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Correlation between immune system activity and the susceptibility to Long COVID symptoms

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Abstract

With an alarmingly high rate of Long COVID, it is essential to address the lack of understanding of the pathogenesis and susceptibility to Long COVID. This study aims to investigate how symptoms and immunological markers of Long COVID are correlated in order to develop better biomarkers and inform strategies to accurately identify patients at high risk for developing Long COVID. It has been found that underlying health conditions increase the likelihood of developing Long COVID. Additionally, certain symptoms during COVID-19 infection have been linked to Long COVID. However, the immune system activity has not been well investigated for its correlation to the susceptibility and severity of Long COVID symptoms. In our study, human subjects from the United States and United Kingdom will be followed for a year, where they will be tested weekly for the presence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), draw blood for immune system activity analysis, and report symptoms bidaily. Reverse-transcription quantitative real-time polymerase chain reaction (RT-qPCR) will be used to detect and quantify the presence of SARS-CoV-2. Qualitative information about participants will be collected via the ZOE Health Study app to enable the collection of clinical metadata such as symptoms. This study's unique and sizeable prospective cohort will provide critical knowledge about the susceptibility of patients to Long COVID by pre-existing symptomatology and immune markers. Moreover, we will better understand the possible mechanisms and pathogenesis of the disease.

Keywords

Research proposal, COVID-19, Long COVID, immune system

Background

COVID-19's Global Impact:

Coronavirus disease 2019 (COVID-19) is an infectious disease caused by SARS-CoV-2. Over 603 million global cases of COVID-19 were reported as of September 2022 (World Health Organization 2022), with over 94 million cases in the United States alone (Centers for Disease Control and Prevention 2022). Declared a pandemic in 2020 only months after the initial outbreak, COVID-19 is at risk of becoming endemic in the coming years due to its high contagion and the inability to fully eradicate the disease. Because of the virus' persistent nature, it is critical to explore the long-term effects it may have.

Long COVID Symptoms and Risks:

Of individuals who experienced COVID-19, 30% developed Long COVID (Yoo et al. 2022). Individuals infected with SARS-CoV-2 experience varying severity of symptoms, ranging from fatal to asymptomatic. Approximately 30% of people who have had COVID-19 develop post-acute sequelae SARS-CoV-2 infection (PASC), also known as Long COVID. Individuals with Long COVID experience new or persistent symptoms after 8-12 weeks of the initial infection (Yoo et al. 2022). In fact, even asymptomatic individuals during the initial SARS-CoV-2 infection can still develop Long COVID symptoms (Munker et al. 2022). Symptoms of Long COVID include nausea, vomiting, skin rash, joint pain, heart palpitations, anosmia, tiredness, headaches, and post-exertional malaise (PEM) (Raman et al. 2022, Tan et al. 2022).

Long COVID is characterized by inflammation caused by prolonged elevated levels of innate immunological markers: Interferon-beta (IFN-β), pentraxin 3 (PTX3/TSG-14), interferon-lambda-1 (IFN-λ1/IL-29), interleukin 6 (IL-6), and interferon-gamma-induced protein 10 (IP-10/CXCL10). With regards to the adaptive immune system, Long COVID patients display higher levels of CD8+ and CD4+ T cells in their blood (Peluso et al. 2021, Phetsouphanh et al. 2022). Other studies have shown that changes in the gut microbiota are associated with individuals who developed Long COVID. In patients who developed Long COVID, there was a higher enrichment of *Ruminococcus gnavus* and *Bacteroides vulgatus* and a lower abundance of *Faecalibacterium prausnitzii* compared to individuals who did not develop Long COVID (Liu et al. 2022). In a retrospective study of 366 adults who had COVID-19, older individuals (≥40) were more likely to develop Long COVID (Yomogida et al. 2021). A study of 4,182 people found that if an individual experiences more than 5 symptoms from the initial SARS-CoV-2 infection, they are more likely to develop Long COVID (Sudre et al. 2021). The most accurate symptom predictors for developing Long COVID are fatigue and shortness of breath. 31.4% of patients with Long COVID experienced fatigue as an initial symptom, and 15.4% of patients with Long COVID experienced shortness of breath during the initial infection (Yoo et al. 2022). In a study of 384,137 individuals who had COVID-19, women were 1.52 times more likely than men to develop Long COVID (adjusted hazard ratio (aHR) = 1.52). Out of all minority groups, Black Afro-Caribbean ethnic groups saw the most significant risk of 1.21 times more likely than whites (aHR = 1.21). Individuals in a lower socioeconomic class were more likely to

develop Long COVID by 11% than those less socioeconomically disadvantaged. Former and current smokers were 1.12 times more likely to get Long COVID than nonsmokers (aHR = 1.12). Those classified as obese by BMI metrics (+30 kgm-2) were 10% more likely to get Long COVID compared to those in the healthy range (18.5 kgm−2 to 25 kgm−2) (Subramanian et al. 2022).

