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Correlation between SARS-CoV-2 infection and autoimmune rheumatic diseases: a comprehensive analysis of blood biomarkers in COVID-19 patients

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Abstract. – OBJECTIVE: This study aimed to better understand the link between SARS-CoV-2 infection and autoimmune rheumatic diseases (ARDs) development by analyzing blood samples from 200 registered participants, and measuring biomarkers, such as cell-free DNA, MPO (myeloperoxidase), cathepsin S, and complement type 2 receptor.

PATIENTS AND METHODS: Blood samples were collected from 200 participants (100 females and 100 males; age range: 17-55 years). Participants were divided into five groups based on their COVID-19, SARS-CoV-2 nucleocapsid-specific IgG, and COVID-19 vaccination status. The Enzyme-Linked Immunosorbent Assay (ELISA) was used to identify the biomarkers.

RESULTS: ANOVA and *t*-tests revealed that the group of COVID-19 positive, SARS-CoV-2 nucleocapsid-specific IgG negative, and non-vaccinated individuals had the greatest average value for cathepsin S ($p = 0.03$). This suggests a correlation between the presence of COVID-19 and autoimmune disorders. The group with the greatest average value of cell-free DNA was the COVID-19-negative, SARS-CoV-2 nucleocapsid-specific IgG-negative, vaccinated group ($p = 0.03$). This may suggest that even though they had not contracted the disease, they may have had a stronger immune response to the virus since vaccines can stimulate an immune response even in individuals who have not been infected by the virus. Furthermore, the SARS-CoV-2 nucleocapsid-specific IgG+ and COVID-19 positive groups had higher levels of myeloperoxidase, a neutrophil protein linked to inflammation and tissue damage, suggesting a higher risk of autoimmune rheumatologic illnesses ($p = 0.02$).

CONCLUSIONS: The study suggests a correlation between COVID-19 status and the development of autoimmune disorders, as well as a potential link between a COVID-19 infection and a strong immune response. However, this study had limitations – the selection of participants and a small sample size, which offers potential for further research and examination.

Key Words:

COVID-19, Nucleocapsid protein, IgG, Cell-free DNA, MPO, Cathepsin S, CD21, SARS-CoV-2, Immune system, Inflammation, Rheumatic, Autoimmune rheumatic disease (ARD's).

Introduction

SARS-CoV-2, a virus that has claimed millions of lives globally causes respiratory infections in patients. It has already been associated with autoimmune diseases, as in a review of 64 articles concluded by Sheikh et al[1] (2021). The authors mentioned the Guillain-Barre syndrome (GBS) as one of the manifestations of COVID-19[1]. Another study by Yazdanpanah et al[2] (2021) concluded the possible involvement of COVID-19 in ADs by presenting 33 post-COVID patients with autoimmune disorders (ADs – GBS, autoimmune hemolytic anemia, immune thrombocytopenia – ITP, Kawasaki disease – KD, subacute thyroiditis)[2]. The ADs linked with COVID demand an early diagnosis along with an expeditious induction of treatment measures for successful rehabilitation and reduced mortality rate. This quantitative analysis was done using 4 biomarkers, namely, cathepsin S, cell-free DNA, myeloperoxidase, and complement receptor 2.

Cathepsin S (CTSS) is a lysosomal cysteine protease which is involved in the degradation of the extracellular matrix and the activation of proinflammatory cytokines[3-5]. CTSS can be found in macrophages which contain elastase activity. It is known to play roles in tissue destruction and invasion. Previously, CTSS has been known to be a biomarker for chronic obstructive pulmonary disease (COPD), sepsis, autoimmune diseases such as rheumatoid arthritis (RA), osteoarthritis, and systemic lupus erythematosus (SLE)[6,7].

