplant patients, and patients on immunomodulatory therapy, were excluded from enrollment. The use of steroids as a part of COVID-19 treatment in doses recommended by ICMR guidelines was allowed and not considered as an exclusion criterion. Out of 123 screened patients, a total of 70 patients who gave written and informed consent were enrolled. The reason for the higher screen failure rate was widespread fear and panic among the patients as significant uncertainty about treatment protocols and their efficacy prevailed in society. Five patients were dropped out from the analysis after enrollment for various reasons.

Study Procedure

The patients were enrolled between the 10th and 14th day of their illness (counted from the day of symptom onset), and they were selected from two distinct clinical groups classified as per ICMR guidelines [13]. Group A consisted of mild cases, and Group B consisted of severe cases. A mild case of COVID-19 was defined as having upper respiratory tract symptoms and/or fever without shortness of breath or hypoxia, whereas a severe case was defined as a patient with a respiratory rate of more than 30/min or significant hypoxia (SpO_2 <90% on room air). Most severe cases required oxygen supplementation and had extensive radiological infiltrates or evidence of thromboembolic phenomena. On day 14 of the illness, a repeat throat swab was collected for RT-PCR with primers and probes targeting the E (Envelope) gene and RdRp {Ribonucleic Acid (RNA)-dependent RNA polymerase} gene specific to SARS-CoV-2. A blood sample was collected and evaluated for total COVID-19 specific antibodies (IgM and IgG), CRP levels, and D-dimer levels. Nasopharyngeal swabs and blood samples were collected by trained medical staff. The Ct value (Cyclic threshold value) of the E gene and RdRp gene in RT-PCR was considered as a parameter for viral clearance, with a value more than 34 considered as negative [16]. COVID-19 specific antibody titers signified the degree of immune response against the virus, and CRP/D-dimer values were markers of inflammation.

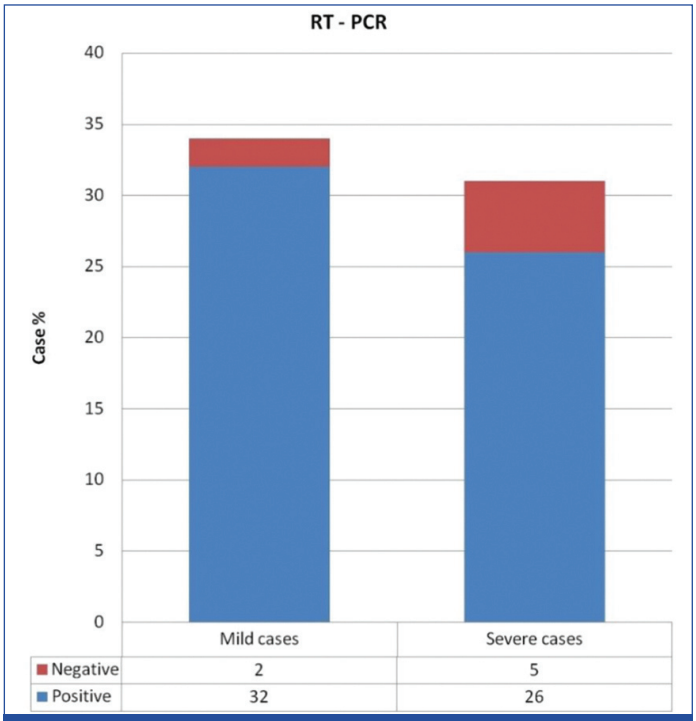
STATISTICAL ANALYSIS

MedCalc (version 20.305) and Epi Info (version 7.2) software were used for data processing and analysis. A Chi-square test was used to assess the association between severity and RT-PCR results, and Fisher's-exact test was used when more than 20% of the cells had an expected value <5. A p-value of less than 0.05 was considered statistically significant.