Causes of Long COVID :

Three causes of Long COVID dominate the discussion (Couzin-Frankel 2022). One is that a cytokine storm during SARS-CoV-2 infection causes lasting, excessive blood clotting, which leads to organ failure and fatigue (Pretorius et al. 2021). Another cause is that the virus' persistence in the body leads to the creation of viral ribonucleic acid (RNA) and proteins and thus, the prolongation of symptoms (Gaebler et al. 2021, Zollner et al. 2022). The third is the immune system's inability to return to normal activity levels after SARS-CoV-2 infection. When the immune system remains inappropriately hyperactive, it can cause damage to organs (Phetsouphanh et al. 2022).

In the first study of the causes of Long COVID (Cause 1), it was found that the immune system was too strong when fighting off SARS-CoV-2; the immune system caused lasting damage to the lungs via blood clots restricting oxygen which manifested as difficulty breathing (Pretorius et al. 2021). In the second study (Cause 2), the immune system was too weak to fight off the infection entirely, so the virus persisted in the body; the virus continuously caused damage to the body which manifested as inflammatory bowel disease (Gaebler et al. 2021, Zollner et al. 2022). In the third study (Cause 3), the immune system was neither too strong nor too weak, but rather hyperactive and persistent post infection; the immune system continuously caused damage to the brain which manifested as fatigue (Phetsouphanh et al. 2022). Despite differences, the three causes are connected through the characteristic of irregular immune activity.

The Limitations of Prior Studies:

While numerous studies have investigated the innate and adaptive immune characteristics as well as qualitative symptoms and underlying conditions predictive of Long COVID, it is still unclear what immunological markers are predictive of the symptoms that underlie Long COVID. Given the heterogeneity of Long COVID patients' symptoms, it is crucial to investigate if immunological markers can be accurate predictors of Long COVID symptoms. Additionally, while previous studies have uncovered important insights into the pathogenesis and susceptibility of Long COVID, limited patient cohort sizes may limit the generalizability of such findings. While some studies may have a total sample size of a few thousand people, this study will have three groups each with 3000 people from different countries. It is essential to explore the correlation using a larger and more diverse sample size to increase the data's resistance to outliers and eliminate the chances of artificial geographical biases. Another limitation of prior studies is the length of time subjects were followed. While some studies collect data from their subjects once, this study will collect data from subjects more frequently over an extended period of time. A longer timeframe for

data collection will allow us to examine potential long-term effects and changes in symptoms that simple one-time data collection in other studies cannot uncover.

The Research Question and the Hypothesis:

This proposal will utilize a longitudinal, multimodal approach to investigate immunological predictors of the pathogenesis of Long COVID by collecting self-reported, serological, and immunological data. To comprehensively determine the relationship between immunological markers and symptoms of Long COVID, the data collected will be statistically analyzed for correlation.

We hypothesize that individuals will have the highest levels of both innate immunological markers (IFN-β, PTX3/TSG-14, IFN-λ1/IL-29, IL-6, and IP-10/CXCL10) and adaptive immunological markers (CD8+ and CD4+ T cells) when experiencing COVID-19 concurrently. Individuals experiencing Long COVID are expected to display persistently elevated levels of immunological markers; however, those levels may not be as elevated as those displayed in COVID-19 patients. Individuals with Long COVID who experience symptoms associated with inflammation (nausea, joint pain, skin rashes, etc.) are expected to display elevated levels of innate immunological markers, such as IFN-β, PTX3/TSG-14, IFN-λ1/IL-29, IL-6, and IP-10/CXCL10. Individuals with Long COVID who experience symptoms associated with organ failure (heart palpitations, anosmia, tiredness, headaches, etc.) are expected to display elevated levels of adaptive immune markers such as CD8+ and CD4+ T cells. Individuals with COVID-19 are expected to display the most elevated levels of immune markers, while individuals who do not develop Long COVID after infection may display relatively normal immune system activity.

