Differences in the concentration of anti-SARS-CoV-2 IgG antibodies as a result of post-covid or/and post-vaccination immunization

Andrzej Tretyn  
Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Torun, Poland

Joanna Szczepanek (szczepanekjoanna@gmail.com)  
Centre for Modern Interdisciplinary Technologies, Nicolaus Copernicus University, Torun, Poland

Monika Skorupa  
Faculty of Biological and Veterinary Sciences, Nicolaus Copernicus University, Torun, Poland

Joanna Jarkiewicz-Tretyn  
Non-Public Health Care Centre, Cancer Genetics Laboratory, Torun, Poland

Dorota Sandomierz  
Non-Public Health Care Centre, Cancer Genetics Laboratory, Torun, Poland

Joanna Dejewska  
Non-Public Health Care Centre, Cancer Genetics Laboratory, Torun, Poland

Karolina Ciechanowska  
Non-Public Health Care Centre, Cancer Genetics Laboratory, Torun, Poland

Aleksander Jarkiewicz-Tretyn  
Polish-Japanese Academy of Information Technology, Warszawa, Poland

Wojciech Koper  
The Voivodeship Sanitary-Epidemiological Station in Bydgoszcz, Bydgoszcz, Poland

Krzysztof Pałgan  
Department of Allergology, Clinical Immunology and Internal Diseases, Collegium Medicum, Nicolaus Copernicus University, Bydgoszcz, Poland

Research Article

Keywords: COVID-19, Comirnaty, humoral response, mRNA vaccine

DOI: https://doi.org/10.21203/rs.3.rs-388040/v2

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Abstract

At the end of 2020, population-based vaccination programs with new generation mRNA-based vaccines began almost all over the world. The aim of the study was to evaluate the titer of anti-SARS-CoV-2 IgG antibodies against the S1 subunit of the "spike protein" as a marker of the humoral response in 477 patients and the concentration of gamma interferon as an indicator of cellular response in 28 individuals. In our studies, we used the serological enzyme-linked immunosorbent assays. IgG were measured in weeks 2 and 3 after the first dose and 1–5 weeks after the second dose of mRNA vaccine in seropositive and seronegative individuals, as well as in symptomatic and asymptomatic convalescents. High levels of antibodies are observed in 98% of our vaccinated cohort, and the presence of protective T cells was confirmed in all the studied groups. The humoral immune response is diversified and is visible as early as 2–3 weeks after the first dose of mRNA vaccine. The level of protection increased significantly after the second dose, with the increase being much greater in pre-vaccine healthy subjects and not much in convalescents.

Introduction

The severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), a member of the subgenus Sarbecovirus, causes coronavirus disease 2019 (COVID-19). The first cases occurred in late 2019\(^1\). On 11 March 2020, the World Health Organization (WHO) declared the SARS-CoV-2 outbreak a pandemic - the gravest global health crisis of our times. Now almost 2,800,000 deaths and 128,000,000 cases of SARS-CoV-2 infection have been reported worldwide as of March 31st\(^2\). An understanding of immune responses to SARS-CoV-2 and coronavirus vaccines is necessary, due to the rapid spread of the pandemic across the globe.

In response to SARS-CoV-2 infection, humans produce specific antibodies, CD4\(^+\) T and CD8\(^+\) T cells\(^3,4,5\). High-affinity antibodies are produced by B cells, which are activated by CD4\(^+\) T cells. The role of CD8\(^+\) T cells is to destroy infected cells\(^6\). SARS-CoV-2 specific antibodies are directed against spike protein (S) and nucleocapsid (N). Special role play neutralizing antibodies against the S1 subunit with receptor-binding domain (RBD). RBD binds to angiotensin-converting enzyme 2 (ACE2), which make it easier for the virus to penetrate host cells. After SARS-CoV-2 infection, antibodies can be detected in patients after 3 days, when symptoms occur and seroconversion in most of them appear within 7–14 days. In the acute phase of the disease, IgM antibodies develop and peak at 14 to 35 days, then begin to decline over the next 21 to 35 days. IgG antibodies peak at around 21 to 49 days after infection occurs and, together with neutralizing antibodies, may persist for up to four months\(^7,8\). CD4\(^+\) T and CD8\(^+\) T cells specific for SARS-CoV-2 infections recognize peptides associated with nucleocapsid, spike protein, membrane proteins (M) of the virus and are present in most COVID-19 patients\(^7\). Specific CD4\(^+\) T cells differentiate into Th1 and Tfh cells. In addition to activating B cells, Tfh cells are crucial for the proper functioning of neutralizing antibodies, while Th1 cells produces IFN\(\gamma\) and cytokines. Furthermore CD4\(^+\) T cells help CD8\(^+\) T cells respond to infection and high concentration of specific CD8\(^+\) T cells gives a better prognosis for COVID-
19 patients, because of their function of killing infected cells. The determination of the required antibody titre and the duration of the immune system response, including the cellular response, are the basis for further research into better understanding the protective mechanisms, pathogenesis and prognostic factors of COVID-19 disease. This knowledge is also important for the development of effective treatments and vaccines.