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Human complement receptor type 2 (CR2, CD21) is a multifunctional surface glycoprotein (GP) that adheres to many target molecules, such as the type I cytokine interferon-alpha (IFN-alpha), C3 components iC3b, C3dg, and C3d. It is most commonly present on follicular dendritic cells (DCs) and during different phases of B-lymphocyte maturation. C3d ligand also regulates the cell cycle of B cells. As compared to acute infections of Epstein-Barr virus (EBV), soluble CD21 has been recorded in lower quantities in patients having autoimmune diseases, including RA, SLE, Stevens-Johnson syndrome (SjS), and multiple sclerosis (MS). A rise in CD21low B cells has been reported in various conditions linked to prolonged immunological activation, such as immunodeficiency or autoimmune illness. A substantial decline has been noted in the SLE patients' B cells' CR1/CR2 density as well as in synovial B cells in RA patients. According to recent data[8], CD21-/low B cells were found in abundant quantities in SjS, SLE, and RA patients' blood samples as compared to healthy donors' blood. An increased amount of this cell type was also found in the blood of individuals having chronic viral infections (human immunodeficiency virus – HIV, hepatitis C virus – HCV) along with malaria patients. In SLE patients, a positive link between the increasing presence of these cells and the rising severity of the disease has been established as well. In humans, B cell CR1/CR2 levels are halved, if not less, in SLE patients and might be inversely related to the disease severity. In regard to COVID-19, research indicated that reduced levels of CD21 expression can be used as a marker to predict the occurrence of hemophagocytic lymphohistiocytosis in COVID-19 patients. Henoch-Schoenlein Purpura (HPS) is an immune-mediated life-threatening disease culminating from defective NK cells and CTLs[9].

Myeloperoxidase, also known as MPO, is a member of the heme-containing peroxidase class that is primarily generated by polymorphonuclear neutrophils (PMNs). The MPO gene, which is found on chromosome 17's long arm, encodes MPO. By means of degranulation/exocytosis, it is released outside the cell. It is the only variety of peroxidase that utilizes hydrogen peroxide to oxidize various halides as well as pseudo-halides to produce various HOX. Thus, MPO produces reactive oxygen and nitrogen molecules as part of its antimicrobial actions. For MPO to function effectively, regulated MPO production at the infection location is crucial. An unrestrained degranulation enhances the inflammatory response and can result in tissue injury despite the lack of any inflammation[9]. Multiple inflammatory illnesses and neurodegenerative disorders have been associated with raised MPO levels, such as Alzheimer's, rheumatoid arthritis, MS, and Parkinson's[10].

Most endogenous DNA is located inside the cell in the nuclei as well as organelles like mitochondria. cf-DNA or cell-free DNA refers to all non-encapsulated DNA in the bloodstream. It is secreted into the circulation following cell death, caused by various methods (apoptosis, pyroptosis, etc.). Because of its rapid breakdown and lack of access to DNA sensors inside the cell, cf-DNA usually does not cause inflammation under physiological circumstances. However, the discovery of extracellular, cell-free DNA in a number of clinical states, such as autoimmune disorders, has sparked interest in the study of cf-DNA as a possible biomarker. Cells release significant quantities of cf-DNA in pathological processes in the form of tiny vesicles bound by a membrane or microparticles (MPs)[11,12]. In the presence of microbial nucleic acid, usually plasmacytoid dendritic cells (pDCs) react powerfully but fail to elicit immunological responses in response to cf-DNA. Initially, it was believed that this tolerance to self-DNA was caused by the distinct sequence variations between self and microbial DNA. These carrier proteins, which are frequently enhanced in inflammatory circumstances, can help DNA uptake while also protecting it from deterioration, which encourages the production of pro-inflammatory responses[1].

Patients and Methods

We analyzed blood samples from 200 registered participants (100, 50.0% females; 100, 50.0% males) in this study. Included participants fell under the age distribution of 17 to 55 years. Five groups were formed based on their COVID-19 incidence, SARS-CoV-2 nucleocapsid-specific IgG, and COVID-19 vaccination status (Table I).

COVID-19-positive patients recovered from COVID-19 after paucisymptomatic disease and did not require hospitalization or corticosteroid therapy. Clinical signs and symptoms and a PCR screening for SARS-CoV-2 using a nasopharyngeal swab were used to identify SARS-CoV-2 infection in all subjects. Patients without a clinical history of the illness were considered as COVID-19 negative.

Table I. Study subjects five groups, formed based on their COVID-19 incidence, SARS-CoV-2 nucleocapsid-specific IgG, and COVID-19 vaccination status.

Groups	Cov Status	IgG Status	Vaccination Status
Group 1	Negative	Positive	Vaccinated
Group 2	Negative	Negative	Vaccinated
Group 3	Positive	Positive	Vaccinated
Group 4	Positive	Positive	Unvaccinated
Group 5	Positive	Negative	Unvaccinated

The NovaLisa SARS-CoV-2 (COVID-19) IgG ELISA diagnostic kit (NovaTec Immunodiagnostics GmbH, Dietzenbach, Germany) was used to determine IgG directed against the SARS-CoV-2 nucleocapsid protein. Analytical sensitivity is the likelihood that the assay will lead to a positive result in the presence of the analyte, and in accordance with the manufacturer's directions, the measured positive results correlate to a specificity of 99.53%. The research was carried out in line with the instructions for the ELISA kit.

