Serum Levels of IL-21 and IL-27 Do not Reflect differential Avidity of Anti-SARS-CoV-2 IgG Antibodies in Symptomatic and Asymptomatic COVID-19 Patients

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ABSTRACT

The quantity and quality of anti-Spike (anti-S) antibodies, rapidly elicited by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), are necessary for understanding the immune response induced by infection. Antibody avidity is a good indicator of the quality of antibody response. Interleukin (IL)-21 and IL-27 are two cytokines that play vital roles in the affinity maturation process. Therefore, we decided to investigate whether there are any relationships between the avidities of antibodies against spike and nucleocapsid (N) antigens of SARS-CoV-2 and serum levels of these cytokines in symptomatic and asymptomatic coronavirus disease 2019 (COVID-19) patients.

Forty symptomatic COVID-19 patients and 40 asymptomatic carriers were enrolled. Anti-S and anti-N IgG avidity indices (AIs) were determined using a modified enzyme-linked immunosorbent assay (ELISA). Serum levels of IL-21 and IL-27 were quantified by specific ELISA kits.

AI values of both anti-S and anti-N IgG were lower in the symptomatic group compared to asymptomatic cases, while only that of anti-N IgG was statistically significant. For IL-21 and IL-27 serum levels, no significant difference between the two groups was shown. Also, we could not find any correlations between cytokine levels and antibody AI values. However, an inverse correlation between anti-S AI value and IL-27 serum level was found in asymptomatic patients.

Our study suggests that serum levels of IL-21 and IL-27 cannot predict differences in anti-S and anti-N IgG avidity between symptomatic and asymptomatic COVID-19 patients.

Keywords: Antibody avidity; Interleukin-21; Interleukin-27; Severe acute respiratory syndrome coronavirus 2

INTRODUCTION

At the end of 2019, a highly contagious strain of coronavirus, namely severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), appeared.[1,2] This virus led to a pandemic called 'coronavirus disease 2019' (COVID-19), posing serious risks to public health and safety due to its acute respiratory disease.[3] The spike (S) glycoprotein of the virus is the main cause of infectiousness. The S1 subunit of the S protein binds to

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angiotensin-converting enzyme 2 (ACE2) receptors, which are distributed in different organs.[4]

Patients with COVID-19 usually produce noticeable SARS-CoV-2-specific antibodies several weeks after symptom onset. Within 4 weeks of displaying symptoms, the humoral response triggered by SARS-CoV-2, specifically the S- and nucleocapsid (N)-specific IgM response, shows peaks and is no longer detectable 3 months post symptom onset. Switching to IgG usually occurs around day 14, and IgG remains detectable for up to 36 months.[5]

Antibodies bind to their specific antigens through non-covalent interactions and their affinity can improve through the process of affinity maturation. This process results in the production of high avidity antibodies. As the interaction between the receptor-binding domain (RBD) of the S protein and the ACE2 receptor is driven by a high-affinity, IgG molecules with a high affinity are needed to disrupt this interaction.[6]

Cytokines play essential roles in modulating lymphocyte activity. Recent studies have indicated that two specific cytokines, interleukin (IL)-27 and IL-21, can directly impact T follicular helper (TFH) cell differentiation to act on B cells.[7] Researchers have demonstrated that in naive T cells, IL-21 enhances the regulation of Bcl-6, which acts as a transcriptional regulator of TFH cells. Deficiency in proper IL-21 signaling leads to a lower number of B cells and a reduced level of high affinity antibodies. IL-27 signaling triggers the generation of IL-21 in T cells, acting as an autocrine factor for TFH cell maintenance and high-affinity antibody production. The lack of IL-27 receptor on B cells ultimately results in a selective loss of high-affinity antibodies. [8, 9]

Given the involvement of IL-21 and IL-27 in the formation of high-quality anti-S and anti-N antibodies, as well as the higher avidities of anti-S and anti-N IgG antibodies in asymptomatic in comparison to symptomatic COVID-19 patients, this study aimed to quantify the serum levels of IL-21 and IL-27 and assess potential relationships between IL-21/IL-27 levels and anti-S and anti-N IgG avidity in symptomatic and asymptomatic patients with COVID-19.