During a global pandemic, mRNA vaccines are the fastest available vaccines due to their short production time and low biological requirements. mRNA-based vaccines avoid the risk of integrating viral genetic material into the host cell's genome and are capable of producing pure viral protein. The technology of producing vaccines against COVID-19 in the form of lipid nanoparticles (LNP) enables the delivery of precise genetic information along with an adjuvant effect to antigen presenting cells. The SARS-CoV-2 vaccines are based on the coronavirus mRNA, specifically on the fragment encoding the S protein. It is one of the structural proteins that initiate infection by attaching the virion to the host's cell membrane. Moreover, the S1 subunit of the S protein contains an immunologically relevant receptor binding domain (RBD) which is a key target of neutralizing antibodies. According to clinical studies, after vaccination, subjects developed a strong dose-dependent antibody response to the S protein after the first and second inoculations. Neutralizing antibodies were found in all subjects after the second inoculation, and the antibody titers were equal to or greater than the neutralizing antibody titers of COVID-19 patients.

The mRNA vaccine is a Comirnaty concentrate from Pfizer and BioNTech. One dose (0.3 ml) contained 30 micrograms of the COVID-19 mRNA vaccine. The active substance of the preparation is mRNA encoding the spike protein of the SARS-CoV-2 virus, acting as an antigen. The vaccine also contains 4 types of fats in the form of lipid nanoparticles: ((4-hydroxybutyl) azanediyl) bis (hexane-6,1-diyl) bis (hexyl 2-decanoate) (ALC-0315), 2 - [(polyethylene glycol) -2000] -N, N-ditetradecylacetamide (ALC-0159), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol and auxiliary substances such as potassium chloride, potassium dihydrogen phosphate, sodium chloride, disodium phosphate dehydrate, saccharose, water for injections. Comirnaty is indicated for the active immunization of persons from the age of 16 years for the prevention of COVID-19 disease caused by SARS-CoV-2 virus. The product is administered intramuscularly (deltoid muscle) after dilution as a cycle of 2 doses with an interval of at least 21 days.

The immune response to COVID-19 is poorly understood. There is insufficient knowledge about safe and immunologically effective vaccination strategies against SARS-CoV-2. There is no definite answer to which vaccination strategies will be most effective. Moreover, there is still insufficient information on the short- and long-term effects of vaccination. The aim of the research was to determine the humoral and cellular responses in vaccinated persons and in convalescents.

Results
Characteristic of study group. Serum samples were collected from 477 individual, between February 2 and March 9, 2021 in Toruń, Poland. The main participants of the research were representatives of medical professions, employees and patients of nursing homes, sanitary and epidemiological inspectorates and volunteers. People taking part in the study completed a questionnaire, which included information on: age, date of COVID-19 onset and symptoms of infection, date of administration of vaccine doses and coexistence of chronic diseases. The age of the probands ranged from 18 to 93 years, and samples were taken at different times after confirming the infection of SARS-CoV-2 or vaccinated with mRNA vaccine. 18.2% of participants were people up to 35 years old, 25.4% people aged 36–45, 22.2% people aged 46–55, 23.1% people aged 56–64, and 11.1% of those aged 65 and over. 362 women (75.9%) and 115 men (24.1%) participated in the study. The study group was considered to be: symptomatic and asymptomatic recoveries, persons vaccinated with the first and second dose of mRNA vaccines. Of the 477 serum samples, 356 were among those who were vaccinated between 2 weeks after the first dose and 5 weeks after the second dose. 134 samples were obtained from the vaccinated SARS-recovered. 121 samples were obtained from individual unvaccinated. Healthy people were chosen as the control group. The detailed structure of the study group, broken down into subcategories, taking into account the age and sex of the participants, is presented in the Table 1. The group analysed included 16 people who became ill with COVID-19 within 2 weeks of taking the first dose, 1 who became ill 4 weeks after taking the first dose, and 2 people who tested positive at 4 and 6 weeks after taking the second dose of mRNA vaccines. These people fell ill a few days after the serological test, which showed that they had high antibody titers 262.2 BAU/ml and 1106 BAU/ml, respectively).