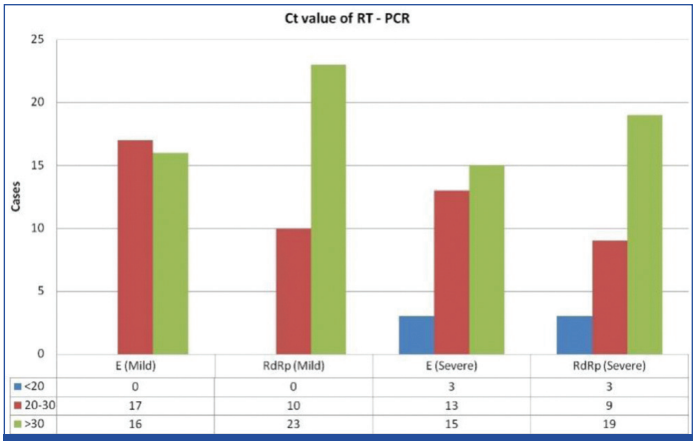
RESULTS

A total of 70 patients were enrolled in the study. Five cases were dropped from the analysis as the day 14 evaluation could not be done due to various reasons such as death, loss of follow-up, unavailability of test kits, etc. Out of the remaining 65 enrolled patients, 34 patients were enrolled in Group A (mild cases) and 31 patients were enrolled in Group B (severe cases). For statistical purposes, Ct values were sub-grouped as <20, 20-30, >30 for RT-PCR; total SARS-CoV antibody titers (IgG+IgM) were sub-grouped as titers <1000, 1000-50000, and >50000; CRP values were sub-grouped as <5, 5-50, and >50 mg/dL, and D-dimer values were sub-grouped as 500, <500-2000, and >2000 ng/mL.

On day 14 of the illness, two out of 34 patients (5.88%) tested RT-PCR negative in Group A compared to five out of 31 patients (16.13%) in Group B. RT-PCR results are shown in [Table/Fig-1,2]. The mean antibody titer for mild cases was 35,390 with a 95% CI

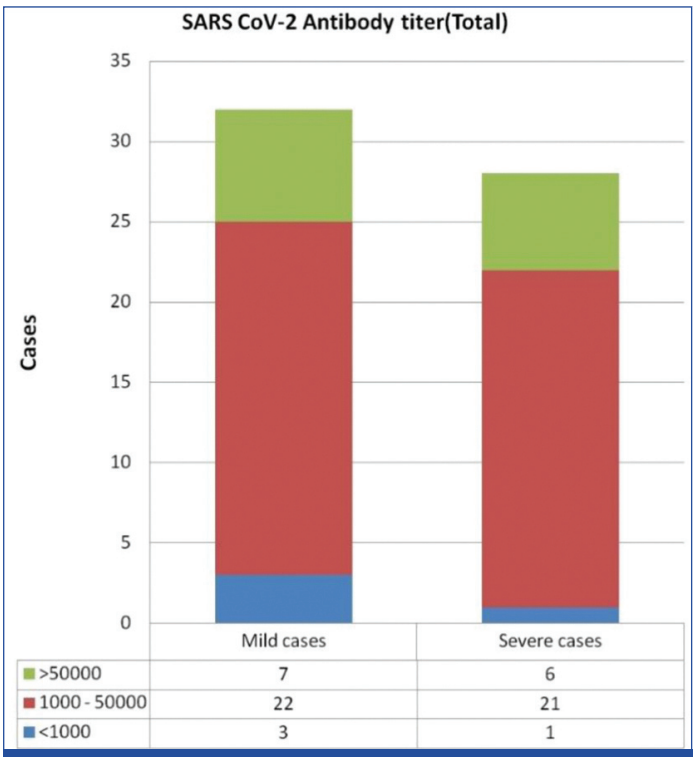


[Table/Fig-1]: Graph showing RT-PCR results at day 14 of illness in enrolled participants.



