PROPERTIES OF RBD SPECIFIC IGG FROM COVID-19 PATIENTS AND SPUTNIK V VACCINATED INDIVIDUALS

Generalova LV[1], Grigoriev IV[2], Vasina DV[2], Tkachuk AP[2], Kruzhkova IS[2], Kolobukhina LV[2], Burgasova OA[1,2], Guschin VA[2] ✉

[1] Peoples' Friendship University of Russia (RUDN University), Moscow, Russia

[2] Gamaleya National Research Center for Epidemiology and Microbiology, Moscow, Russia

SARS-CoV-2 specific antibody response is a generally accepted measure of postinfection and vaccination-induced immunity assessment. The dynamics of avidity maturation and neutralizing activity of virus-specific immunoglobulins G during the SARS-CoV-2–associated coronavirus infection was studied in cohorts of vaccinated volunteers and COVID-19 patients. 4–6 months after vaccination, neutralization activity was low compared to hospitalized patients (medians 57.4% vs 86.4%). On the opposite, the avidity indices in vaccinated volunteers were significantly higher (median 76.7%) than among hospitalized patients (median 61.4%). During the acute phase of the disease (14–16 days PI), post-vaccination patients have also higher avidity indices than primary patients (medians 43.5% vs 20.4%). Our results suggest that in long-term perspective antibody affinity maturation rate is higher after vaccination than after a natural infection. We demonstrated that Sputnik V vaccination leads to formation of high-avidity IgG, which persists for at least 6 months of observation. These results also indicate the presence of protective efficacy markers for at least 4–6 months after the vaccination or a previous illness and gives grounds for the half-year time period chosen for booster immunization with Sputnik V in Russia.

Keywords: antibody avidity, virus neutralization, SARS-CoV-2, immune memory, vaccination, Sputnik-V

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✉ **Correspondence should be addressed:** Vladimir A. Gushchin
Gamaleya, 18, str. 9, Moscow, Russia; wowaniada@gmail.com

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СВОЙСТВА АНТИТЕЛ К RBD У ПЕРЕБОЛЕВШИХ COVID-19 И ВАКЦИНИРОВАННЫХ ПРЕПАРАТОМ «СПУТНИК V»

Л. В. Генералова[1], И. В. Григорьев[2], Д. В. Васина[2], А. П. Ткачук[2], И. С. Кружкова[2], Л. В. Колобухина[2], О. А. Бургасова[1,2], В. А. Гущин[2] ✉

[1] Российский университет дружбы народов, Москва, Россия

[2] Национальный исследовательский центр эпидемиологии и микробиологии имени Н. Ф. Гамалеи, Москва, Россия

Исследование свойств антител, участвующих в нейтрализации вируса, после перенесенного заболевания COVID-19 и применения профилактических препаратов, остается актуальной задачей, от которой зависит выработка стратегий первичной и повторной иммунизации. Измерение уровня антител к антигенам SARS-CoV-2 — один из основных способов оценки иммунитета, однако не дает достаточной информации о количественных и качественных показателях иммунного ответа. Целью работы было исследовать свойства антител IgG к RBD у переболевших COVID-19 и вакцинированных препаратом «Спутник V». На когортах пациентов (18–80 лет; соотношение мужчин и женщин — 47 : 53), переболевших COVID-19, и вакцинированных добровольцев изучена динамика созревания аффинности и изменения нейтрализующей активности IgG к RBD. Нейтрализующая активность сывороток крови у добровольцев через 4–6 месяцев после вакцинации снизилась по сравнению с образцами переболевших пациентов (медианы — 57,4 и 86,4% соответственно). Индекс авидности у вакцинированных добровольцев, напротив, был значительно выше, чем у перенесших COVID-19 (76,7 и 61,4% соответственно). В острую фазу заболевания (14–16 дней от появления симптомов) ранее вакцинированные пациенты имели более высокий индекс авидности, чем первичные пациенты (43,5 и 20,4% соответственно). В долгосрочной перспективе степень созревания аффинности вируспецифичных IgG после вакцинации может быть выше, чем после естественно перенесенной инфекции. Показано, что вакцинация «Спутником V» приводит к формированию высокоавидных IgG, сохраняющихся по крайней мере 6 месяцев. Продемонстрировано наличие уровней антител, коррелирующих с протективным иммунитетом, на протяжении 4–6 месяцев после вакцинации или перенесенной инфекции.

