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# The Role of N-Acetylcysteine as Adjuvant Therapy on TGF-β and II-6-Mediated Immune Response and Subsequent Fibrosis in Covid-19 Patients Predicted by Crp and D-Dimer Levels

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**ABSTRACT**

**Background** Despite vast amount of completed and ongoing researches, the role of immune system in COVID-19 remains unclear. Widespread lung damage as the result of the disease also often causes pulmonary fibrosis as a sequela. Antioxidant property of N-Acetylcysteine (NAC) has prompted its use as adjuvant therapy in COVID-19, both in immune system regulation and prevention of pulmonary fibrosis. We analyze the role of high dose NAC (>1200 mg/day) in COVID-19 immune response, mediated by major pro-inflammatory COVID-19 cytokines IL-6 and TGF-β, and in preventing subsequent pulmonary fibrosis, predicted by CRP and D-dimer, known parameters of lung damage in COVID-19. Premature increase of TGF-β may play a major role in immune system dysregulation in COVID-19.

**Method** This is a non-equivalent experimental study in confirmed COVID patients admitted in our hospital between June 2020 to July 2021. Patients were randomly divided unto NAC and control group, and IL-6, TGF-β, CRP and D-dimer levels were measured at admission and after 7 days of administration of NAC.

**Result** Compared to control group, there are significant decreases of IL-6, CRP, and D-dimer levels (p=.001, .000, and 0.001, respectively) after NAC administration. TGF-β level increases in both control and NAC group, although not significantly.

**Conclusion** NAC is a beneficial adjunctive therapy in alleviating immune response in COVID-19 as it lowers IL-6 level. NAC also lowers both CRP and D-dimer levels, which suggests that NAC may prevent post COVID-19 pulmonary fibrosis by mitigating lung damage caused by the disease.

**KEYWORDS:** COVID-19, Pulmonary Fibrosis, NAC, TGF-β

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## INTRODUCTION

Global significant health challenges are posed by the *Coronavirus Disease* 2019 (COVID-19) pandemic.[1] The pandemic began in Hubei province, Wuhan, China, in early December 2019. Since January 30, 2020, COVID-19, caused by the *severe acute respiratory syndrome coronavirus 2* (SARS CoV-2), has been declared a worldwide health emergency.[2] Since then, the number of confirmed COVID-19 patients from 215 countries has surpassed 9 million, with increasing deaths.[3] The COVID-19 pandemic puts the geriatric population, especially those with severe symptoms or comorbidities, at risk for post-COVID-19 fibrosis. In addition, fibrosis can be evident clinically in most COVID-19-infected patients; therefore, it is something to be aware of as a major long-term complication of COVID-19.[4]

*N-acetylcysteine* (NAC) works via various mechanisms mediated by glutathione precursors (GSH). According to the literature, *Reactive Oxygen Species* (ROS) play an essential role in the pathogenesis of lung injury, where the alveolar epithelial layer in patients with *Acute Respiratory Distress Syndrome* (ARDS) will experience GSH deficiency.[5] The thiol group of GSH acts as a significant ROS reductant.[6] Because of its antioxidant properties, NAC was chosen as the initial treatment for COVID-19.7 NAC may prevent pulmonary inflammation and fibrosis by breaking disulfide bridges, decreasing angiotensin II, and increasing angiotensin 1-7.[8] A study has shown favorable results on the administration of high-dose NAC in the management of post-COVID-19 pulmonary fibrosis.[9] Based on the literature, we intend to examine the effect of administering NAC as

adjuvant therapy on the immune response to COVID-19 to prevent post-COVID-19 fibrosis.

## METHODS

This study employed *Experimental Nonequivalent Control Group Design* method in quantitative approach. Study time was from May 2020 to February 2022. Subjects were patients who tested positive for COVID-19 and were admitted to either the non-intensive or the intensive room and were split into two groups: the treatment/NAC group (receiving standard-of-care therapy and at least 5000 mg/72 hours of NAC) and control group (receiving standard-of-care therapy only). Standard-of-care therapy was in accordance with the guidelines in effect at the time of the research. The treatment group consisted of total of 75 samples, while the control group consisted 16 samples. On the first day (upon admission) and the eighth day, the four variables CRP, D-dimer, IL-6, and TGF-β were examined in both groups. SPSS version 26 was used for data analysis.