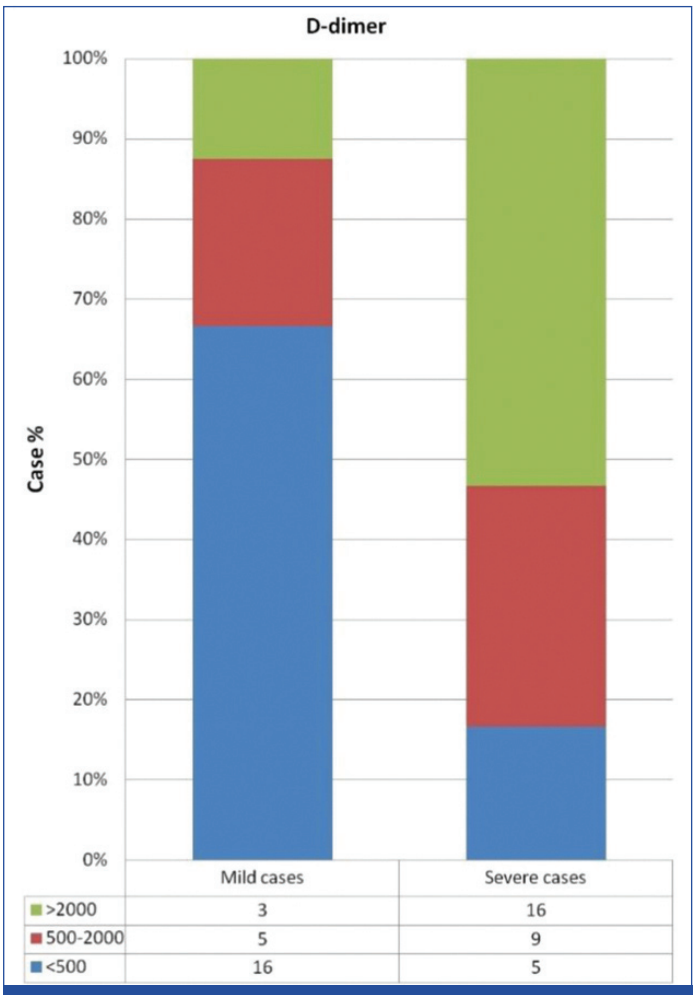
[Table/Fig-2]: Graph showing Ct values of RT-PCR (E and RdRp genes) at day 14 of illness in enrolled participants.

of 23,752 to 47,021, compared to 36,426 for severe cases with a 95% CI of 24,300 to 48,552. The difference was statistically insignificant [Table/Fig-3].



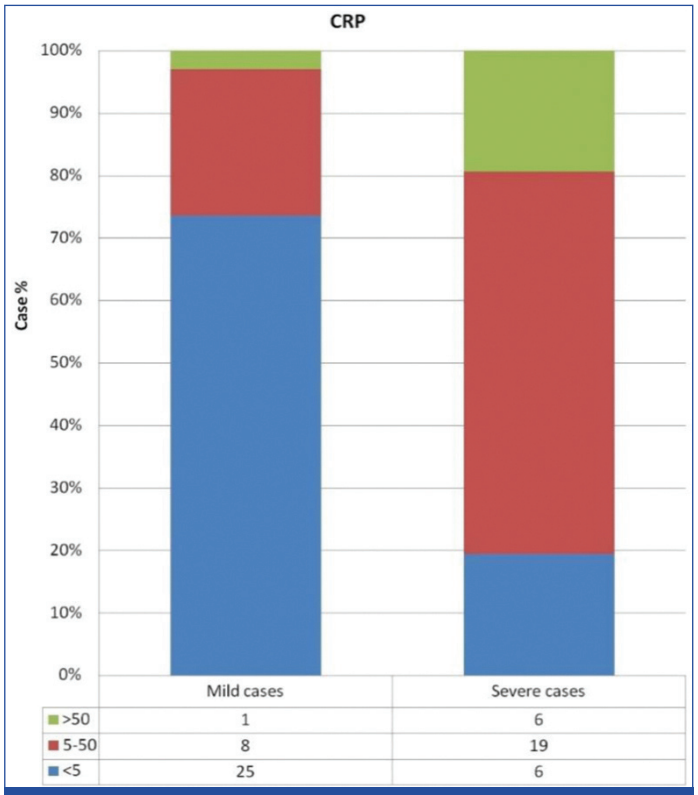
[Table/Fig-3]: Graph showing total COVID-19 antibody titre values at day 14 in enrolled participants.