Ключевые слова: авидность антител, вирус-нейтрализация, SARS-CoV-2, иммунная память, вакцинация, Спутник V

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✉ **Для корреспонденции:** Владимир Алексеевич Гущин
ул. Гамалеи, 18, стр. 9, г. Москва, Россия; wowaniada@gmail.com

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SARS-CoV-2 specific antibody response is a generally accepted measure of postinfection and vaccination-induced immunity assessment. However, the protective efficacy of virus specific antibodies and their ability to withstand the individual's reinfection may be influenced not only by antibody quantity but also their quality including neutralizing activity, binding affinity, isotypes spectrum etc., which are not characterized well enough. In general, SARS-CoV-2 antibody avidity (% of antibodies with high affinity) correlated with duration of infection and higher neutralizing titers [1]. Indeed, high avidity antibodies but not the level of spike-binding antibodies has been previously associated with positive clinical outcomes [2]. At the moment there is no published information about the antibody affinity maturation after the Sputnik V vaccination and in recovered patients, as well as antibodies functional transformations during the prolonged period of observation. This study aims to provide the longitudinal assessment of antibody responses dynamics in patients recovered from COVID-19 and Sputnik V vaccinated individuals with or without further infection and to characterize the patterns of sustaining long-term immunity.

METHODS

Stydy participans

During the study we enrolled 41 participants divided into 3 groups (Table 1): 23 patients, hospitalized in Moscow with different disease severity, were sampled upon the admission to the hospital (acute phase) and 4–6 months after the hospital discharge; 9 vaccinated patients sampled during their hospitalization and 9 healthy vaccinated volunteers at different times from the complete vaccination. Median symptom durations in hospitalized patients before the first sampling were 14 days among unvaccinated individuals and 10 days among vaccinated individuals. For vaccinated volunteers, the absence of COVID-19 was confirmed by the lack of anti-Nc IgG seroconversion.

We characterized antibodies from the three groups of participants. The group of inpatient volunteers was recruited in November through December 2020 at the First Moscow Infectious Diseases Hospital (Moscow, Russia); vaccinated patients were hospitalized and enrolled in March through April 2021 in the same source. Healthy volunteers vaccinated with Sputnik V vaccine were recruited in September through December 2020. Eligible volunteers were adults aged 18–80 years.

All patients were either diagnosed with SARS-CoV-2 infection by RT-PCR of nasopharyngeal swabs or confirmed with CT scanning. Disease severity in hospitalized patients was determined in accordance with NEWS [3]. Group of vaccinated patients included hospitalized participants diagnosed with SARS-CoV-2 infection who received a second vaccine dose at least two weeks before the infection. The vaccinated healthy volunteers received two doses of Sputnik, free of symptoms of COVID-19 for at least 14 days before the sampling. No statistical methods were used to predetermine sample size.

Blood sample processing and storage

Blood samples were collected by venipuncture to vacutainers with clot activator and shipped to the laboratory at +4 °C. Centrifugation at 3000 rpm for 10 minutes was applied to obtain serum, which was further aliquoted and stored at –30 °C.

Anti-nucleocapsid and anti-RBD IgG antibody detection

The anti-nucleocapsid (Nc) and anti-RBD IgG antibodies were measured using in house ELISA test-systems and expressed in positivity index (p.i., S/CO). Briefly, for antibody detection we used recombinant receptor-binding domain fragment of S1 SARS-CoV-2 Spike protein (RBD № 8COV1; HyTest, Russia), expressed in eukaryotic cells and recombinant SARS-CoV-2 Nucleocapsid (Nc) protein, expressed in Escherichia coli and purified in our laboratory.