Materials and Methods

Experimental Design:

In order to discover any correlation between symptoms and immunological markers, the symptoms and quantity of immunological markers will be evaluated across individuals with COVID-19, individuals who have fully recovered from COVID-19 without developing Long COVID, and those with Long COVID. The individuals with COVID-19 will be used as a positive control group. The individuals who fully recovered will be used as a negative control group.

Data collection will take place over the course of a year. Symptoms will be reported bidaily, and blood will be drawn weekly for immunological analysis. The independent variable will be the status of subjects: having COVID-19, fully recovered, or having Long COVID. The dependent variable is the type and severity of symptoms subjects report, as well as the quantity of immunological markers associated with Long COVID. If there is a correlation, there will typically be a higher or lower level of specific immunological markers in those who suffer from the same symptoms.

Patient Selection:

The experiment will consist of 3 groups of approximately 3000 people from the United States and the United Kingdom. Approval by the United Kingdom's King's College London (KCL) and the United States Partners Human Research Committee for the use of human test subjects, as well as patient consent, will be received prior to study initiation (Sudre et al. 2021). The three groups will be individuals who are currently infected with SARS-CoV-2 (Group 1), individuals who have been infected but have fully recovered without the development of Long COVID (Group 2), and individuals who have been diagnosed with Long COVID (Group 3). Individuals asymptomatic during COVID-19 infection are more likely unaware of being infected. Because of the high likelihood that a person has unknowingly acquired COVID-19 beforehand, patients may be assigned to Group 1 irrespective of previous SARS-CoV-2 infection history to avoid false association between first infection, immunological markers, and symptoms.

Because testing ends after 1 year (Round 1 Testing), long enough for those in Group 1 to potentially develop Long COVID, those who do develop Long COVID will be followed for an additional year after Round 1 Testing is complete (Round 2 Testing). It will be noted that they developed Long COVID and their data from that point forward will be shared by Group 1 and Group 3. If any individual is reinfected with SARS-CoV-2 during the study, their data will be added to Group 1 data for the time they have COVID-19. Essentially, this will offer insight into the development of symptoms post-COVID-19 by tracking the same individuals rather than comparing different individuals. It also adds more data to each group on top of the large sample size to further increase the generalizability of our findings.

Collection of Qualitative Data – Symptoms:

Each group will report symptoms bidaily over the course of the year using the ZOE Health Study app (formerly known as the COVID Symptom Study app). Although ZOE is a nutritional research company, they have recently expanded their fields of interest to COVID-19 as well. The ZOE Health Study app has been cited in papers countless times, including in the study that contributed to finding the symptoms of COVID-19 (Menni et al. 2020), the study of symptoms for the omicron and delta SARS-CoV-2 variants (Menni et al. 2022), and an exploration of the risks and side-effects of the SARS-CoV-2 vaccine (Antonelli et al. 2021). The ZOE Health Study app asks users to enter general health information such as age and underlying conditions, daily reports of changes and persistence of symptoms, and baseline wellness to compare the relative severity of symptoms. Since data is only available to KCL, KCL has granted permission to access the participants' data in this experiment. In this particular study, the information collected from all groups will focus on what symptoms they experience, changes in symptoms, and symptom severity.

Collection of Quantitative Data - Quantity of Immunological Markers:

RT-qPCR tests will be conducted weekly over the 1-year period to detect the RNA-dependent RNA polymerase (RdRp) gene of SARS-CoV-2 (Chung et al. 2021). The presence of the gene will indicate the persistence of the virus in the body. Participants will use a nasal swab to collect a sample for use in the RT-qPCR test; samples will be dropped off at the lab and kept at -80°C prior to the test for preservation (Chan et al. 2020). This data will help classify which group individuals in the study belong to as the study progresses.

Blood will be collected longitudinally (weekly) for immunological profiling over the 1-year period. Using SepMateTM-50 kits from STEMCELL Technologies, peripheral blood mononuclear cells (PBMCs) and serum will be extracted via centrifugation. 15 mL of LymphoprepTM, a density gradient medium, from STEMCELL Technologies will be pipetted into the designated holes in the SepMateTM tubes. Blood samples collected will be diluted with an equal volume of 2% Fetal Bovine Serum (FBS) Phosphate Buffered Saline (PBS) from STEMCELL Technologies. The blood sample solution will be pipetted down the side of the SepMateTM tube and centrifuged for 10 minutes at 1200 g-forces. This will separate the plasma containing serum and PBMCs which will be transferred to a different container for analysis (STEMCELL Technologies 2018). These samples will be used to analyze both innate and adaptive immune markers.

