**HIGH LEVEL PRODUCTION OF SARS-COV-2 ANTIBODIES ALSO AFFECTED THE FORMATION OF ANTI-NUCLEAR AUTOANTIBODIES**

Excess SARS COV-2 antibody and ANA

Faruk Dişli1, MSc; Sedat Yıldız1, PhD

1 İnönü University, Faculty of Medicine, Department of Physiology

\*Corresponding author: Prof. Dr. Sedat YILDIZ

İnönü University,

Faculty of Medicine,

Department of Physiology

Battalgazi/Malatya

TÜRKİYE

[sedat.yildiz@inonu.edu.tr](mailto:sedat.yildiz@inonu.edu.tr)

**Conflict of interest**

None of the authors have a conflict of interest to disclose

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**Data Availability**

Data will be made available on reasonable request

**Ethical appproval**

Ethical approval was obtained from the Inonu University Health Sciences Non-Invasive Clinical Research Ethics Committee (2022/3802).

**ABSTRACT**

**Background:** In SARS-CoV-2 infection, in addition to the production of virus-specific antibodies, autoantibodies can also be produced, which attack self-structures and worsen the prognosis. We hypothesized that overproduction of virus-specific antibodies may also trigger autoantibody production.

**Methods:** To test this hypothesis, the antinuclear antibody (ANA) positivity rate was examined in samples with high and low (i.e., negative) anti-SARS-CoV-2 antibody levels. A quantitative ELISA test kit with a dynamic measurement range of 1-475 RU/mL was used to determine Sars-CoV-2 antibodies. In the high SARS-CoV-2 antibody criterion, it was required to have the last quarter antibody level (>235 RU/mL) in the ELISA calibration curve. For low antibody levels, the requirement was <15 RU/mL. Anti-SARS-CoV-2 antibody levels of 1222 samples were examined and 62 (33 men, 29 women) samples were determined to have high antibodies (high group; HG). Among the samples with low antibody levels, 62 gender-matched samples were selected by randomization (low group; LG). ANA positivity was analyzed with 3 different commercial ELISA test kits (anti-dsDNA, anti-ENA, anti-Hep-2 nucleus; Y immunoTEK, Turkey). Total IgG levels were also measured to evaluate the difference in total antibody levels.

**Results:** Anti-SARS-CoV-2 antibody levels were 413 ± 72 RU/mL and 3.8 ± 1.4 RU/mL for HG and LG, respectively (p<0.001). The ANA positivity rate was found to be significantly higher in HG than in LG (anti-dsDNA 9/62, 14.5% - 19/62, 30.7%; anti- ENA 10/62, 16.1% - 22/62, 35.5%; anti- Hep-2 nükleus 8/62, 12.9% - 20/62, 32.3% respectively). There was no difference between total IgG levels (HG; 11.1 ± 3.0 and LG; 10.6 ± 3.4 mg/mL) (p>0.05).

**Conclusions:** As a result, it was determined that high levels of SARS-CoV-2 antibody production were associated with the formation of ANA. This suggests that SARS-CoV-2 antibody and ANA production have similar mechanisms or pathways.

**Key words:** Autoantibody, ANA, SARS-CoV-2 antibody, Covid-19

**1. INTRODUCTION**

The coronavirus disease (Covid-19), caused by the SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus Disease 2) virus identified in 2019, turned into a global health problem and caused the death of more than 6 million people (1). Although the severity of symptoms in Covid-19 varies from person to person, the immune system plays a critical role in overcoming the disease. Following the viral infection, immune defense occurs both at the cellular level and at the humoral level by producing antibodies (2). These antibodies produced by the B cells bind to viral pathogens, neutralize them and help to overcome infection (3).

During the immune defense Covid-19, apart from antibodies specific to the SARS-CoV-2 virus, 'autoreactive' antibodies targeting the individual's own structures might be observed, (4). Especially antinuclear antibodies (ANA) are the most common of these autoantibodies (5). ANA enters the cell and attacks structures such as DNA, histone, and centromere in the cell nucleus, causing widespread tissue damage and inflammation. It has been determined that ANA, which is also very common in autoimmune diseases, is an important finding affecting the prognosis of Covid-19 (6). It has been observed that ANA develops more frequently, especially in those with severe disease, compared to those with mild disease, and it has been accepted as one of the reasons for worsening prognosis and delayed recovery (4, 7). Moreover, it has been reported that the heart tissue damage that can be seen in coronavirus patients may be related to ANA (8).