To perform anti-RBD and anti-Nc ELISAs, 96 well high binding plates (Costar 2592; Corning, USA) were coated overnight with 100 µl of 1 µg/mL recombinant protein solution in PBS. Next day the plates were blocked for 2 hours at room temperature with a blocking buffer, containing 0,5% casein. Serum samples were diluted 1 : 100 with universal ELISA buffer S011 (XEMA; Russia). Sera of PCR+ reconvalescent was used as a positive control and a pool of pre-COVID samples, collected in 2019, was used as negative control. ELISA plates with 100 µl of diluted samples were incubated 1 hour at 37 °C and washed 3 times with PBS, containing 0,1% Tween-20. After washing, wells were incubated with 100 µl of HRP-conjugated anti-human IgG (Novex A18823; USA) for 1 hour at 37 °C and washed 6 times. After adding 100 µl of the HRP substrate solution, containing 3,3',5,5'-Tetramethylbenzidine (R055; XEMA, Russia) per well, the colour reaction was developed for 10 minutes at room temperature and then stopped by 10% HCl. Optical density (OD) was measured at 450 nm using Multiscan FC (Thermo Scientific; USA).

Neutralization assay (Inhibiting assay)

Detection of IgG neutralizing antibodies was performed with commercial "SARS-CoV-2-anti-RBD-ELISA" kit (MT-

Table 1. Study Cohorts. Values are reported as the medians with the range in parentheses

Characteristics	Patients (*n* = 23)	Vaccinated volunteers (*n* = 9)	Volunteers (*n* = 9)
Age median years, IQR	59 (54–65)	72 (69–79)	34 (33–39)
Sex, %	52% male, 48% female	33% male, 67% female	56% male, 44% female
Time from symptom onset to Visit 1 (Me days min-max)	14 (9–17)	10 (5–19)	NA
Time from symptom onset to Visit 2 (Me days min-max)	135 (116–159)	16 (11–24)	NA
Time from initial vaccination to Visit 1 (Me days min-max)	NA	62 (30–88)	147 (100–263)
Time from initial vaccination to Visit 2 (Me days min-max)	NA	68 (35–95)	261 (161–349)