MATERIALS AND METHODS

Ethics Statement

After obtaining written informed consent from participants, the results of this study, which were approved by the Research Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1401.955), were obtained. No diagnostic or therapeutic procedures were imposed on any of the study participants. Demographic data, clinical, and laboratory conditions were collected for each patient.

Study Population

Real-Time PCR was conducted on 80 individuals diagnosed with SARS-CoV-2 infection, and the results were evaluated by a specialist clinician according to clinical protocols. The study subjects were divided into two groups: 40 symptomatic and 40 asymptomatic. The two groups showed no statistically significant differences in terms of age and sex. The symptomatic group showed lung involvement and had been admitted to Sina Hospital from September to December 2021, affiliated with Hamadan University of Medical Sciences. The study also consisted of an asymptomatic group, which did not exhibit any symptoms but whose SARS-CoV-2 RT-PCR tests were positive. These subjects were selected from symptomatic patients' family or relatives. Exclusion criteria included the presence of autoimmune or immunodeficiency diseases or having a history of any inflammatory or infectious diseases during the past 6 months.

Serological Assays
Avidity Assay

The avidity index of anti-S IgG and anti-N IgG antibodies in serum samples was measured by a modified enzyme-linked immunosorbent assay (ELISA) procedure (Pishtaz Teb, Iran).[13] To optimize the test, urea solutions with concentrations of 2, 4, 6, and 8 M were tested to measure avidity index (AI), and based on our experience, using 6 M urea provided the most suitable results. To begin the experiment, 100 µL of standards, controls, and samples (that were previously diluted at a ratio of 1 to 101) were added to the wells. The wells were then covered and incubated at 37 ℃ for 30 minutes. For each sample (tested for measuring AI of each antibody), two sets of wells were designated. Following incubation and washing step, one set was filled with 300 µL PBS ("intact" wells). In the second set of wells, 6 M urea (300 µL) was added ("denaturation" wells). Then, the plates were incubated for 10 minutes at room temperature. Afterwards, the wells were washed with the washing solution followed

by addition of 100 µL of conjugate solution. The wells were covered and incubated at 37 ℃ for 30 minutes again, and then washed five times. Subsequently, 100 µL of chromogen solution was added. The plate was kept in the dark at room temperature for 15 minutes. Finally, stop solution (100 µL) was added and OD was read at 450 nm. AI values for each antibody were calculated based on the formula presented below:

AI= (OD in the "denaturation" well/OD in the "intact" well)×100%.

Previous research has established that AI values of≤40% are considered as low, AI values between 40% and 50% are considered as intermediate, and AI values≥50% are designated as high avidity.[7]

ELISA for cytokines
To assess IL-21 and IL-27 levels in samples, specific ELISA kits were purchased (Zellbio GmbH, Germany). The kits had a standard of original concentration that was diluted to make standard samples of lower concentrations. At the beginning, 40 µL of sample and then 10 µL of IL-21 antibody were added. For standard wells, 50 µL of standards and 50 µL of streptavidin-HRP were added. The plate was incubated for 60 min at 37 ℃. The liquid in the wells was then drained and washed five times with the washing solution which required a 30-second soaking time. Then, 100 µL of chromogen solution was added to each well, with an incubation time of 20 minutes at 37 ℃ away from light for color development. To stop the reaction, 50 µL of stop solution was added. Absorbance was read at 450 nm. The same procedure was followed for IL-27 measurement.

Statistical Analysis
Statistical analysis was performed using GraphPad Prism (version 8.4.3) and SPSS (version 25) software. Normal distribution was evaluated by Anderson–Darling, D'Agostino and Pearson, Shapiro-Wilk, and Kolmogorov–Smirnov tests. A T-test or Mann-Whitney test was used to compare the data between the two groups. Pearson or Spearman correlation test was used to determine correlations between cytokine concentrations and AI values. Graphs were produced with GraphPad Prism (version9). A p value≤ 0.05 was considered statistically significant.