Although it remains unclear how autoantibodies are produced, some theories have been proposed (9). One of these theories is overactivity of B cells (10-12). It is thought that overactive B cells produce large amounts of antibodies and mediators, which disrupts the immunomodulatory mechanism and leads to autoreactive antibody production (11, 13). However, the knowledge confirming this theory is quite insufficient and the overactivity of B cells is still an important research subject in the immunopathology of autoimmune diseases (14). In some cases of Covid-19, overproduction of virus-specific antibodies may be associated with overactivity of B cells (15, 16). However, it is not yet known how this situation affects the production of antinuclear autoantibodies. In this context, the aim of the our study is to determine how high level of antibody production against SARS-CoV-2 virus affects ANA production.

**2. METHODS**

Before the study, ethical approval was obtained from the Inonu University Health Sciences Non-Invasive Clinical Research Ethics Committee (2022/3802). The research was carried out retrospectively using existing serum samples collected from people aged 18-60 during the pandemic period between 2020-2022. Samples of people with a chronic disease or a history of autoimmune disease were not included in the study. In addition, samples that were not kept in appropriate storage conditions were excluded from the study.

**2.1. Determination of anti-SARS-CoV-2 IgG level**

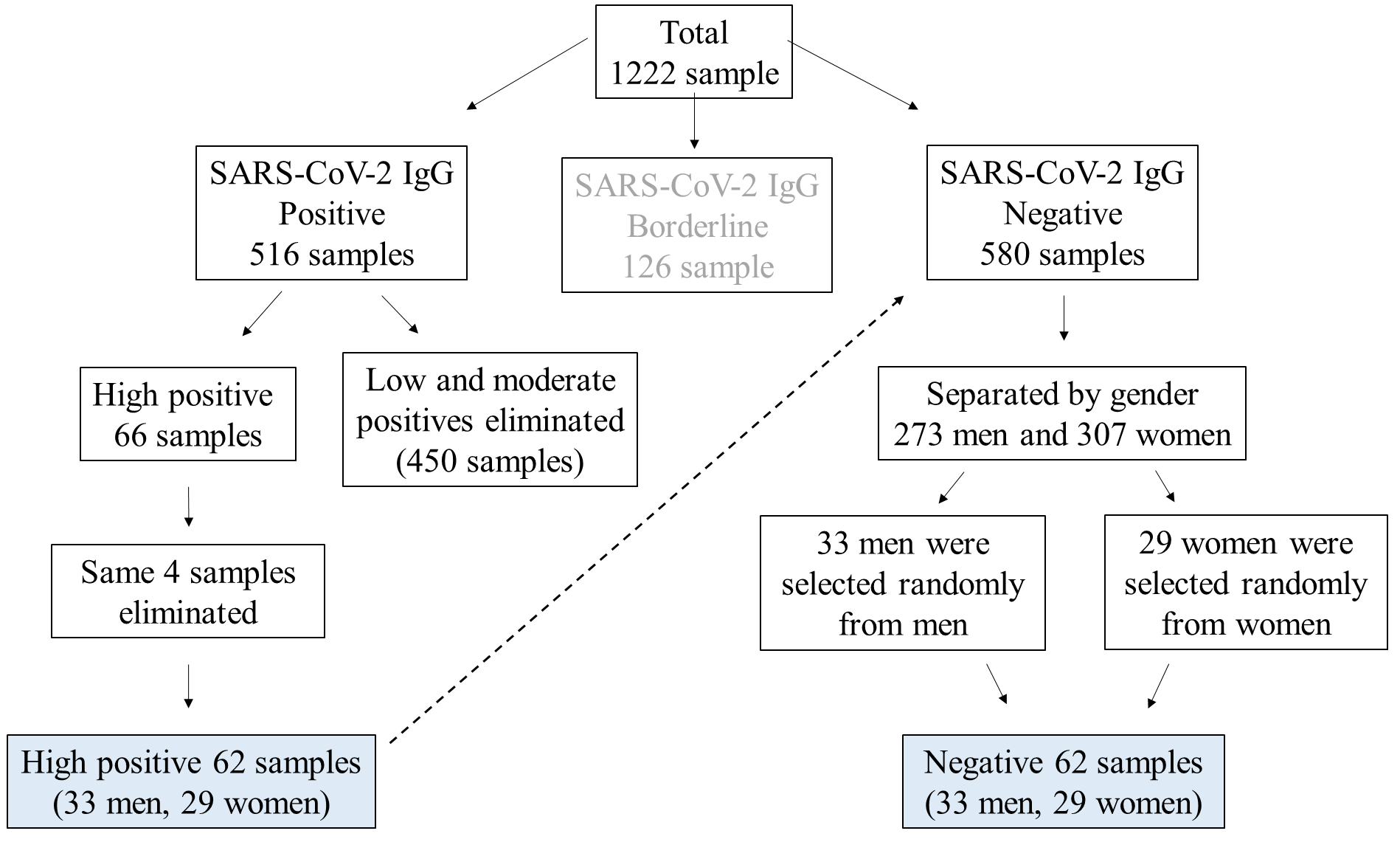
Anti-SARS-CoV-2 IgG levels of the samples were measured with quantitative commercial enzyme-linked immunoassay (ELISA) test kit (QuantiCOR, Y Immunotek A.S. Malatya, Türkiye) (17). The reference values of the test kit measuring in the range of 1 to 450 RU/ml are given in Table 1.