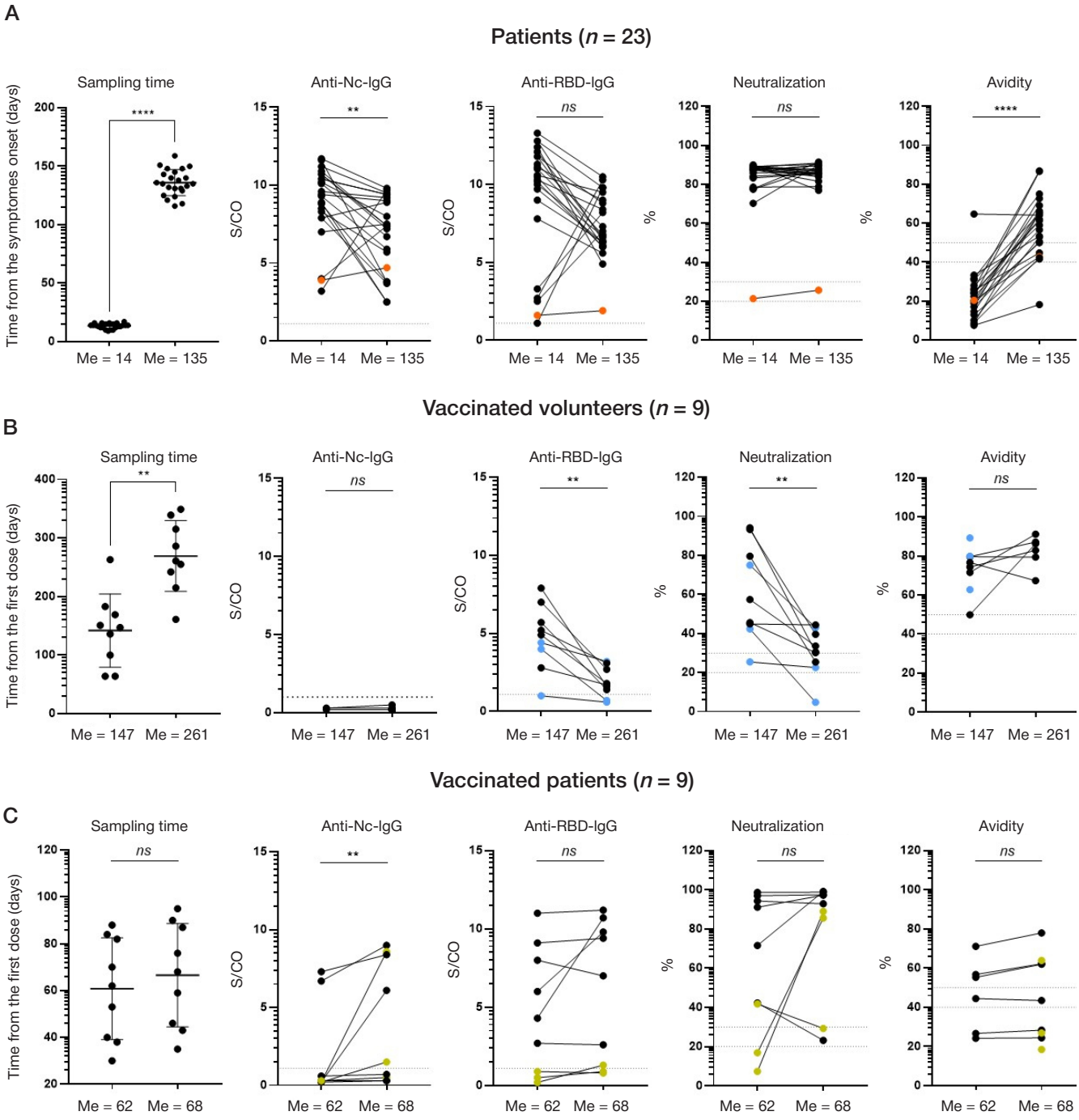

Fig. 1. Immune responses to SARS-CoV-2 in the studied groups. (**A**) Cohort of hospitalized patients (**B**) Cohort of vaccinated patients (**C**) Cohort of healthy vaccinated volunteers. Significant differences are shown as asterisks (Wilcoxon signed-rank test). Me — median time of sampling in groups from the symptoms onset or the initial vaccination as indicated in the axis

И-С1-04.192; MedipalTech, Russia) in accordance with manufacturer's instructions. Briefly, serum samples were mixed with biotinylated recombinant human ACE-2 receptor and added to the ELISA plate, precoated with recombinant RBD. After incubation and wash, streptavidin-conjugated HRP was added to the wells. Reaction was visualized by adding an HRP-substrate solution. Optical density in wells was inversely proportional to the concentration of antibodies, able to block ACE-2 binding to RBD. Inhibition coefficient (IC) was calculated as ratio of sample OD to negative control OD, subtracted from 1. IC = (1 — ODsample/ONneg) — 100%. The antibody neutralization criteria were assessed as high neutralizing if serum sample inhibited binding of soluble RBD to ACE-2 on plate by > 30%; intermediate one, if the inhibition rate was between 20 and 30%; and a low level for samples with inhibition < 20%.

Avidity assay

The IgG avidity assay targeted the spike protein RBD of SARS-CoV-2 was performed with "SARS-CoV-2-ELISA-IgG plus" kit (No MT-*И*-С1-03.192; MedipalTech, Russia) in accordance with manufacturer's instructions. Briefly, duplicate anti-RBD IgG positive serum samples were incubated in wells of ELISA plates, precoated with recombinant RBD. Then either PBS (non-denaturing conditions) or urea solution (denaturing agent, DA) was added to the wells. After washing, HRP-conjugated anti-human IgG was added and, finally, reaction was visualized by

Table 2. Antibody levels and characteristics in cohorts of patients with different disease severity