Comparisons of antibody levels between subgroups. Neutralizing antibody concentrations ranging from 0 to 38 400 BAU/ml were analysed in the study. Anti-SARS-CoV-2 antibody levels were variable (Fig. 1, Table 1). Concentrations below 25.6 BAU/ml (negative result) were found in people who were not vaccinated and did not suffer from SARS-CoV-2 infection, 12 SARS-recovered (infection confirmed in October and November 2020), as well as in 3 people, who had only taken the first dose of mRNA vaccine. Relatively low primary humoral immunity was found in 3 patients 2 weeks after taking the second dose (78 BAU/ml for an 86-year-old woman, 89 BAU/ml for an 80-year-old woman, and 106.02 BAU/ml for a 46-year-old man, respectively).

In seronegative subjects, in the third week after immunization with the preparation, the mean level of antibodies was higher than in seropositive subjects without vaccination, which confirms the effectiveness of the vaccines in inducing a humoral response. 10 to 14 days after the second dose, a 10-fold increase in neutralizing antibodies is obtained.
Table 1
Median, maximum and minimum concentration of anti-SARS-CoV-2 IgG antibodies in the compared subgroups.

| SARS-recovered and vaccinated subgroups                                      | IgG_concentration (BAU/ml) |
|------------------------------------------------------------------------------|----------------------------|
|                                                                              | N  | Median | Minimum | Maximum |
| uninfected, unvaccinated                                                      | 24 | 3.57   | 3.20    | 8.56    |
| asymptomatic SARS-recovered, unvaccinated                                     | 15 | 94.95  | 3.20    | 296.40  |
| symptomatic SARS-recovered, unvaccinated                                      | 82 | 124.33 | 13.63   | 1920.00 |
| 2 weeks after the first vaccine dose, uninfected                             | 8  | 46.98  | 3.55    | 404.45  |
| 3 weeks after the first vaccine dose, uninfected                             | 11 | 348.00 | 67.62   | 7680.00 |
| 3 weeks after the first vaccine dose, SARS-recovered                          | 9  | 3989.00| 1666.00 | 10068.00|
| 1 week after the second vaccine dose, uninfected                             | 17 | 1747.20| 106.02  | 7045.00 |
| 1 week after the second vaccine dose, SARS-recovered                          | 10 | 11973.25| 2280.30 | 19200.00|
| 2 weeks after the second vaccine dose, uninfected                             | 39 | 2563.20| 78.00   | 12650.50|
| 2 weeks after the second vaccine dose, SARS-recovered                          | 22 | 10375.75| 198.70  | 19638.00|
| 3 weeks after the second vaccine dose, uninfected                             | 24 | 3039.60| 880.00  | 8677.00 |
| 3 weeks after the second vaccine dose, SARS-recovered                          | 36 | 5516.00| 168.50  | 19865.00|
| 4 weeks after the second vaccine dose, uninfected                             | 55 | 2379.50| 235.50  | 7805.00 |
| 4 weeks after the second vaccine dose, SARS-recovered                          | 31 | 4196.50| 210.50  | 10391.50|
| 5 weeks after the second vaccine dose, uninfected                             | 68 | 2294.50| 166.00  | 9836.50 |
| 5 weeks after the second vaccine dose, SARS-recovered                          | 26 | 3096.25| 160.00  | 10135.50|

The highest levels of neutralizing antibodies were found in vaccinated probands who underwent SARS-CoV-2 infection, both after the first and second doses, regardless of the week of vaccination (Table 1, Fig. 3). Two weeks after the first dose of vaccination, the median level of antibodies in seronegative subjects was lower than in seropositive subjects without vaccination. In the third week after taking the first dose, vaccinated convalescents had a titer of IgG antibodies more than 11-fold higher than in those who had not received the first dose in the same period (348.00 BAU/ml vs. 3989.00 BAU/ml; p = 0.008). The median (as well as the mean, data in the supplement) individual seropositive anti-SARS-CoV-2 IgG concentrations after the first dose was even higher than the median (and mean concentrations) of seronegative subjects each week after the second dose of mRNA vaccine (Fig. 3).

Correlation of the antibody titer with age and gender. The correlation between the concentration of IgG antibodies and the age of the study participants and their gender was analysed. There was no significant
correlation (p > 0.05) of the titre of antibodies against the S protein, although a lower concentration of antibodies of this class was noticeable in men compared to women (Fig. 2B,C; Table in the supplement) in each of the analysed subgroups. Considerable disproportion in the size of the groups should be taken into account. Similarly, for each of the compared categories, no significant correlation was found between age and the concentration of anti-SARSs-CoV-2 IgG (p > 0.05). Nevertheless, a noticeable trend was the highest concentration values for people aged 36–45 yrs. and 46–55 yrs. (Fig. 2A). In people over the age of 65, slightly lower antibody titers were found, but the difference was noticeable after the age of 80 and in those with chronic diseases (especially: diabetes, thyroid disease, ulcerative enteritis; data not shown).