Statistical Analysis

ELISA diagnostic kit for routine analysis was used for quantitative measurement of myeloperoxidase (MPO, FineTest Biotech Inc., Boulder, USA), cell free DNA (cf-DNA, FineTest Biotech Inc., Boulder, USA), cathepsin S (CTSS, FineTest Biotech Inc., Boulder, USA) and complement receptor 2 (CD21, FineTest Biotech Inc., Boulder, US). In accordance with the protocol unique to the ELISA kit, the research was conducted.

Statistical analysis employed SPSS v.28.0.1.1 software (IBM Corp., Armonk, NY, USA), with intra-group comparisons determining *p*-values; significance was established at $p<0.05$.

Results

CTSS

To analyze all the groups for discrepancies of CTSS, single-factor ANOVA was performed. The statistical analysis revealed significant differences among the groups, as evidenced by a *p*-value of 0.03, which is below the conventional significance level of 0.05. The *F*-statistic exceeded the critical *F*-value (*F*stat > *F*crit), leading to the rejection of the null hypothesis (Ho) that cathepsin S is not a biomarker of COVID-19-related autoimmune diseases. This rejection supports the alternative hypothesis (H1) that cathepsin S is indeed a biomarker of COVID-19-related autoimmune diseases. To further examine the specific variations between individual groups, *t*-tests were conducted to assess differences in the COVID-neg/pos, IgG-neg/pos, and vaccinated/non-vaccinated groups. These additional analyses aimed to provide a detailed understanding of the discrepancies within each subgroup, shedding light on the significance of Cathepsin S as a potential biomarker in the context of COVID-19-related autoimmune diseases.

The COVID-neg IgG-neg vaccinated group has an average of 34.04 ng/ml, SD= 4.56 (max – 39.8 ng/ml). The COVID-neg IgG-pos vaccinated has an average of 33.83 ng/ml, SD= 6.36 (max – 41). The COVID-pos IgG-pos vaccinated group has an average of 32.22 ng/ml, SD= 40 (max – 40). The COVID-pos IgG-pos non-vaccinated group has an average of 33.55 ng/ml, SD= 5.01 (max – 41). The COVID-pos IgG-neg non-vaccinated group has an average of 36.06 ng/ml, SD= 6.9 (max – 49).

The results of *t*-tests between IgG neg/pos with a *p*-value of 0.02 showed that IgG pos individuals had an average of 33.20 ng/ml whereas IgG neg individuals had an average of 35.05 ng/ml which could indicate that IgG neg individuals who did not have any memory immunity had higher levels of CTSS ($p=0.02$). *t*-tests between COVID-positive and COVID-negative were also performed, which showed that the COVID-positive group had an average of 33.94 ng/ml and the COVID-negative group had an average of 33.94 ng/ml ($p=0.9$). *t*-tests between vaccinated groups that had an average of 33.36 g/ml and that of the non-vaccinated were 34.81 with a *p*-value of 0.08.

cf-DNA

ANOVA test was also performed for cell-free DNA wherein the *p*-value was 0.003 (*p*-value <0.05), which means that the null hypothesis was rejected. In order to prove an alternate hypothesis, *t*-tests were also performed among the groups that showed us that the COVID-neg IgG neg vaccinated group had a much lower average of 45.225 than COVID-pos IgG pos vaccinated and COVID-pos IgG pos non-vaccinated, average of 59.47 ng/ml ($p<0.007$). Furthermore, *t*-tests

between groups COVID negative and COVID positive had no significant difference as p ($T \leq t$) 0.78. There was also no significant difference between vaccinated and non-vaccinated groups with p ($T \leq t$) 0.24. But on the other hand, the *t*-test of IgG positive (62.725 ng/ml) and IgG negative (50.81 ng/ml) groups showed a statistically significant result with a p-value of 0.006 ($p<0.05$).

CD21

To analyze all the groups for discrepancies, single factor ANOVA was performed in excel which implied that all the groups were different or had a different pattern of readings as the p=0.01 (<0.05) (Fstat > Fcrit). Subsequently, to further scrutinize the precedent result, *t*-tests assuming unequal variances were conducted within the groups yielding more specific insights. Fascinatingly, statistically significant variations between groups were observed concerning COVID neg/pos, IgG neg/pos, and vaccinated/non-vaccinated statuses.