RESULTS

Demographic Characteristics
In total, we analyzed samples of 40 symptomatic and 40 asymptomatic patients. There was no significant difference for gender and age between the two groups (p>0.05). The demographic characteristics of the individuals are summarized in Table 1.

Table 1. Demographic data of the study populations

	Symptomatic	Asymptomatic	p
Female/ Male	22/18	17/23	0>0.05
Age (years)	55±9	49±15	0.069
Dose of vaccine (0/1/2)	10/11/19	1/18/21	0<0.05
Type of vaccine			0<0.05
1. Sinopharm	26	25	
2. Sputnik	0	6	
3. AstraZeneca	2	7	
4. Barakat	1	1	
5. Pastocovac	1	0	

Evaluation of Anti-S and Anti-N IgG Avidity in SARS-CoV-2 Symptomatic and Asymptomatic Individuals

Avidities of anti-S and anti-N IgG (in AI%) were determined by a modified ELISA method, using urea as a chaotropic agent to separate antibodies with lower avidities. In this method, AI is calculated by the proportion of OD from urea-treated wells and OD of intact wells. The avidity evaluation assay revealed that, although median AI values for both anti-S and anti-N antibodies were higher in asymptomatic individuals compared to the symptomatic ones, only the difference

in anti-N IgG AI was found to be statistically significant (*p*=0.038) (Figure 1A and 1B).

Determining the Levels of Serum IL-21 and IL-27 in Symptomatic and Asymptomatic Groups

Serum levels of IL-21 and IL-27 were measured in symptomatic and asymptomatic patients using ELISA as stated in the "Materials and methods" section. The median values for IL-21 and mean values for IL-27 were not found to be statistically different between the two groups (*p*>0.05 for both) (Figure 1C and 1D).

Correlation Analyses

We analyzed correlations of serum levels of IL-21 and IL-27 with anti-S and anti-N AI values in the two groups. In the symptomatic group, there was no correlation between cytokine levels with anti-S IgG AI