Enzyme-Linked Immunosorbent Assay (ELISA) kits from Thermo Fisher Scientific will be purchased and used to quantify the immunological markers of the innate immune system (IFN-β, PTX3/TSG-14, IFN-λ1/IL-29, IL-6, IP-10/CXCL10) associated with Long COVID. Specifically, the Invitrogen IFN beta Human ELISA Kit will be used to measure the quantity of IFN-β; the Invitrogen Human PTX-3 ELISA Kit will be used to measure the quantity of PTX3; the Invitrogen IL-29 Human ELISA Kit will be used to measure the quantity of IFN-λ1; the Invitrogen IL-6 Human ELISA Kit will be used to measure the quantity of IL-6; the Invitrogen IP-10 (CXCL10) Human ELISA Kit will be used to measure the quantity of IP-10. These sandwich ELISA kits are pre-coated with the antigen that the desired markers will bind to, and will be used according to the manufacturer's instructions to ultimately produce a visible color change if the presence of the desired molecule. In order to quantify the desired molecule, the absorbance of color will be analyzed at 450 nm wavelength using an ELISA plate reader. To quantify the immunological markers of the adaptive immune system (CD8+ and CD4+ T Cells) associated with Long COVID, immunomagnetic separation will be conducted via EasySepTM kits for CD8+ and CD4+ from STEMCELL Technologies (STEMCELL Technologies 2016). The kits will be used in accordance with the manufacturer's instructions to ultimately isolate the desired cell for quantification.

Once data collection has been completed, the analysis will begin. The goal is to compare immune activity against different experimental groups among those with similar underlying conditions. Those in Group 1 who later developed Long COVID will be compared against themselves for a more robust comparison with a more negligible risk of influence of confounding variables. Overall, we will compare immune activity with symptoms at the

three stages (infected with SARS-CoV-2, fully recovered, and suffering from Long COVID) to determine the greatest predictors of symptoms via immune markers.

Discussion

Expected Results:

We expect Long COVID patients with inflammation-related symptoms (nausea, joint pain, skin rashes, etc.) to exhibit elevated levels of innate immunological markers compared to individuals who fully recovered. The innate immune system is the body's first line of defense. Inflammation is indicative of white blood cells and macrophages engulfing foreign particles, such as SARS-CoV-2. If we assume the immune system remains hyperactive post-infection (Cause 3), inflammation from COVID-19 should persist in those with Long COVID, and innate immune markers such as IFN-β, PTX3/TSG-14, IFN-λ1/IL-29, IL-6, and IP-10/CXCL10 should still be present. Therefore, greater severity of inflammation-related symptoms may correlate with elevated levels of innate immune markers.

On the other hand, we expect Long COVID patients with organ failure-related symptoms (heart palpitations, anosmia, tiredness, headaches, etc.) to exhibit elevated levels of adaptive immunological markers compared to individuals who fully recovered. Neutrophils of the innate immune system can cause damage to the body and also release molecules that enhance T cell proliferation (Kalyan and Kabelitz 2014, Minns et al. 2021). Neutrophils, among many other factors, can cause tissue damage which manifests as symptoms reported by participants. Since neutrophils also increase the proliferation of T cells, elevated levels of CD8+ and CD4+ T cells should be associated with increased neutrophil activity and thereby, organ damage. Thus, greater severity of organ failure-related symptoms may see a more elevated quantity of adaptive immune markers.

We expect individuals with COVID-19 to have the most symptoms and the greatest concentration of immunological markers. Individuals who fully recover are expected to experience no symptoms and display the lowest concentration of immunological markers.

If these findings are confirmed, this would indicate that a hyperactive immune system post-infection is the primary driver of Long COVID. To find elevated immunological markers, the molecules must have been activated during the initial COVID-19 response and persisted for over 8-12 weeks into the Long COVID timeframe. If these findings are proven false through the study, this could indicate that another method for acquiring Long COVID is more prominent. If the initial immune response is too strong (Cause 1), we would find similar levels of immunological markers in COVID-19 patients instead of Long COVID patients, yet still distinguished by inflammation-related or organ damage-related symptoms. If the primary method for acquiring Long COVID is that the immune system is too weak to fight off the infection entirely (Cause 2), we may see lower quantities of the immunological markers both in COVID-19 and Long COVID patients compared to individuals who are fully recovered.