## RESULTS

**Table 1** shows the demographic characteristics of the samples. Individuals in the NAC group were mainly < 60 years old (68.0%), male (58.7%), and had moderate COVID-19 (44.0%). Individuals aged < 60 (62.5%), male (68.8%), and complaints of fever, Dyspnea, and cough (100%) dominated the control group. The most common complication in the NAC group was *acute respiratory distress syndrome* (ARDS), which was experienced by 21 subjects (28.0%). The most prevalent complications among participants who did not get NAC were ARDS and respiratory failure (3.0%). Patient outcomes were good, with 63 subjects receiving NAC (84.0%) and 15 subjects (93.8%) not getting NAC improving and being discharged. The baseline characteristics of the two groups did not differ significantly. However, fever was observed to be significantly higher (69.3%; p=0.009), and the length of treatment was significantly shorter in the group of patients who received NAC (13,56±4,226 vs. 13,87±6,649; p=0,001).

**Table 1. Demographic Characteristics of Research Subjects.**

| Variable | NAC group (n=75) | Control group (n=75) | p |
|---|---|---|---|
| **Age** | | | |
| <60 years | 51 (68,0%) | 10 (62,5%) | 0,671 |
| ≥60 years | 24 (32,0%) | 6 (37,5%) | |
| **Gender** | | | |
| Male | 44 (58,7%) | 11 (68,8%) | 0,454 |
| Female | 31 (41,3%) | 5 (31,3%) | |
| **Severity** | | | |
| Moderate | 33 (44,0%) | 3 (18,8%) | 0,526 |
| Severe | 21 (28,0%) | 11 (68,8%) | |
| Critical | 21 (28,0%) | 2 (12,5%) | |
| **Presenting symptoms** | | | |
| Fever | 52 (69,3%) | 16 (100%) | 0,009* |
| Dyspnea | 62 (82,7%) | 16 (100%) | 0,114* |
| Cough | 71 (94,7%) | 16 (100%) | 1,000* |
| Chest pain | 9 (12,0%) | 0 (0,0%) | 0,352* |
| Hemoptysis | 1 (1,3%) | 0 (0,0%) | 1,000* |
| Decreased consciousness | 3 (4,0%) | 0 (0,0%) | 1,000* |
| Gastointestinal complaint | 42 (56,0%) | 0 (0,0%) | 0,000 |
| **Comorbidities** | | | |
| Yes | 34 (45,3%) | 6 (37,5%) | 0,567 |
| None | 41 (54,7%) | 10 (62,5%) | |
| **Smoking history** | | | |
| Yes | 34 (45,30%) | 7 (43,8%) | 0,908 |
| No | 41 (54,70%) | 9 (56,3%) | |
| **Complications** | | | |
| Shock | 2 (2,7%) | 2 (13,3%) | 0,128* |
| ARDS | 21 (28,0%) | 3 (20,0%) | 0,751* |
| Respiratory failure | 18 (24,0%) | 3 (20,0%) | 1,000* |
| MODS | 12 (16,0%) | 2 (13,3%) | 1,000* |
| **Patient outcome** | | | |
| Alive | 63 (84,0%) | 15 (93,8%) | 0,451* |
| Dead | 12 (16,0%) | 1 (6,3%) | |
| **Length of Treatment** | 13,56±4,226 | 13,87±6,649 | 0,001 |

**Fischer's exact test*

**Table 2** shows the levels of laboratory markers between the two groups at the start of the study before treatment. According to the table, There is no significant differences between the treatment and control groups (p>0.05) in the laboratory markers IL-6, CRP, D-dimer, and TGF-β.

**Table 2**. **Differences in laboratory markers in the treatment and control groups at the beginning of the research**.

| Variable | NAC group (n=75) | Control group (n=16) | p |
|---|---|---|---|
| IL-6 (pg/mL) | 120.72 (7.09 – 1058.32) | 39.98 (4.08 - 419.78) | 0.102 |
| CRP (mg/dL) | 6.17 (0.18 – 38.87) | 8.32 (2.10 – 16.60) | 0.271 |
| D-dimer (mg/L) | 2.19 (0.38 – 8.37) | 0.60 (0.20 – 1.15) | 0.066 |
| TGF-β (pg/mL) | 6.55 ± 3.25 | 5.47 ± 3.13 | 0.572 |

The results of laboratory marker analysis in the group of subjects who received NAC on the first day (before NAC administration) and the eighth day (after seven days of NAC administration) can be seen in Table 3. There was a significant decrease in the mean values of IL-6 (120.72 vs. 16.29; p = 0.001) and CRP (6.17 vs. 2.73; p = 0.000) after NAC administration. On the other hand, there was an increase in mean TGF-β after NAC administration but not significantly (6.55±3.25 vs. 6.92±3.23; p=0.293) (**table 3**).