Firstly, COVID-neg IgG neg vaccinated group turned out to be different from the COVID-pos IgG neg non-vaccinated group (p=0.025), the former one (9.03 ng/ml) had a higher average value than the latter (7.4 ng/ml). When all of the participants were compared after tuning down to a single variable, i.e., vaccinated/non-vaccinated *via t*-test, the vaccinated group was found to have an increased mean as compared with the non-vaccinated (9.13 ng/ml; 8.17 ng/ml) [p-value = 0.01 (one-tailed); 0.02 (two-tailed)]. Further, considering COVID neg/pos as a variable, another *t*-test revealed that COVID positive group (8.45 ng/ml) had lower levels of CD21 than the COVID-negative group (9.18 ng/ml) (p-value = 0.013;0.03). These findings suggest that lower levels of CD21 are seen in non-vaccinated participants when compared with vaccinated ones and in COVID-positive patients as compared to COVID-negative ones. These CD21 levels might be lower because of ongoing infection and the absence of IgG antibodies, implying a lack of attainment of long-term immunity or vaccination.

Secondly, discrepancies were found in the COVID-pos IgG pos non-vaccinated and COVID-pos IgG neg non-vaccinated groups (p=0.03). The former had a higher average value (8.9 ng/ml) than the latter (7.4 ng/ml). Since both the groups are COVID-positive and non-vaccinated, this outcome might imply that the presence of IgG is related to a rise in CD21 levels, facilitating an improved immunity against the infection. To further strengthen this point, separate *t*-tests were performed after dividing the whole population into IgG negative/positive, which showed that the IgG positive group (9.09 ng/ml) had a higher average value of CD21 levels than the negative group (8.23 ng/ml) (p=0.01; 0.02). Thirdly, with an average of 9.33 ng/ml, the COVID-neg IgG pos vaccinated group appears to be different from the COVID-pos IgG neg non-vaccinated group, which has an average of 7.4 ng/ml (p=0.0016). The presence of IgG in the former group might be due to past infection or vaccination. An increased level of CD21 favors the earlier finding that IgG presence and vaccination might be the reason behind this elevation. On the other hand, the COVID-infected non-vaccinated participants with an absence of IgG have lower levels of CD21. Lastly, the COVID-pos IgG pos vaccinated group turned out to be different from the COVID-pos IgG neg non-vaccinated group (p=0.0026), with the former one having an elevated average of 9.015 and the latter one, 7.43 ng/ml. In addition, strengthening the precedent result, this finding is suggestive of the statement that vaccinated people, despite being COVID-positive, have higher levels of CD21 than their non-vaccinated COVID-positive counterparts (Confidence level 95%).

After contemplating the results and the analysis, it can be implied that CD21 levels alter with the aforenamed variables, i.e., COVID negative/positive, vaccinated /non-vaccinated, and IgG pos/neg. Being COVID-positive or vaccinated or having IgG antibodies was linked with an increased CD21 level. It was noticed that the presence of IgG spiked the levels irrespective of COVID-19 positivity, as both groups were COVID-positive and non-vaccinated. Also, no relevant correlation was seen with the other biomarkers.

MPO

An ANOVA test between the above-mentioned 5 groups was performed; the p-value within the group was 0.111615 ($p<0.05$), implying that the hypothesis was rejected. As the hypothesis is rejected, we consider each group in the *t*-test. The p-value of IgG neg and IgG pos *t*-test came out 0.0269. It means that IgG may be considered as one of the reasons for elevated MPO levels. Another *t*-test about COVID-neg and COVID-pos p-value became 0.0239, which means COVID-19 can be the cause of elevated MPO. In the *t*-test between vaccinated and non-vaccinated, the p-value came out to be 0.177; thus, vaccination does not measure MPO level. Besides, *t*-test is also done among groups that differ only by

COVID status. For instance, the *p*-value between groups 1 and 4 was 0.0292, while the *p*-value between groups 1 and 3 was 0.0155. Moreover, the *p*-value for groups 2 and 3 was 0.58.