**Table 1.** Reference range of QuantiCOR anti-SARS-CoV-2 IgG ELISA assay

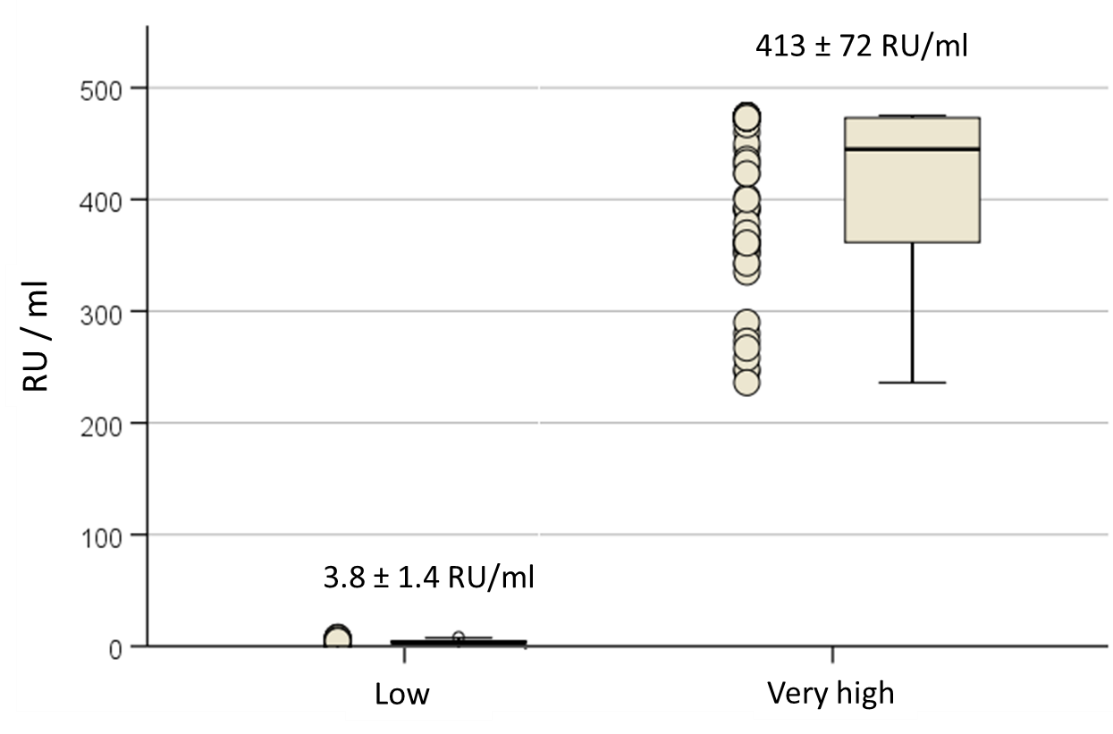
|  |  |
| --- | --- |
| **Reference range** | **Result** |
| > 15 RU/mL | Positive |
| 10 –15 RU/mL | Border line |
| < 10 RU/mL | Negative |