**Table 3**. **Laboratory marker changes in the treatment group following NAC administration.**

| Variable | n | NAC administration | | p |
|---|---|---|---|---|
| | | Before (H1) | After (H8) | |
| | | Mean (min-max) | Mean (min-max) | |
| IL-6 (pg/ml) | 75 | 120,72 (7,09-1058,32) | 16,29 (1,12-970,15) | **0,001** |
| CRP (mg/dL) | 75 | 6,17 (0,18-38,87) | 2,73 (0,21-33,94) | **0,000** |
| D-dimer (mg/L) | 75 | 2.19 (0.38 – 8.37) | 1.15 (0.11 – 6.43) | **0,001** |
| | | Mean±SD | Mean±SD | |
| TGF-β (pg/ml) | 75 | 6,55±3,25 | 6,92±3,23 | 0,293 |

**Table 4** shows the subgroup analysis on moderate degree COVID-19 patients in the treatment group. Mean IL-6 levels decreased (160,05 [7,09-969,78] vs. 16,33 [1,12-423,92]; p=0,057), and mean CRP levels decreased from 4,16 [0,18-21,20] to 2,73 [0,21-8,11], while D-dimer increased (1,09 [0,38-6,95] vs. 1,12 [0,17-4,72]; p=0,780). Meanwhile, TGF-β levels increased (6,75±3,00 vs. 6,84±3,19; p= 0,922). All biomarker changes in the moderate degree COVID-19 treatment group did not experience significant changes (p>0,05).

**Table 4**. **Laboratory marker changes in the treatment group in moderate COVID-19.**

| Variable | n | NAC administration | | p |
|---|---|---|---|---|
| | | Before (H1) | After (H8) | |
| | | Mean (min-max) | Mean (min-max) | |
| IL-6 (pg/ml) | 33 | 160,05 (7,09 – 969,78) | 16,33 (1,12 – 423,92) | 0,057 |
| CRP (mg/dL) | 33 | 4,16 (0,18 – 21,20) | 2,73 (0,21 – 8,11) | 0,397 |
| D-dimer (mg/L) | 33 | 1,09 (0,38 – 6,95) | 1,12 (0,17 – 4,72) | 0,780 |
| | | Mean±SD | Mean±SD | |
| TGF-β (pg/ml) | 33 | 6,75±3,00 | 6,84±3,19 | 0,922* |

Subgroup analysis of severe degree COVID-19 patients in the treatment group (**table 5**) revealed that mean IL-6 level reduced significantly (p=0,339) from 96,24 [16,34-364,04] pg/ml to 7,68 [2,73-713,15] pg/ml. CRP levels significantly decreased (6,17 [0,50-38,87] vs. 0,57 [0,09-33,94]; p=0,002). The increase in D-dimer levels from 1,78 (0,11-7,11) mg/L to 1.47 (0.11-6.43) mg/L was not significant (p=0,981). TGF-β level increased, though non-significantly (6,29±3,47 vs. 6,77±3,63; p=0,717).

**Table 5. Laboratory marker changes in the treatment group in severe COVID-19.**

| Variable | n | NAC administration | | p |
|---|---|---|---|---|
| | | Before (H1) | After (H8) | |
| | | Mean (min-max) | Mean (min-max) | |
| IL-6 (pg/ml) | 21 | 96,24 (16,34 – 364,04) | 7,68 (2,73 – 713,15) | **0,039** |
| CRP (mg/dL) | 21 | 6,17 (1,32 – 38,87) | 0,57 (0,09 – 33,94) | **0,002** |
| D-dimer (mg/L) | 21 | 1,78 (0,11 – 7,11) | 1,47 (0,11 – 6,43) | 0,981 |
| | | Mean±SD | Mean±SD | |
| TGF-β (pg/ml) | 21 | 6,29±3,47 | 6,77±3,63 | 0,717 |