The mean D-dimer levels were 895 ng/mL for mild cases with a 95% CI of 225.52 to 1564.48, compared to 2896.57 ng/mL with a 95% CI of 1807.79 to 3985.34 for severe cases (p<0.05) [Table/Fig-4].



[Table/Fig-4]: Graph showing D-Dimer values at day 14 in enrolled participants.

The mean CRP levels were 4.92 mg/dL for mild cases with a 95% CI of 2.96 to 6.87, compared to 31.01 mg/dL with a 95% CI of 18.77 to 43.25 for severe cases (p<0.05) [Table/Fig-5]. All the results have been summarised in [Table/Fig-6]. The results suggested that there was a higher chance of viral clearance in patients with a stronger inflammatory response, though the results were not statistically significant.



[Table/Fig-5]: Graph showing CRP values at day 14 in enrolled participants.

Variables	Mild	Severe	p-value
RT-PCR result			
Negative	2	5	0.183
Positive	32	26	
Ct value of E gene			
<20	0	3	0.22
20-30	17	13	
>30	16	15	
Ct value of RdRp gene			
<20	0	3	0.082
20-30	10	9	
>30	23	19	
COVID-19 Antibody titre (total)			
<1000	3	1	0.7469
1000-50000	22	21	
>50000	7	6	
CRP			
<5	25	6	**<0.001**
5-50	8	19	
>50	1	6	
D-dimer			
<500	16	5	**<0.001**
500-2000	5	9	
>2000	3	16	

[Table/Fig-6]: Table illustrating various variables and *statistical results of the study. *Statistical analysis was done using Chi-square test and Fisher's-exact test to assess the variables. A p-value of less than 0.05 was considered significant

DISCUSSION

When the pandemic came into the limelight in January 2020, there were a lot of questions and doubts regarding the virus. Healthcare workers were not immune to these challenges either. There was limited data regarding the duration of viral shedding after acquiring the virus and the mean duration for viral RNA detection. As the days passed, case reports revealed patients testing positive for SARS CoV-2 even up to 70-90 days from the onset of symptoms, even though they became asymptomatic with clinical resolution [17,18]. Patients remained isolated for prolonged durations because of the fear of transmission. Doctors and nursing staff of COVID-19 care centres who tested positive faced a similar issue. They remained quarantined for weeks before they tested negative, disrupting the workforce of the hospital in the fight against the pandemic.

During this period, a clinical pattern was observed which suggested that patients with mild disease remained positive for a longer period compared to patients with severe disease, who tended to become RT-PCR negative relatively quickly, even though their disease morbidity and hospital stay were longer. This led to a hypothesis suggesting an inverse relationship between the severity of the disease and viral clearance. However, there was still inadequate understanding of the various immune responses. The inflammatory response leading to a cytokine storm frequently occurred in the second week of illness and was a destructive immune response. A protective immune response, helping in viral clearance, was thought to be the antibody response, although the results of studies treating severe COVID-19 cases with convalescent plasma were not very promising [19,20]. This suggested that the immune response was distinctly acting on two different domains: the inflammatory response and the protective response. The link between them was missing, and it was unclear whether the antibody response protected against clinical worsening. Some studies indicated that immune evasion might play a significant role in the disease, suggesting that the production of antibodies may not be linked to the clearance of the virus [21-23]. Other studies found that SARS-CoV-2 RNA fragments may integrate into the human genome, which may explain the persistence of positive RT-PCR results in some patients [24,25]. The persistence of RT-PCR and infectivity may not go hand in hand.

van Kampen JJA et al., reported that COVID-19 patients shed SARS-CoV-2 RNA for 10-20 days [26]. However, the detection of viral RNA does not necessarily mean that a person is infectious and able to transmit the virus to another person. The findings of Glans H et al., suggested that the presence of SARS-CoV-2 specific antibodies in the serum may indicate a lower risk of viral shedding, which may indicate a possible link between viral clearance and the body's immune response [27].