**2.2. Study design**

Two independent experimental groups, high positive and negative, were formed according to their anti-SARS-CoV-2 IgG levels. For this purpose, anti-SARS-CoV-2 IgG levels of 1222 samples were examined. It was determined that 516 of these samples were positive (100 ± 123 RU/ml), 580 were negative (4.1 ± 2.1 RU/ml), and 126 samples were borderline (12.3 ± 1.7 RU/ml). Of the 516 positive samples, 66 were found to be highly positive (410 ± 74 RU/ml). Four of them were excluded from the study because they were duplicate samples of the same person, and a total of 62 (33 males, 29 females) samples were included in the high positive group. The negative group was formed according to the stratified randomization method so that the number of men and women in the groups was equal. First, negative samples were separated by sex. Then, a negative group (n=62) was formed by randomly selecting 33 samples from the male group and 29 samples from the female group (Figure 1). SARS-CoV-2 IgG levels of the groups are shown in Figure 2.



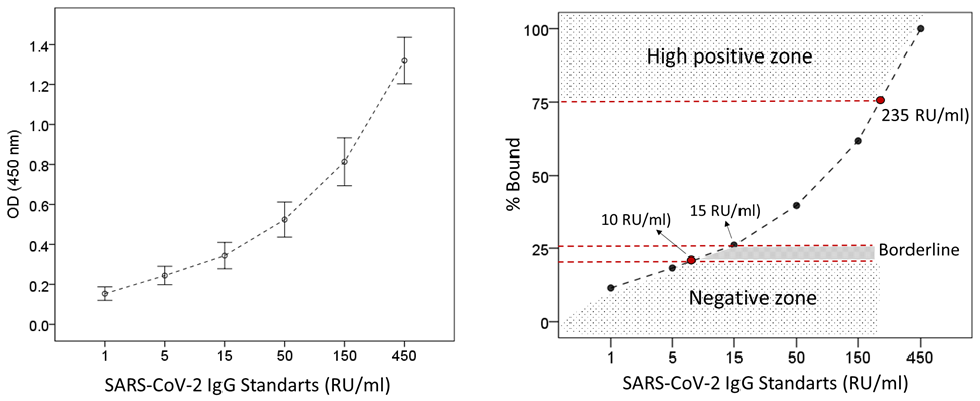
**Figure 1.** Selection of experimental and control groups



**Figure 2.** SARS-CoV-2 IgG levels of the experimental groups.

**2.3. Anti-SARS-CoV-2 IgG high positivity criterion**

In the SARS-CoV-2 IgG ELISA test, the criterion of high positivity was determined according to the % binding level in the standard curve ((OD/Standard 1) x100). Samples binding above 75% (>Q3-last quarter) were considered high positive (Figure 3).



**Figure 3.** % Bound – SARS-CoV-2 IgG Standards curve. According to the graph, 235 RU/ml was calculated to correspond to 75% binding level. Samples with antibody levels >235 RU/ml were considered high positive.

**2.4. Determination of ANA positivity of groups**

ANA was measured with three different commercial ELISA test kits: anti-dsDNA, anti-ENA and anti-Hep-2 nucleus (Y immunoTek, Turkey). The procedures were performed according to the kit protocol and serum samples were diluted 1/100 and added to the wells together with negative and positive control samples. Then, anti-human IgG with biotin and streptavidin peroxidase were added respectively. Up to this step the plates were washed 3 times (0.05% tween) before each solution was added. Finally, a chromogenic substrate (tetramethyl benzidine (TMB)) was added and the resulting color was stopped with 11% H₂SO₄. Plates were read in a spectrophotometer at 450 nm.

**2.5. Calculation of Results**

The cut-off value was determined according to the negative OD x 1.5 formula determined in protocols. Obtained OD values were converted to Ab index according to Ab index= Sample OD / Cut off OD formula. Those with an index value of <1.0 were considered ANA IgG negative, and those with an index value >1.0 were considered ANA IgG positive. The test was considered valid provided that the Ab index of the positive control was >1.1 and the negative control was <0.9.

**2.7. Determination of total IgG**

Total IgG levels of the groups were determined by the previously developed and validated ELISA test. Plates were coated with 1 ug/ml human IgG (Sigma I4506) and blocked with 1% BSA. After adding standard (100-20-5-1-0.2 ug/ml) and samples (1/5000) to the wells, anti-human IgG with biotin was added and incubated for 45 minutes. Then, streptavidin peroxidase and TMB were added, respectively. The resulting color was stopped with 11% H₂SO₄ and read at 450 nm.