Subgroup analysis of critical stage COVID-19 patients in the treatment group (**table 6**) showed mean levels of IL-6 (103,63 [29,64-1058,32] vs. 16,29 [1,17-970,15]; p=0,002) and CRP (7,60 [0,50-29,87] vs. 1,04 [0,12-2,72]; p=0,000) which significantly decreased on the eighth day of treatment. In the subgroup of critical COVID-19 patients, the rise in D-dimer was likewise statistically significant (1,97 [0,19-8,37] vs. 1,61 [0,26-5,77]; p=0,022). After the eighth day of treatment, mean TGF-β did not significantly rise (6,49±3,53 vs. 6,98±3,30; p=0,511).

**Table 6. Laboratory marker changes in the critical degree treatment group.**

| Variable | n | NAC administration | | p |
|---|---|---|---|---|
| | | Before (H1) | After (H8) | |
| | | Mean (min-max) | Mean (min-max) | |
| IL-6 (pg/ml) | 21 | 103,63 (29,64 – 1058,32) | 16,29 (1,17 – 970,15) | **0,002** |
| CRP (mg/dL) | 21 | 7,60 (0,50 – 29,87) | 1,04 (0,12 – 2,72) | **0,000** |
| D-dimer (mg/L) | 21 | 1,97 (0,19 – 8,37) | 1,61 (0,26 – 5,77) | **0,022** |
| | | Mean±SD | Mean±SD | |
| TGF-β (pg/ml) | 21 | 6,49±3,53 | 6,98±3,30 | 0,511 |

Changes in laboratory markers were also performed on 16 control group subjects who did not receive NAC. Evaluation of laboratory markers was performed on the eighth day of treatment. The analysis showed that IL-6 levels increased (39,98 [4,08-419,78] vs. 59,58 [7,81-515,74]) and CRP levels decreased (8,32 [2,10-16,60] vs. 0,80 [0,31-16,50]). Meanwhile, the mean D-dimer level did not change after day eight. All laboratory markers showed no statistically significant changes (p>0,05). TGF-β did not increase significantly on day eight (5.61±3.13 vs. 5.47±3.51; p=0,518) (**table 7**).

**Table 7. Laboratory marker changes in control group.**

| Variable | n | Day 1 of treatment | Day 8 of treatment | p |
|---|---|---|---|---|
| | | Mean (min-max) | Mean (min-max) | |
| IL-6 (pg/ml) | 16 | 39,98 (4,08-419,78) | 59,58 (7,81-515,74) | 0,408 |
| CRP (mg/dL) | 16 | 8,32 (2,10-16,60) | 0,80 (0,31-16,50) | 0,374 |
| D-dimer (mg/L) | 16 | 0,60 | 0,60 | 0,249 |

| | | (0,20-1,15) | (0,32-7,50) | |
|---|---|---|---|---|
| | | Mean±SD | Mean±SD | |
| TGF-β (pg/ml) | 16 | 5.61±3.13 | 5.47±3.51 | 0.518 |

**DISCUSSION**

Subjects aged <60 years were found to be more common in this research, with 68,0% in the NAC group and 62,5% in the group without NAC, which was insignificant, indicating that the age characteristics were similar in both groups. This finding is consistent with a prior trial in which the mean age in the NAC group was 59 years old, and the placebo group was 58 years old.[10] Based on symptoms, all patients without NAC (100%) had a fever, shortness of breath, and cough. However, only fever was substantially different in both groups ($p<0.05$). In addition, the most prevalent symptoms in the group with NAC were similar to those in the group without NAC: cough (94,7%), shortness of breath (82,7%), and fever (69,3%). These findings are in line with a study of COVID-19 confirmed population profiles in Indonesia, which found that cough (77,7%), fever (42,8%), and shortness of breath (34,8%) were the most prevalent COVID-19 symptoms.[11] In this research, 56,0% of subjects in the NAC group experienced gastrointestinal disorders. Because SARS-CoV-2 interacts with ACE-2 receptors in the gastrointestinal system, 3–39% of COVID-19 patients experience gastrointestinal symptoms. It has been noted that prolonged positive nucleic acid detection results and more severe pneumonia correlate with gastrointestinal symptoms.[12]