Monitoring of the humoral response in the first weeks after vaccination. For the 8 vaccinated persons (COVID laboratory employees), who had not been infected with SARS-CoV-2, regular weekly determinations of IgG antibody levels were performed to understand the dynamics of immunization. Last assay was performed at an interval of 1 month. All of them showed a several-fold increase in the level of neutralizing antibodies compared to the previous measurement until the second week after receiving the second dose of the mRNA vaccine. The greatest changes in IgG concentration were noted in the first and second weeks after taking the second dose (5–10 fold increase). Between the second and third weeks after the second dose of the vaccine (6 weeks after the first dose of vaccine), all probants had a significant decrease in anti-SARS-CoV-2 antibody titers (Fig. 3). In the third week after the first dose, the mean concentration was 998.89 BAU/ml (range 83.83–2845 BAU/ml). In the second week after the second dose, it was highest with a mean of 6,056.18 BAU/ml (range 1889-12650.50 BAU/ml). After 10 weeks of vaccination, antibody levels had dropped to a mean level of 1758.66 BAU/mL (range 320.00-3840.00 BAU/ml).

Humoral immunity and cellular immunity. Cellular immunity analysis was performed for selected patients. Th cell activity was analysed indirectly by measuring the concentration of interferon gamma secreted by activated lymphocytes after 24 h in vitro stimulation. The mean concentration of INF-γ in the non-antigen stimulated samples was 18.17 mlU/ml (range 0.50–89.08 mlU/ml). After stimulation with S1 antigen, the concentration of interferon gamma in the samples increased significantly (p < 0.001). The mean concentration was 1625.00 mlU/ml (range 11.75–2499.25 mlU/ml). In convalescents, the mean INF-γ was 1210.53 (range 91.56–2498.53 mlU/ml), and there was a correlation between the determined amount of gamma interferon and the time after the onset of COVID-19. The lowest concentrations were obtained for the sick in October and November 2020. In people who received 2 doses of mRNA vaccine, the mean concentration of INF-γ was similar to the level described in convalescents and amounted to 1172.73 mlU/ml (range 11.75–2485.92 mlU/ l). The highest concentration of a marker of lymphocyte activity was 1854.52 mlU/ml (range 168.41-2499.25 mlU/ml) marked in vaccinated SARS-recovered. Despite the high titer of antibodies and the concentration of interferon gamma (Fig. 4), 3 people in this group were infected with the SARS-CoV-2 virus. One of these patients was reinfected with the virus within 6 months of COVID-19 disease and 4 weeks after receiving the vaccine dose, which confirmed the lack of sufficient immune protection.
Discussion

Covid vaccines produced by Pfizer/BioNTech use mRNA technology. According to the characteristics of vaccine, the minimum time needed to obtain full immunity after the second dose for the Comirnaty vaccine is 7 days. Onset of protection observed approx. 14 days after vaccination. Clinical trials have shown almost 95% of effective in preventing COVID-19 in people without prior infection. After the first dose, the effectiveness of the preparation was estimated at approx. 52%. How long immunity induced by SARS-CoV-2 infection remains unclear at this stage, but antibodies are expected to last for least six months (as in the case of a COVID-19) to potentially several years. There is also insufficient information on protection against the emerging new variants of coronaviruses.

In this study, we analysed the elements of the immune response primarily in vaccinated (seropositive and seronegative) individuals and compared them with determinants of immunity in convalescents (vaccinated and unvaccinated). The basic determinant of immunization as a result of disease or vaccination in our study was the analysis of the humoral response expressed by the concentration of IgG antibodies against the S protein of the SARS-CoV-2 virus. The purpose of this analysis was to screen the primary immune response and thus cross-sectional analysis of the dynamics of B cell responses in each of the 16 compared patients groups. In the second stage, the activation of T lymphocytes was analysed in selected people (in 3 representative groups). Based on the obtained results, a high degree of heterogeneity of immune responses was found. After vaccination, the parameters of the humoral response were measurable in all probands, which confirmed the effectiveness of the mRNA vaccine in activating B lymphocytes to produce antibodies and T lymphocytes to secrete gamma interferon.

Additional assessment of the cellular immune response (detection of interferon gamma, including the determination of pathogen-responsive T-cell activity), confirmed post-vaccination and post-covid immunization at the cellular level in all subjects. The response was variable, but we did not observe such wide differences as in the case of neutralizing antibodies.