(*p* values for IL-21 and IL-27 were 0.06 and 0.59, respectively and r values were 0.21 and 0.08, respectively). Similarly, no statistically significant correlation was found between anti-S IgG avidity with serum IL-21 level in asymptomatic group (*p*=0.76, r=0.04). Interestingly, we found a significant, inverse correlation between anti-S AI and serum level of IL-27 in asymptomatic cases (*p*=0.03, r=-0.32). In addition, we could not find significant correlations between anti-N IgG AIs and IL-21 (*p* values for symptomatic and asymptomatic groups were 0.92 and 0.82, respectively and the corresponding r values in symptomatic and asymptomatic groups were calculated to be 0.01 and 0.03, respectively) and IL-27 levels (*p* values in symptomatic and asymptomatic groups were 0.41 and 0.07, respectively with the corresponding r values of 0.13 and -0.28, respectively) in any of the groups.
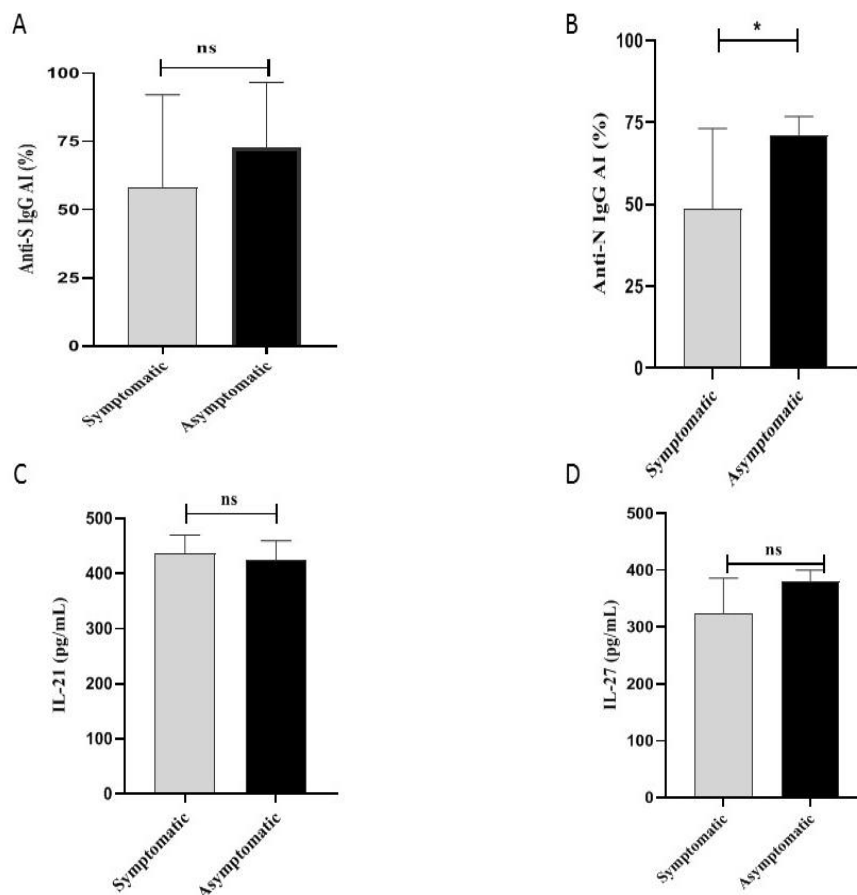
Figure 1. Comparison of anti-S IgG (A), anti-N IgG (B), AI values and serum levels of IL-21 (C), and IL-27 (D), in symptomatic and asymptomatic groups. Data are depicted as bar graphs with medians (for AI values and IL-21) or means (for IL-27). AI: avidity index; ns: not significant. (*: *p*=0.038)

DISCUSSION

During SARS-CoV-2 infection or vaccination, humoral immune responses play a key role in preventing virus propagation. The kinetics of anti-SARS-CoV-2 IgG avidity maturation plays a vital role in interpreting anti-SARS-CoV-2 IgG avidity results properly, and it is also suggested as an indicator of antibody quality and protection against further infection.[8] Antibody avidity develops because of the B cell somatic hypermutation in the germinal centers (GCs).[9, 10] It is well established that the two cytokines, IL-21 and IL-27, are essential for a T cell-dependent antibody response in mouse models. These two cytokines lead to antibody affinity maturation, act as regulators of TFH function in GCs,[11] and directly enhance GC B cell activities.[12] Antibody profiles in symptomatic and asymptomatic individuals change during the infection. This encouraged us to investigate whether or not there are correlations between serum levels of IL-21/IL-27 with avidity of anti-SARS-CoV-2 antibodies.

Antibody avidity is defined as the strength of bivalent or multivalent interactions between an antibody and its epitope. Based on the evidence, antibody binding strength increases during the course of infection and remains stable for a few months.[13]

Although the findings of our study showed that the avidity of antibodies were higher in asymptomatic patients than symptomatic cases, the difference was only significant for anti-N IgG. The previous study by Hajilooi et al on a larger sample size reported significantly higher anti-S and anti-N IgG antibodies in asymptomatic versus symptomatic SARS-CoV-2 infected cases.[13] Several studies have shown that incomplete avidity maturation in SARS-CoV-2 infection may lead to later re-infection.[14] Hendriks et al suggested that in COVID-19 patients, antibodies with high titers but low avidity are associated with the severity of the disease.[7]Previous studies on varicella-zoster virus (VZV) infection have reported that out of 52 infected patients, 41 had high avidity antibodies and had been previously vaccinated. Regardless of vaccination status, clinical severity was lower in high avidity cases compared to low avidity patients.[15] A notable point in our study is the absence of a significant difference in anti-S IgG avidity between the two groups.