Compared to the group of patients who did not get NAC, the length of stay for COVID-19 patients who received NAC was significantly shorter (13,56±4,226 vs. 13,87±6,649; p=0,001). According to prior reports, administering NAC to COVID-19 patients may affect how long they stay in the hospital. According to Faverio et al., patients who got NAC reported a mean length of hospitalization of 15 (10–24) days as opposed to 17 (12–30) days for patients who did not get NAC.[13] Similar findings were also reported in a study of COVID-19 patients who received high doses of NAC. Patients without comorbidities experienced a mean hospitalization time of 12 days with N-acetylcysteine 1200-5000 mg, whereas patients with comorbidities experienced a mean hospitalization time of 14 days.[14]

IL-6 is one of COVID-19's primary inflammatory regulators. Critical situations like sepsis, ARDS, and COVID-19 have been found with higher IL-6 concentrations. Along with IL-1 and TNF-α, IL-6 has been discovered to be elevated in patients with severe COVID-19. N-acetylcysteine (NAC) has been reported to reduce ROS in the form of TNF-α and IL-6 in type II alveolus cells infected with influenza A/B virus and *respiratory syncytial virus* (RSV).[15,16] N-acetylcysteine functions as an antioxidant, anti-inflammatory, and mucolytic. As an anti-inflammatory, NAC prevents the release of pro-inflammatory cytokines (IL-6) on immune cell proliferation during disease progression.[17]

In this research, COVID-19 patients who received NAC had a substantial drop in mean IL-6 on the eighth day of treatment, from 120,72 pg/mL to 16,29 pg/mL. A severity-based degree investigation discovered that IL-6 levels were decreased significantly in severe and critical COVID-19 patients. This study's findings align with the prior study by Hanum and Hanifa, who discovered that all severe COVID-19 patients given large doses of NAC (1200 - 5000 mg/24 hours) exhibited a drop in IL-6 on day 12 of treatment.[14] Studies in SARS-CoV-2 infected hamster models reported high dose NAC (500 mg/kg/24 hours) can reduce the expression of pro-inflammatory gene IL-6 compared to low dose NAC (150 mg/kg/24 hours). The decrease in IL-6 could be up to 4,2 times when high-dose NAC was administered together with remdesivir. The study also reported that although NAC could not reduce *viral load*, it could suppress the inflammatory process and slow down lung tissue damage.[18]

*Transforming Growth Factor-β* (TGF-β) is vital in lung tissue repair and fibroproliferation, which is characterized by collagen synthesis.[19] TGF-β has been found to be elevated in various lung illnesses, including pulmonary fibrosis, emphysema, bronchial asthma, and lung cancer.[20] NAC can inhibit the synthesis of pro-fibrotic factors like TGF-β.[21] Furthermore, NAC can diminish TGF-β disulfide links, transforming the bioactive dimers form of TGF-β into the inert monomeric form. NAC also changes TGF-β receptor binding activity in hepatic stellate cells, suggesting it may directly modulate TGF-β function and signaling.[22]

In this research, there was no significant change in TGF-β, either in the group that received NAC or not. It could be due to the characteristics of the COVID-19 disease phase. In the early phase of the disease, IgG provides systemic protection against the SARS-CoV-2 pathogen, while IgA protects mucosal surfaces. TGF-β factor has been found to contribute to antibody formation by seroconverting plasmablasts into IgA1 and IgA2. Serum IgA1 and IgA2 titer levels in severe to critical COVID-19 patients will be detected after the seventh day of treatment and will rise over time. These findings suggest that SARS-CoV-2 triggers chronic inflammation, which TGF-β upregulates.[23] TGF-β, a fibrosis mediator, is not elevated in the early phase of COVID-19, mainly if lung damage is still ongoing.[24]

C-*reactive protein* is an infection *bio*marker (acute phase protein) that is used as an indicator of the extent of inflammation in the body.[25] Serum CRP levels strongly correlate with COVID-19 progress, with CRP increasing

markedly in the early stages of inflammation. In addition, CRP has been found to be an independent predictor of COVID-19 severity in previous studies.[26]