Primary humoral immunity, one of the indicators of which is the presence of IgG antibodies, appeared 2 weeks after receiving the mRNA vaccine in 66.8% of people vaccinated with the first dose of Comirnaty, and after 3 weeks in all 11 people in our study group. Researchers working on the clinical trials for the Comirnaty vaccine observed a vaccine effectiveness of 52% between the time of the first and second doses, which is a 21-day period. Based on independent UK studies, it is estimated that the Pfizer/BioNTech vaccine may be more effective after the first dose than previously thought. In this study, it was observed that the effectiveness of the first dose of the vaccine 15 days after receiving it was actually closer to 89 to 91 percent. Researchers University of Sheffield and University of Oxford, in cooperation with the UK Coronavirus Immunology Consortium (UK-CIC), tentatively conclude, based on observational studies conducted in the UK, in which healthcare workers were vaccinated against COVID-19, that the first dose of the vaccine may provide immune protection against a severe course of COVID-19. The study was conducted on a group of 237 people, some of whom had previously been infected with SARS-CoV-2, and some had never suffered from COVID-19. Above mentioned researchers, similar to our observations, obtained the strongest immune response in those who had have been infected SARS-CoV-2
before vaccination. After one dose of the Pfizer/BioNTech vaccine, the levels of T-cells in the plasma clearly increased compared to the levels seen in people who had been vaccinated but previously uninfected to coronavirus\textsuperscript{17}.

Based on the concentration of anti-SARS-CoV-2 antibodies, it was found that patients who experienced symptomatic SARS-CoV-2 infection, both after receiving the first and second doses, regardless of the week of vaccination, have significantly higher antibody titer compared to seronegative people. Our observations are consistent with studies Angyal et al.\textsuperscript{17}, who published data showing that in people who got vaccinated after contracting COVID-19, antibody responses after the first dose of Pfizer/BioNTech vaccine were 6.8 times higher, and T cell responses 5.9 times higher than in people who had never had the disease before. In contrast, among those who did not get sick but received a single dose of mRNA vaccine, the level of protection was similar or higher than that observed after natural infection. These researchers also did not find any correlation between age and the intensity of the humoral or cellular response. Our observations are consistent also with the results published by Krammer et al.\textsuperscript{18} and Saadat et al.\textsuperscript{19}. Both our and available studies show that the titre of antibodies in seropositive people after the first dose of the vaccine is about ten times higher than in vaccinated people who have never had the disease. Based on these results, one can assumed that a prior SARS-CoV-2/COVID-19 infection triggers the immune system to a very strong response to a single dose of COVID-19 vaccine. The first dose of the vaccine, given in to people whose immune systems are already stimulated by the natural infection, has a similar effect as when given as a second 'booster' dose. Moreover, administration of the second dose of the vaccine in seropositive persons does not significantly increase the antibody concentration in these individuals. Confirmation of these observations could constitute a premise for the optimization of the vaccination program, in which decisions about taking vaccine doses should be based on the analysis of primary indicators of immune immunity. Another important aspect pointed out by Krammer et al.\textsuperscript{18} is that taking first dose of mRNA vaccines by seropositive people could protect people formerly suffering from COVID-19 from the negative effects of taking the second dose of the preparation (these people suffer the most from the second dose). It appears that the added benefit of delaying or eliminating the second dose in highly immunized individuals would also be to increase the distribution of vaccine stocks among multiple individuals. Nevertheless, this approach requires further research including the analysis of factors influencing overall immunity or vaccine efficacy. Current FDA recommendations recommend adherence to a dosing schedule that has been tested in clinical trials\textsuperscript{20}.

The group of SARS-recovered patients included both those with high serum levels of antibodies and those whose IgG titre may suggest a loss of immunity acquired after COVID-19, and thus indicate the need for vaccination. Antibody levels below < 35.2 BAU/ml (negative or uncertain result) were detected in symptomatic convalescents (10 patients) who had been ill 5–6 months prior to serological examination. This observation in accordance with the reports contained in ECDC Technical Report\textsuperscript{21}. The currently available results of cohort studies confirm that the protective effect of natural SARS-CoV-2 infection ranges from 81–100%, begins on day 14 after infection, and lasts for a period of five to eight months\textsuperscript{21}.\textsuperscript{22}. Unfortunately, relatively low titers of IgG antibodies were also determined in 5 asymptomatic survivors
after a period of several weeks after the positive test results, which may indicate a high risk of viral reinfection. People who have had COVID-19 should be vaccinated to ensure long-term and strong immunity. Chia et al. 23, noted that in convalescents group it is possible to distinguish five different patterns of the dynamics of neutralizing antibodies, and their modelling may influence the prediction of individual immunity longevity in convalescents, and thus the decision to vaccinate within this group of patients. Persistence of neutralizing antibodies in SARS-recovered related to the severity of the disease (we also observed this relationship in our study) and the sustained levels of proinflammatory cytokines, chemokines and growth factors. Chia et al.23 also observed that despite the different dynamics of neutralizing antibodies in the different groups, the T-cell responses were similar. Therefore, it seems likely that analogous dynamics of the humoral and cellular responses may also apply to the post-vaccination immune response.