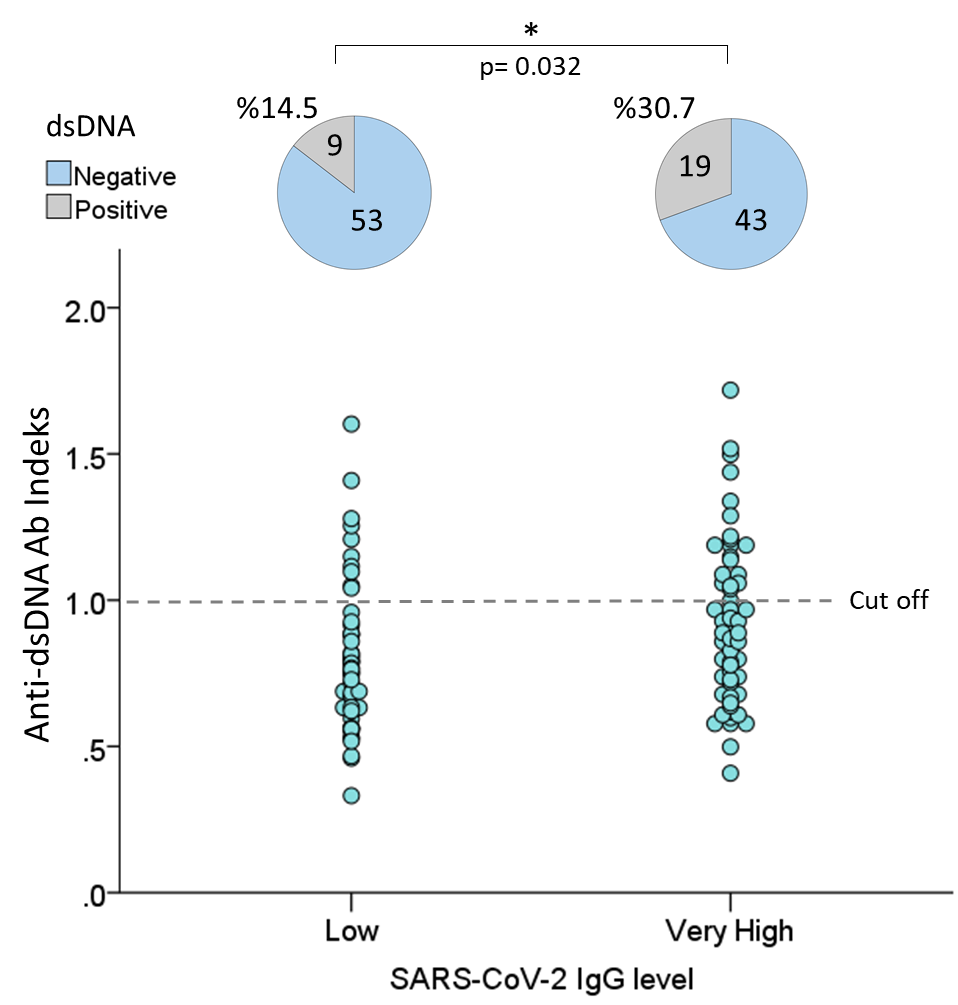
**2.8. Statistical analysis of data**

Chi-square test was used to compare ANA results of experimental groups, and independent T test was used to compare Total IgG. Results with a P value below 0.05 were considered statistically significant.