The mean CRP in COVID-19 patients who got NAC was found to decrease significantly from 6,17 mg/dL to 2,73 mg/dL in this research. A substantial decrease in CRP was also found in the group that received NAC at severe and critical degrees. This finding aligns with previous research that found a significant decrease in CRP and an increase in oxygen saturation in patients with severe COVID-19 pneumonia who received NAC 1200-1500 mg i.v. The decrease in CRP was accompanied by a reduction in lung lesions, demonstrating CRP as a mediator of lung tissue fibrosis.[27] Another study that examined the efficacy of NAC 600 mg/12 hours for 14 days in COVID-19 patients discovered that CRP decreased dramatically, as did the risk of ARDS, the need for mechanical ventilation, and mortality.[28] Administration of NAC, as an addition to the standard protocol of therapy for COVID-19 patients, was able to support a decrease in CRP levels.

D-dimer is a fibrin degradation product that is frequently utilized as a thrombosis *bio*marker. COVID-19 has been linked to coagulation abnormalities; hence, D-dimer could be used to assess prognosis and predict the severity of COVID-19.[29] The risk of thrombosis complications such as pulmonary embolism and deep vein thrombosis is associated with increased mortality in COVID-19 patients with elevated D-dimer.[30]

In this research, D-dimer levels experienced significant changes in all patients who received NAC, especially in patients with critical degree COVID-19. The decrease in D-dimer after the administration of NAC was not substantial in COVID-19 patients with moderate and severe disease. This finding is in line with a prior study by Assimakopoulos et al., who discovered a significant decrease in D-dimer on days seven and 14 after patients took NAC 600 mg/12 hours orally.[28] Another study analyzing the efficacy of NAC on critical COVID-19 patients admitted to the ICU also found that NAC administration can significantly reduce D-dimer levels after 14 days (186,37 ± 410,23 vs. 1339,04 ± 2183,87 ng/mL; p=0,03).[31] This suggests that NAC administration is beneficial for critical COVID-19 patients, especially in the potential to reduce thromboembolism events through reducing D-dimer.

Correlation tests revealed a significant correlation between TGF-β and several *bio*markers, namely IL-6, CRP, and D-dimer. Previous research showed a correlation between IL-6 and TGF-β, in which IL-6 triggers TGF-β production and vice versa via fibroblasts. Both cytokines also interact through the same pathway (STAT3).[32] With this correlation, it is statistically shown that with the decrease in IL-6 that occurs with the administration of NAC in our research, the value of TGF-β will also decrease.

The correlation between CRP as a lung damage parameter used in this research and TGF-β suggests a possible indirect causative relationship due to the relationship between CRP and IL-6 (where IL-6 directly triggers CRP production) and the previously mentioned relationship between IL-6 and TGF-β.[33] However, the correlation intensity among CRP and TGF-β suggests a probable direct relationship in which lung damage directly triggers fibrogenesis via a *common pathway* mediated by TGF-β. This correlation is also in accordance with the theory, which states that lung damage in COVID-19 is mediated by intense inflammation.[4,34] The same thing can also be shown by the correlation between D-dimer and TGF-β, where D-dimer also plays a role as a parameter for the occurrence of inflammation in COVID-19.[35] In this case, the role of the D-dimer indicates that the damage that occurs to the endothelium due to NETosis plays a role in the formation of lung parenchymal damage in post-COVID-19 fibrosis.

Our research has several limitations. Because this research only used subjects from one Health Facility, the number of subjects was limited; therefore, research with a larger sample size and from *multi-center* Health Facilities is required. This research used the same dose of NAC for all subjects, so it is impossible to determine the dose-effect relationship between NAC and *bio*marker levels. Therefore, research with various dosages of NAC is required to discover the optimal dose of NAC on inflammatory *bio*markers in COVID-19. Furthermore, in this research, there was no analysis of confounding factors that could influence the impact of NAC on *bio*markers, such as age, gender, comorbidities, and so on. As a result, it is crucial to analyze the impact of NAC on *bio*markers while accounting for confounding factors that may affect the research's findings.

NAC is a beneficial adjunctive therapy in alleviating immune response in COVID-19 as it lowers IL-6 level. NAC also lowers both CRP and D-dimer levels, which suggests that NAC may prevent post COVID-19 pulmonary fibrosis by mitigating lung damage caused by the disease.

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