Our research results shows that in convalescents the humoral immune response induced by natural infection is significantly enhanced by a single dose of vaccine, and vaccination significantly improves the extent of the immune cell responses that appear after infection. Moreover, studies by Angyal et al.17 show that the first administration of the vaccine strengthens the cellular response, as well as in vitro it strengthens the neutralizing properties in relation to the variant B.1.351 (South African). This aspect is pointed out by Skelly et al.24, who tested the neutralization strength of antibodies resulting from natural SARS-CoV-2 infection and immunization with the Pfizer/BioNTech vaccine. They noted that there is a difference in the humoral (decreased neutralization) and, to a lesser extent, cellular responses to variants of the B1.1.7 (UK) and B1.351 lines. The authors attribute these differences to the strength of the homotypic antibody responses. Thus, it is speculated that the new SARS-CoV-2 variants may avoid the protective neutralizing responses resulting from natural infection, and to a lesser extent immunization. Hence, as the authors emphasize, there is a need to induce a vaccine immune response.

In the serological test, 3 people patients had significantly lower IgG anti-SARS-CoV-2 antibodies compared to the other vaccinated persons, the level of IgG antibodies (78-106.02 BAU/ml). Based on these results, it can be concluded that these people have not acquired significant humoral immunity and may still be at risk of coronavirus infection despite vaccination. Hence the need to conduct special serological surveillance in people aged 65 + and/or people with coexisting chronic diseases, and perhaps to consider the need to take further doses of the preparation ensuring protective properties. Worrying is also the fact of confirmed cases in 3 despite vaccination, high rates of cellular and humoral responses. However, no variant of the virus has been identified that overcame the immune response mechanisms in these individuals. SARS-CoV-2 infections and vaccine infections have been reported sporadically25,26, but raise important issues regarding the duration of immunity after natural infection and the extent of protection after vaccination, as well as the transmission of the virus by these individuals.

Testing the concentration of antibodies to S protein in both convalescents and vaccinated patients enables the analysis of the course of the humoral immune response in COVID-19. Quantitative testing of anti-SARS-CoV-2 IgG antibodies allows to determine whether the patient has responded to vaccination,
and if so how intensely. It also enables the assessment of the humoral immunity acquired after undergoing SARS-CoV-2 infection. By testing anti-SARS-CoV-2 antibodies, it is possible to determine the concentration of antibodies that provide protection against infection, as well as to make rational decisions about booster doses of COVID-19 vaccines. The greatest benefit of the research is the ability to quantify the acquisition of humoral immunity to SARS-CoV-2 as a result of coronavirus infection and/or vaccination. Antibody testing is not required in the context of vaccination, but knowledge of the immune status before and several weeks after the last dose of vaccination may nevertheless allow inferring the immune response to immunization and provide an indication of the degree of immunity obtained against COVID-19. Due to the lack of data on the persistence of immunity acquired as a result of a vaccine reaction, it is also important to monitor the level of antibodies over time (especially among healthcare professionals, people over 65 years and chronically ill), as the future may also indicate the need (or lack thereof) taking a booster dose. The results of the conducted research could be useful in the future for the development of new recommendations of the vaccination program, hence the need to continue them on a larger scale. The diversity of immune responses shows the need for research, the inherent element of which will be immunological monitoring of the durability of disease resistance or protection against its severe course in vaccinated people and/or susceptibility to reinfection in COVID-19 convalescents. By analysing the level of antibodies, it is possible to identify people who are already immunized as well as people who have not acquired immunity as a result of vaccination, and those who may have lost the acquired immunity after contacting SARS-CoV-2. It seems that such a test should be an integral part of the assessment of immunological parameters, especially before making an informed decision about vaccination or its delay in convalescents, as well as the assessment of the durability of immune protection, important from the perspective of making a decision to take booster doses a few months after the initial administration of the vaccine against COVID-19.

Methods

Human subjects. The research group consisted of 477 adult volunteers, 362 women and 115 men. All human subjects were assessed for medical decision-making capacity using a standardized, approved assessment, and voluntarily gave informed consent prior to being enrolled in the study. The patients represented research subgroups (Table 1): 24 healthy and unvaccinated individuals; 15 persons with confirmed SARS-CoV-2 infection but no symptoms (unvaccinated); 82 persons who had COVID-19 and had multiple symptoms of infection (unvaccinated); 19 persons who were 2 or 3 weeks after the first dose of the vaccine (did not have COVID-19); 203 persons who were vaccinated with the second dose within 1–5 weeks before blood sampling for analysis (did not have COVID-19); 124 patients who were confirmed to be infected with SARS-CoV-2 and who were vaccinated at corresponding time points.
Table 2
Characteristics of the frequency of the studied subgroups, taking into account the sex and age of the patients.