Our study highlighted that IL-21 serum level was higher in symptomatic subjects in comparison to asymptomatic ones, while higher serum level of IL-27 was found in asymptomatic subjects. These differences, however, were not statistically significant. Ozturk Acet and colleagues in 2021, demonstrated that IL-21 cytokine levels were higher in patients with COVID-19 pneumonia compared to non-COVID-19 pneumonia cases and they presented this cytokine as a negative prognostic factor.[16] Further, in 2022, another study was conducted to investigate cytokine profile in COVID-19 patients and the control group. The results indicated that the serum levels of all investigated cytokines, including IL-21, were higher in patients compared to healthy individuals.[17]

In another study conducted in 2021, Tamayo-Velasco et al investigated and compared serum cytokines in COVID-19 patients. They showed that high levels of hepatocyte growth factor (HGF) and IL-1α along with low levels of IL-27 could be used as biomarkers related to the poor prognosis of the disease.[18] In a 2022 study by Klingler et al, serum samples from 52 convalescent COVID-19 patients were compared with 21 healthy individuals. It was observed that IL-4 and IL-27 were significantly higher in hospitalized patients.[19]

There are some contradictions between the findings of this study and the results of our previous study in terms of lack of statistically significant difference in serum anti-S IgG avidity between the symptomatic and asymptomatic groups. We could not find any statistically significant differences in the serum levels of IL-21 and IL-27 between the two groups. Correlation analyses of anti-S and anti-N IgG antibody avidity with serum levels of IL-21 and IL-27 showed that there were no expected correlations, and even in the case of IL-27 in asymptomatic patients, there was a unexpected inverse correlation. All these discrepancies could be due to several reasons. Among these reasons, we can mention: small sample size, primary/secondary infection, existence of different subgroups based on the type of vaccine and vaccine dose and the presence or absence of comorbidities and the type of underlying disease in some patients. We speculate that first, serum levels of IL-21 and IL-27 cytokines increase, and after affecting GC reactions and completion of affinity maturation, serum levels of these cytokines would decrease again. Therefore, it is suggested that if the sampling was done earlier, maybe we could have found the expected results. It is also plausible that secretion of IL-21 and IL-27 required for antibody affinity maturation is restricted mainly to GCs and these

cytokines act in combination with surface T cell-B cell interactions occurring in lymph nodes or spleen without apparent changes in serum IL-21 or IL-27 levels. Ideally, future studies should perform more comprehensive analyses including flow cytometry tests and intracellular staining for the mentioned cytokines or cell marker assays on lymph node or spleen cells (that are performed in animal studies) and immunohistochemical/immunocytochemical assays on lymph node tissue samples to potentially achieve different findings. Such experiments are suggested to support the concept provided in our study.

As the main limitation of our study, only a single sample from the subjects was available, which limited the possibility of performing follow-up experiments over time and conducting further analyses.

Altogether, there was no significant difference of anti-S IgG AI and serum levels of IL-21 and IL-27 between the symptomatic and asymptomatic patients but anti-N IgG AI was significantly higher in asymptomatic cases. In addition, the expected correlations between anti-S and anti-N IgG avidity and the serum levels of these two cytokines were not observed. Overall, it can be concluded that measuring and comparing serum levels of IL-21 and IL-27 cannot serve as an indicator of anti-S/anti-N IgG avidity difference between symptomatic and asymptomatic COVID-19 patients.

Therefore, conducting more studies on a larger number of samples as well as sampling at different time intervals are highly recommended to confirm our hypothesis.

STATEMENT OF ETHICS

Informed consent forms were obtained from all participants. All methods were done in accordance with the relevant guidelines and regulations.

The study was approved by the Research Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.REC.1401.955)

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

The analyzed data obtained during this study can be available from the corresponding author upon reasonable request (E-mail: m.rastegaripouyani@umsha.ac.ir).

AI ASSISTANCE DISCLOSURE

No AI tool was used for preparing this paper.

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