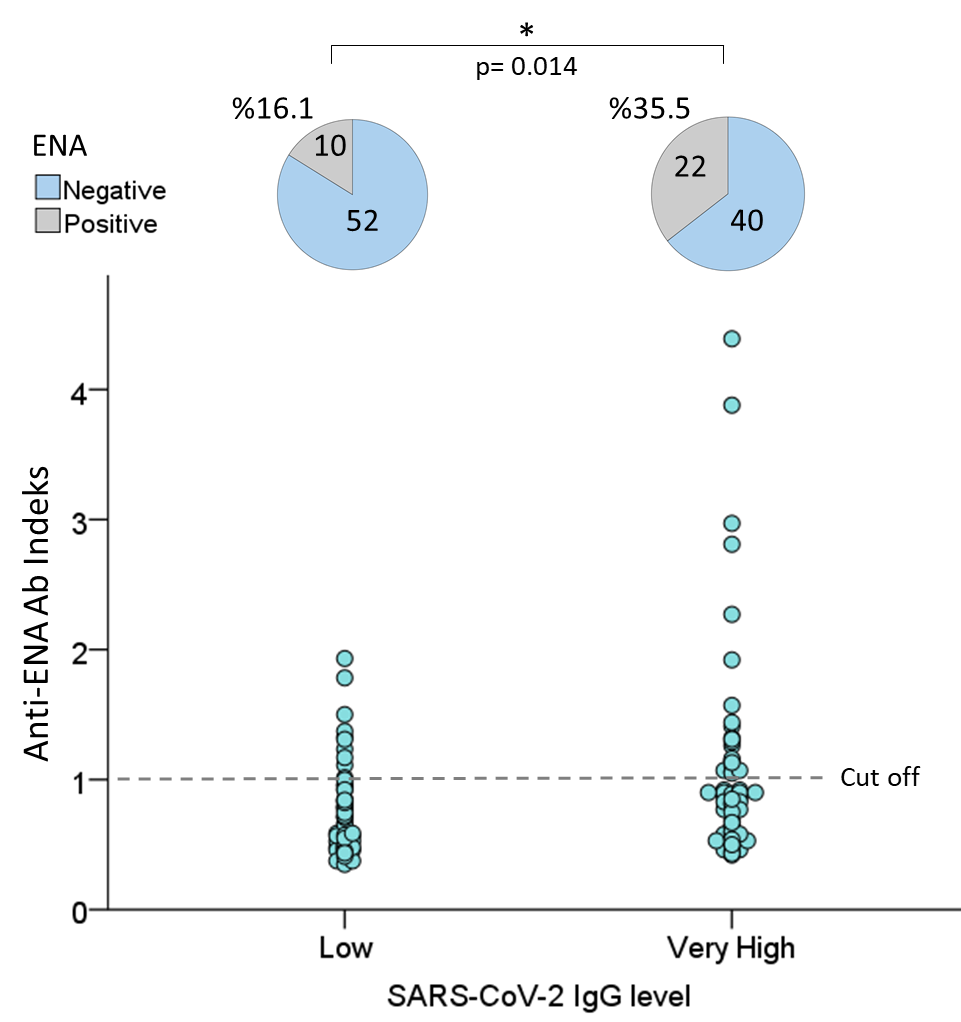
**3. RESULTS**

**3.1. Association between high level of SARS-CoV-2 IgG and ANA positivity**

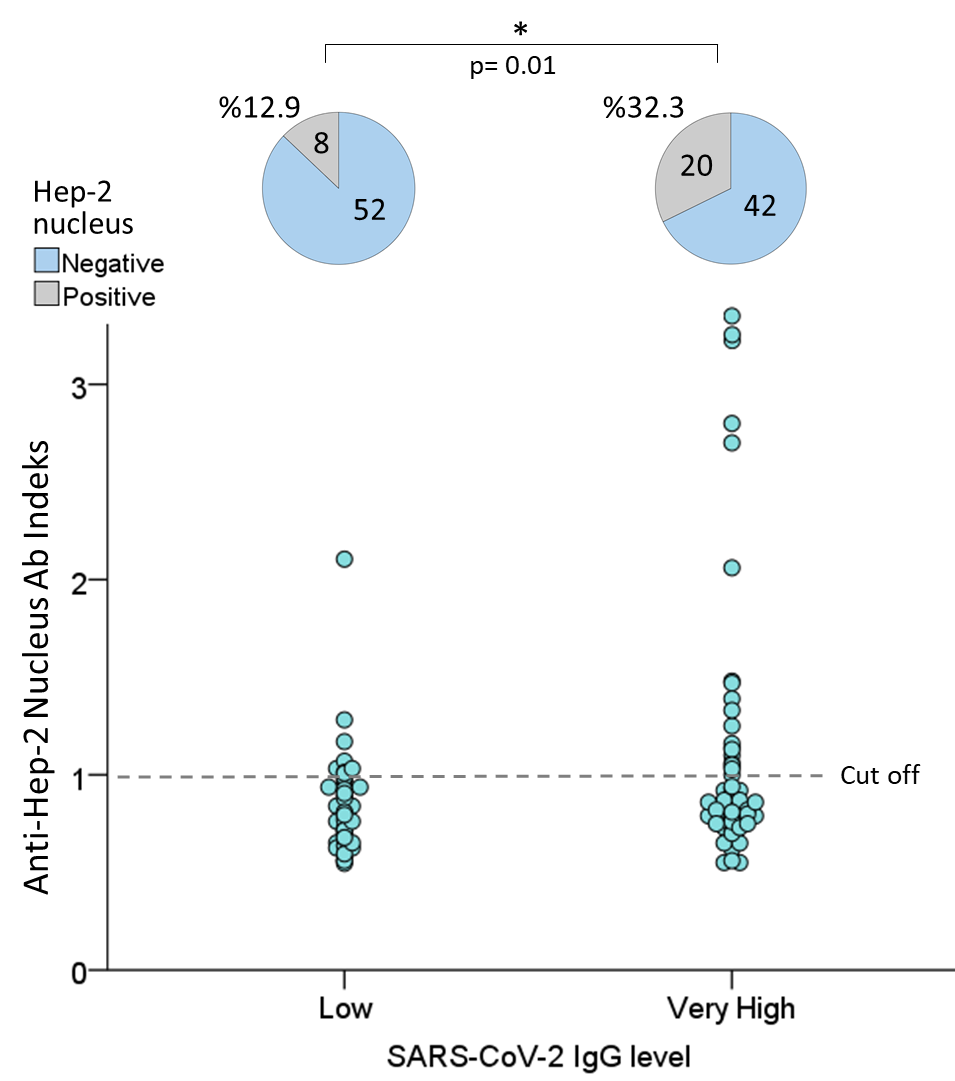
As a results, in the anti-dsDNA test, 9 samples (9/62, 14.5%) in the LG group and 19 samples (19-62, 30.7%) in the HG group were found positive (p<0.05) (Figure 4). In the anti-ENA test, 10 samples (10/62, 16.1%) in the LG group and 19 samples (19/62, 35.5%) in the HG group were found positive (p<0.05) (Figure 5) . In the anti-Hep-2 nucleus test, 8 samples (8/62, 12.9%) in the LG group and 20 samples (20/62, 32.3%) in the HG group were found positive (p<0.05) (Figure 6).



**Figure 4.** dsDNA levels in SARS-CoV-2 IgG high and negative groups.



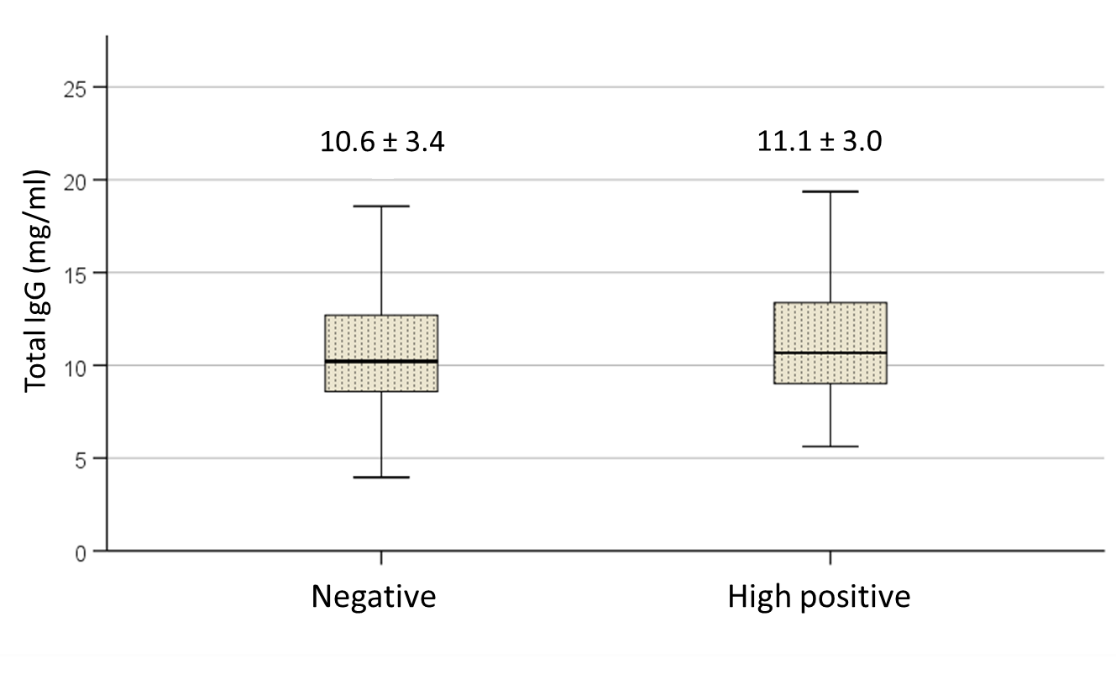
**Figure 5.** ENA levels in SARS-CoV-2 IgG high and negative groups.