		Patients			
		Total (*n* = 23)	Mild (*n* = 10)	Moderate (*n* = 6)	Severe (*n* = 7)
Visit 1, median (IQR)					
1	RBD-specific IgG, S/CO	10.5 (7.8–11.8)	10.4 (7.6–11.9)	8.9 (1.5–11.5)	11.3 (10.2–12.8)
	Negative, *n* (%)	1 (4.3)	0 (0)	1 (20)	0 (0)
	Reactive, *n* (%)	22 (95.6)	10 (100)	5 (80)	7 (100)
2	N-specific IgG, S/CO	9.4 (7.9–10.7)	9.9 (8.8–11.3)	7.45 (3.7–8.55)	10.4 (8.8–10.7)
	Negative, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
	Reactive, *n* (%)	23 (100)	10 (100)	6 (100)	7 (100)
3	Neutralization, %	87.7 (83.3–88.7)	87.5 (78.4–88.9)	87.1 (58.1–88.5)	87.8 (87.3–89.0)
	Low, *n* (%)	1 (4.3)	0 (0)	1 (20)	0 (0)
	High, *n* (%)	22 (95.6)	10 (100)	5 (80)	7 (100)
4	Avidity, %	20.4 (14.6–26.3)	19.5 (15.28–25.7)	22.2 (17.4–25.95)	22.4 (8.7–31.6)
	Low, *n* (%)	22 (95.6)	9 (90)	6 (100)	7 (100)
	High, *n* (%)	1 (4.3)	1 (10)	0 (0)	0 (0)
Visit 2, median (IQR)					
1	RBD-specific IgG, S/CO	6.8 (6.1–9.0)	6.5 (6.1–8.7)	6.6 (4.7–9.2)	7.3 (6.8–9.8)
	Negative, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
	Reactive, *n* (%)	23 (100)	10 (100)	6 (100)	7 (100)
2	N-specific IgG, S/CO	7.4 (4.7–9.2)	7.3 (3.5–9.3)	6.7 (5.5–9.1)	7.4 (3.8–9.4)
	Negative, *n* (%)	0 (0)	0 (0)	0 (0)	0 (0)
	Reactive, *n* (%)	23 (100)	10 (100)	6 (100)	7 (100)
3	Neutralization, %	86.4 (84.3–89.5)	86.2 (80.9–88.8)	87.1 (69.7–90.1)	85.8 (85.3–89.5)
	Low, *n* (%)	1 (4.3)	0 (0)	1 (20)	0 (0)
	High, *n* (%)	22 (95.6)	10 (100)	5 (80)	7 (100)
4	Avidity, %	61.4 (50.0–66.2)	64.7 (52.3–70.0)	55.7 (42.4–62.6)	59.0 (42.2–75.0)
	Low, *n* (%)	6 (26.1)	1 (10)	3 (50)	2 (28.6)
	High, *n* (%)	17 (73.9)	9 (90)	3 (50)	5 (71.4)

adding HRP-substrate solution. Avidity index (a.i.), proportional to antibody denaturation resistance, was calculated as ratio of OD-450 in DA and PBS wells. The antibody avidity criteria were as follows: avidity index (a.i.) > 50% — high avidity; between 40 and 50% — intermediate avidity; < 40% — low avidity.

Data analysis

The results are reported as medians with min-max range and the parameters were compared between groups by the Mann–Whitney nonparametric *t* test and at different time points using the paired Wilcoxon rank test. *P* value < 0.05 was considered statistically significant.

RESULTS

All antibody assays were provided for 100% of samples. Samples of 23 hospitalized patients with confirmed COVID-19 demonstrated a common Ab dynamics pattern. Two weeks after the symptoms onset all samples became IgG positive for both RBD (Me S/CO 10.5, min-max, 1.1–13.3) and Nc (Me, 9.4, min-max 3.2–11.7) (Fig. 1, Table 2). Twenty two out of 23 samples (95.6%) demonstrated high neutralization activity in ACE2-RBD binding inhibition assay and low avidity with median 20% (min-max 7.5%–37.3%) of anti-RBD IgG antibodies. Only 1 sample in this cohort had a.i. of 64.7%, probably due to previous unregistered antigen exposure. As expected, these results propose the presence of unmatured virus-specific IgG [4]. To analyze the longevity of humoral immunity to SARS-CoV-2 in convalescent patients, blood samples were collected 4–6 months later. All samples remained positive for anti-RBD and anti-Nc IgG (Fig. 2, Table 2). Median anti-RBD p.i. dropped from 10.5 to 6.8 S/CO and median anti-Nc p.i. — from 9.4 to 7.4 S/CO. Samples with low anti-RBD IgG levels (S/CO <5) at the first time point were obtained after 10–12 days after the symptom's onset, thus, they had not enough time to reach peak of serum antibody concentrations due to heterogeneity of SARS-CoV-2 incubation period and variability in the individual dynamics of Ab production. However, four months later all patients with one exception had similar levels of anti-RBD IgG (Me S/CO 6.8, IQR 6.3–8.9).