| SARS-CoV-2 and vaccination status | N   | Female |       |       |       |       | Male |       |       |       |       |       |
|-----------------------------------|-----|--------|-------|-------|-------|-------|------|-------|-------|-------|-------|-------|
|                                   |     | <=35   | 36-45 | 46-55 | 56-65 | 65+   | <=35 | 36-45 | 46-55 | 56-65 | 65+   |       |
| uninfected, unvaccinated          | 24  | 4      | 2     | 3     | 5     | 0     | 2    | 4     | 2     | 1     | 1     |       |
| asymptomatic SARS-recovered,      | 15  | 2      | 0     | 2     | 1     | 1     | 2    | 1     | 4     | 2     | 0     |       |
| unvaccinated                      |     |        |       |       |       |       |      |       |       |       |       |       |
| symptomatic SARS-recovered,       | 82  | 11     | 14    | 12    | 15    | 6     | 2    | 6     | 4     | 5     | 7     |       |
| unvaccinated                      |     |        |       |       |       |       |      |       |       |       |       |       |
| 2 weeks after the first vaccine   | 8   | 2      | 1     | 1     | 0     | 1     | 0    | 1     | 0     | 1     | 1     |       |
| dose, uninfected                  |     |        |       |       |       |       |      |       |       |       |       |       |
| 3 weeks after the first vaccine   | 11  | 4      | 1     | 0     | 2     | 1     | 0    | 1     | 0     | 1     | 1     |       |
| dose, uninfected                  |     |        |       |       |       |       |      |       |       |       |       |       |
| 3 weeks after the first vaccine   | 9   | 1      | 1     | 3     | 3     | 0     | 0    | 0     | 0     | 0     | 0     | 1     |
| dose, SARS-recovered              |     |        |       |       |       |       |      |       |       |       |       |       |
| 1 week after the second vaccine   | 17  | 4      | 1     | 4     | 2     | 2     | 1    | 2     | 0     | 1     | 0     |       |
| dose, uninfected                  |     |        |       |       |       |       |      |       |       |       |       |       |
| 1 week after the second vaccine   | 10  | 1      | 2     | 2     | 3     | 0     | 0    | 0     | 0     | 0     | 0     | 2     |
| dose, SARS-recovered              |     |        |       |       |       |       |      |       |       |       |       |       |
| 2 weeks after the second vaccine  | 39  | 7      | 3     | 6     | 7     | 7     | 1    | 1     | 1     | 3     | 3     |       |
| dose, uninfected                  |     |        |       |       |       |       |      |       |       |       |       |       |
| 2 weeks after the second vaccine  | 22  | 3      | 4     | 4     | 3     | 4     | 1    | 0     | 0     | 0     | 0     | 3     |
| dose, SARS-recovered              |     |        |       |       |       |       |      |       |       |       |       |       |
| 3 weeks after the second vaccine  | 24  | 5      | 4     | 4     | 1     | 2     | 1    | 2     | 2     | 2     | 2     | 1     |
Quantitative determination of IgG antibodies against SARS-CoV-2. The starting material for analyses was serum obtained after centrifuging whole blood in clot tubes for 5 min at 4 000 rpm. The serum was then carefully removed from the cell pellet and used. Depending on the subgroup of patients, sera were diluted 5-fold, 10-fold, 20-fold, 50-fold or 100-fold. An enzyme-linked immunosorbent assay (ELISA) was used to quantify the *in vitro* quantification of human IgG antibodies to SARS-CoV-2. The tests were performed using the commercial automated analyzers EUROIMMUN Analyzer I-2P and the Anti-SARS-CoV-2 QuantiVac ELISA kit (IgG) EUROIMMUN (Lübeck, Germany), according to the manufacturer’s instructions. The test reaction wells were coated with the S1 domain of the SARS-CoV-2 spike protein recombinantly expressed in the human cell line HEK 293. In the first step, reaction wells were incubated with diluted patient samples (1:101 dilutions) for 60 min at 37°C. In the presence of IgG antibodies (also IgA and IgM), bind to the antigens present on the surface of the well. After washing with the washing solution (3 x 450 µl), bound antibodies were incubated for 30 min at 37°C with 100 µl of an anti-human IgG antibody-peroxidase conjugate. After subsequent washing (3 x 450 µl), 100 µl of substrate/chromogen solution was added to each well and incubated 30 min at RT. During the last stem of the procedure 100 µl of stopping solution was added to each well. Photometric determination of the colour intensity was performed at a wavelength of 450 nm. A six-point standard curve (388-3,2 BAU/ml) was performed in parallel and a positive and negative control in the form of human IgG was used. The results were expressed in BAU/ml (BAU = binding antibody units).
Cellular response analysis - cell stimulation. The starting material for analyses was fresh whole blood collected in lithium heparin tubes. Plasma was obtained using the SARS-CoV-2 IGRA stimulation tube EUROIMMUN (Lübeck, Germany), according to the manufacturer's instructions. The kit contains three test tubes for one sample. The first (BLANK) does not contain any activating ingredients for immune cells. The plasma obtained from it is used to determine the individual interferon-gamma background. The second (TUBE) is coated with the components of the S1 domain of the SARS-CoV-2 virus protein. If activation-capable cells are present in plasma, they are stimulated to secrete interferon gamma during incubation in this tube. The third (STIM) is coated with a mitogen that induces non-specific interferon-gamma secretion. The plasma obtained from this tube is used to verify whether the sample contains a sufficient number of cells and whether they have a sufficient ability to activate. 500 µl of whole blood was pipetted into three set tubes and incubated at 37°C for 24 h. After this time, the tubes were centrifuged for 10 min at 12 000 rpm. The plasma interferon-gamma concentration from the BLANK tube represents the individual interferon gamma background and therefore must be subtracted from the plasma concentration obtained in the TUBE and STIM tubes. This BLANK subtraction must be performed individually for the TUBE and BLANK samples of each whole blood sample. After subtracting BLANK, the interferon-gamma concentration in the STIM tube must be significantly higher than the BLANK value alone in order to consider that the immune cell count and stimulation in the whole blood sample are sufficient.