If the results of the study do not agree with any of the proposed outcomes, more research must be done to identify what factors contributed to the incongruity of the data. Uncontrolled variables seemingly unrelated to the study may play a pivotal role in the data observed, thus creating a deviation from the expected results. Erroneous fundamental assumptions may also impact the ability to discern the expected correlation in the data. Perhaps the innate immunological response is not a reliable predictor of inflammatory symptoms as many factors can contribute to lasting inflammation. Similarly, it is possible that the adaptive immunological response is not a reliable predictor of symptoms of organ damage. It is also possible that symptoms cannot be predicted by the immune system in general, but instead a different factor yet to be researched.

Limitations of This Study:

In this study, the medical history (autoimmune diseases, diabetes, allergies, etc.) and lifestyle habits (smoking, drinking, overworking, etc.) of participants were not controlled. Control over this variable was sacrificed in order to optimize the ability to have a large sample size. Medical history and lifestyle habits may affect the data without being tracked. If this had a significant impact on the data, we would not know for sure what the cause is. Additionally, the structure of the study is observational; human subjects were not randomly exposed to different treatment options. This was also a sacrifice that was made since it would be inhumane to force human subjects to be infected with SARS-CoV-2 and potentially die, even though participation is voluntary. It would also be difficult to force people to develop Long COVID or fully recover, as there is no way to control those aspects. However, due to the lack of randomization, indirect and direct biases may arise due to factors both accounted for and not accounted for in the study. Furthermore, other immunological markers, such as immunoglobulins, were not evaluated.

Future Explorations:

With that said, future studies may benefit from a unimodal exploration between underlying health conditions and lifestyle habits and symptoms of Long COVID. Researching the potential correlation between underlying health and symptoms will reveal the statistical significance of underlying health impacting the relationship between immunological markers and symptoms.

A study should also be conducted to determine if the number of times a person has experienced COVID-19 affects their likelihood of developing Long COVID. This study wanted to avoid using the number of reinfections as a factor to consider since data is self-reported and may be inaccurate due to being asymptomatic and unknowledgeable of prior exposure. However, if there becomes a way to determine the number of times reinfected, or if it is determined that self-reporting is an effective enough way to determine the number of reinfections, the scientific community would greatly benefit from understanding the relationship, especially since many people are beginning to experience COVID-19 for the second time.

Our study will collect data on the quantity of the virus present in the body through RT-qPCR. However, this is only to classify patients as either having COVID-19 or Long COVID. In the future, a study should be conducted to determine if the quantity of the virus from the initial infection can predict the symptoms and severity of Long COVID. This may even be able to use the findings from our study. If the quantity of the virus correlates to different quantities of specific immunological markers, the immunological markers may predict certain symptoms. The findings from this study may even be used to verify the results of our study.

Additionally, since there are many other immunological markers, more studies should be done to assess the correlation between severity of Long COVID symptoms and those other markers. Although other studies have discovered through their own rigorous experimentation that a rise in immunoglobulin G (IgG) is associated with moderate COVID-19 symptoms (Su et al. 2022, Wajnberg et al. 2020), it would be benefical to understand the role immunoglobulins play, if any, in the symptoms of Long COVID.

Among the list of other factors that may be able to predict the presence and severity of symptoms is the abundance of specific bacteria in the gut microbiome. Since a higher concentration of *Ruminococcus gnavus* and *Bacteroides vulgatus* and a lower concentration of *Faecalibacterium prausnitzii* are associated with Long COVID (Liu et al. 2022), an investigation may be able to reveal a correlation to symptoms of Long COVID or even immunological markers associated with Long COVID. Additionally, since changes in the gut microbiome are indicative of Long COVID, it will be beneficial to explore whether Long COVID can be treated by artificially adjusting the concentration of the noted gut microbiome bacteria. Likewise, it will be groundbreaking if artificially adjusting the quantity of immunological markers can help treat Long COVID by reducing the likeliness of symptoms potentially seen in our study.

In summary, Long COVID is becoming more and more prominent as cases of COVID-19 continue to rise. It is imperative to explore the relationship between immunological markers and symptoms of Long COVID. These explorations may discover potential ways to treat Long COVID and prevent future severe damage in those who develop Long COVID.

Conflicts of interest

The authors have declared that no competing interests exist.

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