Currently, the required isolation period in India is a minimum of seven days after the onset of symptoms and 72 hours after being asymptomatic, and a repeat throat swab is not required [13]. In the current study, it was observed that very few cases turned RT-PCR negative after 14 days of illness in both the mild and severe groups.

This is one of the very few studies evaluating the relationship between the severity of the disease, immune response, and viral clearance. Other studies testing a similar hypothesis have shown conflicting results. Masiá M et al., showed that the magnitude of the antibody response to SARS-CoV-2 contributed to viral clearance and concluded that the antibody response was directly proportional to the extent of bilateral lung infiltrates [3]. However, authors found an excellent antibody response in both mild and severe cases. On the contrary, Hafez W, in retrospective analysis, showed that the severity of COVID-19 was associated with delayed viral clearance [2]. Zhao H et al., also reached the conclusion that clearance of viral RNA in sputum was delayed in severe COVID-19 patients [28]. These findings were against present clinical observations that led to the proposed hypothesis of present study. Authors found that the clinical severity did not correlate with viral clearance, although there was a statistically non significant trend of early viral clearance in severe cases. Although the hypothesis could not be accepted based on the statistical analysis, there was a trend towards early viral clearance in severe cases. The data will make a useful contribution to any future meta-analysis.

It was also observed that severe cases had significantly higher CRP (p=0.000055) and D-dimer (p=0.000471) values compared to mild cases, suggesting that CRP and D-dimer are apt markers for inflammation and, hence, the severity of the disease. Ali AM et al., had similar findings in their analysis of COVID-19 patients, where severe cases had higher D-dimer and CRP levels and were associated with higher mortality rates [29]. Ullah W et al., also suggested that raised CRP and D-dimer were linked to the need for intensive care and increased in-hospital mortality, which supported present findings [30].

Limitation(s)

The sample size could have been larger if, authors had chosen a lower margin of type 2 error during estimation, thus increasing the power of the study. However, there were many challenges, including limited funds, expensive investigations, lack of manpower, lack of effective treatment, and fear among healthcare workers and patients. These factors were prevalent during the study period due to the nationwide lockdown. Despite these challenges, it was important to evaluate the proposed hypothesis, and hence, the study was conducted as a pilot project.

A limitation of present study is that RNA detection using RT-PCR does not distinguish between viable and nonviable viruses. Therefore, a positive nasopharyngeal swab is not indicative of infectivity or transmissibility and cannot differentiate between live virus and dead viral fragments. In the present study, authors chose the two extremes of severity to look for any meaningful differences. Authors expected an overlap in people with moderate severity, and hence, this category was not considered in the present study.

CONCLUSION(S)

The study results primarily shed light on the pathophysiological immune response to the unmutated COVID-19 virus, and similar studies for the various COVID-19 variants are warranted. The severity of the disease correlated with the inflammatory markers but not the antibody response or viral clearance, which were similar in both mild and severe cases. This suggests that different pathways may be responsible for viral clearance and protective immunity.

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PARTICULARS OF CONTRIBUTORS:
1. Professor, Department of Respiratory Medicine, Dr. D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India.
2. Junior Resident, Department of Respiratory Medicine, Dr. D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India.
3. Senior Specialist, Department of Pulmonary Medicine, Aster RV Hospital, Bengaluru, Karnataka, India.
4. Associate Professor, Department of Respiratory Medicine, Dr. D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India.
5. Professor, Department of Respiratory Medicine, Dr. D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India.
6. Associate Professor, Department of Microbiology, Dr. D.Y. Patil Medical College, Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri, Pune, Maharashtra, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Tushar Ramesh Sahasrabudhe,
Professor, Department of Respiratory Medicine, Dr. D.Y. Patil Medical College,
Hospital and Research Centre, Dr. D.Y. Patil Vidyapeeth, Pimpri,
Pune-411018, Maharashtra, India.
E-mail: dr_tushar_s@yahoo.co.in

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