Cellular response analysis - interferon-gamma ELISA. The level of interferon gamma was determined by an ELISA test performed on a commercial automatic EUROIMMUN Analyzer I-2P with the use of the Interferon-gamma ELISA kit, according to the manufacturer's instructions. The test kit contains a microplate with reaction wells coated with anti-interferon-gamma monoclonal antibodies. In the first reaction step, calibrators, controls and plasma samples diluted in sample buffer (1:5) were added to the coated reaction wells to bind interferon-gamma and incubated for 120 min at RT. After this time, the wells were washed with washing buffer (5 x 450 µl). In the next step of the reaction, 100 µl of biotin-labeled anti-interferon-gamma antibodies were added and incubated again for 30 min at RT. The washing was repeated (5 x 450 µl), then 100 µl of streptavidin-HRP was added and incubated 20 min at RT. Photometric determination of the colour intensity was carried out at a wavelength of 450 nm. The colour intensity was proportional to the interferon-gamma concentration. The results were expressed in mlU/ml.

Statistical analysis. One measurement was performed for each patient, and based on the medical questionnaire, the patient was assigned to the group data according to vaccination and COVID-19 status. The mean, median, minimum and maximum value were determined for each group in the statistical description. In order to determine the significance of differences between experimental subgroups, a one-way analysis of variance (ANOVA, p < 0.05) was performed. Spearman's test (two-tailed) was used to determine the correlation. Statistical analyses were performed using the IBM SPSS Statistics software (version 27.0.1.0)

Declarations
Acknowledgment

We thank the study participants for their support of COVID-19 research.

Funding statement

The research was financed from the own funds of the Non-Public Health Care Centre, Cancer Genetics Laboratory and Nicolaus Copernicus University, Torun, Poland.

Author contributions. AT and JSz designed the study; JSz, MS, DS, JD and KC performed antibody titer tests; JSz and MS performed analyses of cellular immunity; AT, JSz and MS analysed the data; JSz and AJ-T acquired and processed clinical samples; JSz prepare final manuscript version; DS, JD and KC wrote Introduction; MS wrote Methods; JSz wrote Results and Discussion; JJ-T provided access to equipment and reagents; WK coordinated the collection of samples and data from the WSSE; AT and KP carried out a data interpretation check.

All authors had full access to all the data in the study, reviewed the final manuscript, and had final responsibility for the decision to submit for publication.

The authors declare that there are no conflicts of interest

Materials&Correspondence. Joanna Szczepanek, szczepanekj@umk.pl

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Figures

Figure 1

Comparisons of antibody levels between the analysed patients subgroups. Blue colour indicates the concentration of anti-SARS-CoV-2 IgG antibodies in seronegative individuals before vaccination. Individuals with confirmed SARS-CoV-2 infection prior to receiving the mRNA vaccine dose, as well as seropositive unvaccinated individuals, are marked in green.
Figure 2

Distribution of the mean values of anti-SARS-CoV-2 IgG antibodies in relation to age (A) and the distribution of neutralizing antibody concentrations by gender of the study participants (B- for female, C- for male).
Figure 3

IgG-anti-SARS-Cov-2 concentrations in the first 10 weeks after receiving the mRNA vaccine for 8 seronegative individuals.
Comparison of anti-SARS-CoV-2 IgG antibody titers against S1 protein and gamma interferon concentrations released after stimulation of Th lymphocytes in 28 individuals (patients who recovered from SARS, vaccinated persons without previous viral infection, and vaccinated SARS-recovered). The red frame marks 3 people who were infected with SARS-CoV-2 virus despite vaccination.

**Supplementary Files**

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