Although we detected a 1,5-fold decrease of anti-RBD IgG levels of this cohort, the neutralization activity remained at high level without significant difference between first and second time points which indicates that the quantity of antibodies is not the only determinant of the neutralizing activity. Preservation of high neutralization effects can be due to the compensation of decreased antibody concentration by improved quality (specificity and affinity) during this period. On the other hand, the observed effect may be due to the peculiarities of the test system used in the study. Since the measured values are near the limit of quantification of the system it does not allow to reliably assess the changes in neutralizing activity. Additional experiments are required to access neutralization dynamics more precisely. During the same observation period anti-RBD IgG avidity increased significantly, reaching a high avidity level (>50%) in 78% of patients. Median avidity at the second time point was 61.4% (min-max 18.2–86.9%). Remaining

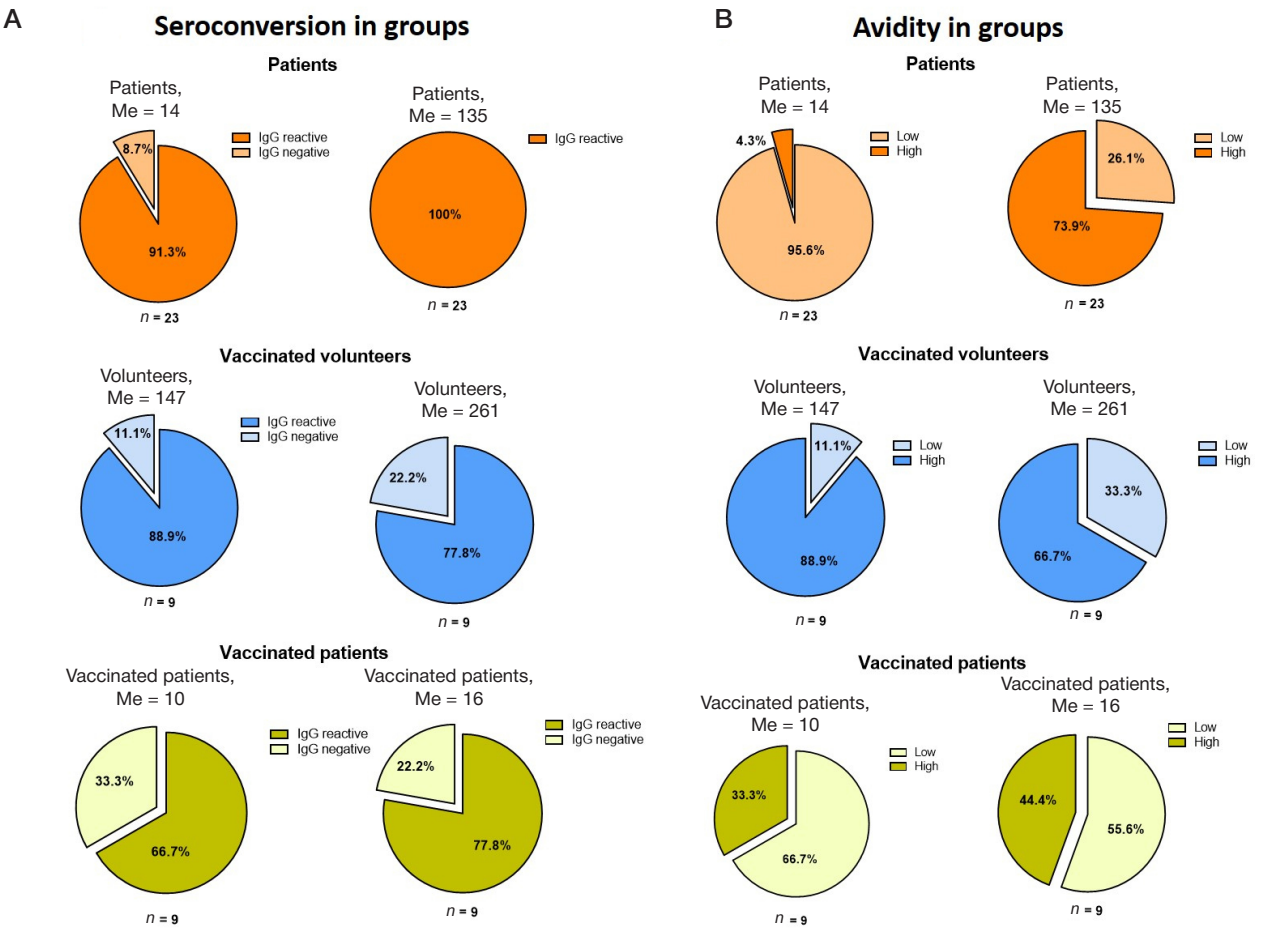

Fig. 2. Anti-RBD IgG conversion (**A**) and avidity dynamics (**B**) in the studied groups

6 patients (22%) had intermediate (40–50%, "maturation zone") or low (<40%) anti-RBD IgG avidity. Importantly, five of low- and intermediate-avidity patients had moderate or severe disease (Fig. 3). One patient (Fig 1A, orange circles) had low levels of anti-RBD IgG and neutralization activity at both time points, but his anti-RBD IgG avidity increased from 20.4 to 42.7%. To the end of the hospitalization period most patients possessed strong IgG response to SARS-CoV-2 antigens and their antibodies were effective at preventing RBD binding to human ACE-2. During the next months affinity maturation of anti-RBD IgG response occurred and overall concentration of anti-RBD and anti-Nc IgG decreased, neutralization activity remained at high level.