Discussion

CTSS

Our findings indicate that non-vaccinated groups had higher levels of CTSS levels. The role of CTSS in the pathogenesis of acute respiratory distress syndrome (ARDS) has been reported[13]. Considering the objective that ARDS caused by COVID-19 differs from ARDS caused by other factors[14], the elevated CTSS might indicate the severity of disease correlating with increased inflammation. Furthermore, our findings support the hypothesis that Cathepsin S could be a biomarker of COVID-19 related autoimmune diseases and suggest a potential relationship between SARS-CoV-2 nucleocapsid protein IgG status, vaccination, and CTSS levels. However, further investigation is required to fully understand the implications of these relationships.

cf-DNA

Our findings demonstrate significant differences in cell-free DNA levels among various groups. Vaccination status, SARS-CoV-2 nucleocapsid protein IgG status, and potentially COVID-19 infection seem to be associated with these variations. While the relationship between cell-free DNA and COVID-19 infection or vaccination might not be straightforward, the statistically significant difference between SARS-CoV-2 nucleocapsid protein IgG positive and negative groups suggests an intriguing correlation between immune responses and cell-free DNA levels. It is noteworthy, that cf-DNA showed a significant correlation with neutrophil to lymphocyte ratio (NLR), ferritin, lactate dehydrogenase (LDH), procalcitonin, and IL-6. A direct correlation between COVID-19 and cf-DNA shall be investigated further. However, cf-DNA might be used as an effective marker of COVID-19 implications and of underlying health conditions and risk factors of severe illness during SARS-CoV-2 infection[15].

CD-21

Our findings illustrate a complex interplay between CD21 levels, COVID status, SARS-CoV-2 nucleocapsid protein IgG presence, and vaccination. These results suggest that CD21 levels can be influenced by these factors, potentially reflecting variations in immune response and long-term immunity. It is noteworthy, that CD21 can indicate the status of B lymphocytes. The significance of B cells alterations in the context of COVID-19 is evident. Lymphopenia and shifts in B cell subpopulations appear to be linked to disease severity and clinical outcomes, reflecting the crucial role of immune responses in COVID-19 pathology[16].

MPO

Our findings provide insights into the relationship between MPO levels, SARS-CoV-2 nucleocapsid specific IgG status, COVID-19 status, and vaccination. Elevated MPO levels might be associated with SARS-Cov-2 nucleocapsid protein IgG positivity and COVID-19 infection. Vaccination, on the other hand, does not seem to significantly affect MPO levels. The variations in *p*-values for different group comparisons suggest nuanced relationships that require further investigation for a comprehensive understanding of their implications. The contribution of MPO in increasing of endothelial glycocalyx shedding in COVID-19 has been reported[17]. Considering these data we assume, that MPO levels might be considered as a prognostic factor of COVID-19 associated or induced coagulopathies.

Conclusions

The data presented in this study revealed significant differences in biomarker levels in different groups of patients with COVID-19 infection, IgG seropositivity, and vaccination status. Specifically, MPO, which is associated with active infection, showed elevated levels in COVID-19 positive patients. On the other hand, IgG positive patients exhibited increased concentrations of all biomarkers, suggesting potential remission or the development of immunity against the infection. The biomarkers analyzed in this study are closely linked to the immune response, supporting the idea that their variations could be indicative of disease progression[13,18,19] and recovery.

The vaccinated group showed higher average values for CD21 and cf-DNA compared to the non-vaccinated group, while the opposite was observed for CTSS. These findings may shed light on the impact of vaccination on the immune response[20] and how it affects the levels of specific biomarkers[19,21].

The results of this research provide valuable insights into the potential roles of these biomarkers in disease progression, recovery, and the

development of post-recovery complications[22], such as autoimmune diseases, in COVID-19 patients. Further investigation and follow-up studies are warranted to fully understand the implications of these findings and to explore the clinical utility of these biomarkers in managing and predicting outcomes in COVID-19 patients.

Data Availability
All data generated or analyzed during this study are included in this published article; the datasets are available from the corresponding author on reasonable request.

Ethics Approval
Ethical approval for this study was obtained from the Bioethics International Committee of the Petre Shotadze Tbilisi Medical Academy (identification code: 22022022/1, Tbilisi, Georgia). All procedures conducted in this study adhered to the Helsinki Declaration of 1975, as revised in 2013. Patients were provided with detailed information regarding the study design and objectives.

Informed Consent
Written informed consent was obtained from all participants prior to their inclusion in the study.

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

Authors' Contributions
All authors equally contribute to the present manuscript preparation.

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