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**Figure 6.** Anti-Hep-2 nucleus levels in SARS-CoV-2 IgG high and negative groups.

**3.2. Comparison of Total IgG levels of the groups**

Total IgG level was found to be 10.6 ± 3.4 mg/ml in the group with high SARS-CoV-2 IgG level and 11.1 ± 3.0 mg/ml in the negative group (Figure 7). When the results were compared statistically, no significant difference was observed between the groups. (p=0.431).



**Figure 7.** Total IgG levels of the groups

**4. CONCLUSIONS**

The relationship between high level antibody production against the SARS-CoV-2 virus and ANA formation was investigated and it was determined that high level SARS-CoV-2 production increased ANA formation.

High levels of antibody production are thought to be an important indicator of the high activity of B lymphocytes (18). It has also been reported that excessive activation of B lymphocytes may be associated with autoantibody production in some autoimmune diseases (10, 19-21). It is assumed that overactive B cells disrupt the immunomodulatory mechanism by increasing the production of antibodies and some mediators. It is thought that this situation negatively affects immune tolerance and triggers autoantibody production (22). It has been reported that B cell overactivity may be associated with autoantibody formation in some Covid 19 cases, as in autoimmune diseases (23). Liu, Yu and colleagues (2021) suggested that the excessive immune activity seen in Covid-19 cases weakens immune tolerance, increases the possibility of cross-reactivity in host cells and leads to the production of autoantibodies (24). Similarly, Germendia et al. reported that autoantibodies produced against nuclear proteins and some cytokines may be associated with excessive activation of B cells (25). In our study, similar to the literature, high levels of SARS-CoV-2 antibody production was accepted as an excessive immune response and it was determined that this situation increased ANA positivity. On the other hand, the similar total IgG levels of the NG and HG groups showed that although the specific IgG level increased, total IgG remained within a certain range. The fact that the specific IgG produced against SARS-CoV-2 is only 1/10,000-20,000 of the total IgG explains the reason for this situation (26).

Another reason for autoantibody production in coronavirus cases is the molecular mimicry theory (27, 28). According to this theory, the presence of molecular mimicry between virus epitopes and self-antigens is thought to increase the likelihood of antibodies against the virus to cross-react with self-antigens and trigger the production of autoantibodies (29-31). In a study, Lucchese and Flöel (2020) discovered that some structures in the respiratory neurons of the brainstem carry common antigenic epitopes with the SARS-CoV-2 virus (32). In the same study, it was emphasized that antibodies produced against the SARS-CoV-2 virus may also affect the respiratory rhythm by cross-reacting with respiratory neurons (32). In another study, Angileri et al. (2020) suggested that autoantibodies resulting from molecular mimicry between self-antigens and the virus may be responsible for the multiple organ failure seen in Covid 19 cases (33). Similarly, Cappello (2020) reported that there are similar epitopes between the SARS-CoV-2 virus and vascular endothelial cells, so antibodies against the virus cross-react with vascular endothelial structures, leading to disseminated intravascular coagulation (DIC) (34). . Based on this and similar literature information, it can be assumed that the more antibodies specific to the SARS-CoV-2 virus are produced, the higher the likelihood of cross-reactivity of these antibodies with their own antigens. As a matter of fact, in our study, the higher rate of ANA in the group with high anti-SARS-CoV-2 antibody levels shows that the molecular similarity theory may have an important role in the Covid-19/ANA relationship.

As a result, it was determined that high levels of antibody production against the SARS-CoV-2 virus were associated with ANA production. This condition has been associated with overactivity of the immune system and molecular similarity theories. In addition, the similarity of total IgG level between groups showed that the increase in specific IgG was not reflected in total IgG.

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**Conflicts of Interest**

The authors report no conflicts of interest related to this study.

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