In a group of healthy Sputnik V vaccinated volunteers (Fig. 1B) 8 out of 9 samples were positive for anti-RBD IgG at the first time point (median of 147 days after the initial vaccination) with 4.9 S/CO (min-max 1–7.9). Four months later (261 days post initial vaccination), the median anti-RBD IgG p.i. dropped significantly to a median level 1.7 S/CO although 7 out of 9 volunteers still remained positive for anti-RBD IgG. None of the volunteers had detectable IgG to Nc, indicating there were no cases of infection with SARS-CoV-2 in this group. The neutralization correlated the IgG dynamics and decreased significantly in all samples from median 57.4% in the first time point to median 30.6% in the second time point. On the opposite, the avidity remained at the same high level indicating that at the first time point (147 days post vaccination), antibody maturation occurred in all vaccinated volunteers. However, for 3 samples (33%) at the second time point it was impossible to estimate the avidity as the level of anti-RBD IgG was too low (Fig. 1B, highlighted in *blue*).

In a group of Sputnik V vaccinated patients with confirmed COVID-19 (Fig 1C, Table 1) the median time from receiving a first vaccine dose was 62 days (ranging from 30 to 88 days) and the median time of sampling from symptoms onset was 10 days (5 to 19 days). At that time 6 out of 9 samples were positive for anti-RBD IgG with median 4.3 S/CO and only two were positive for anti-Nc IgG. All anti-RBD positive samples and one negative sample were positive in the neutralization ELISA (median 71.6%, min-max 7.4%–98.6%). Most of the samples (66.7%) demonstrated low avidity antibodies with median a.i. 27.6% (min-max 0%–71.1%). The second sampling time point in this group was around the 16th day after the symptoms onset. One week after the first sampling, the median S/CO of anti-RBD IgG in general did not change significantly (median 7 S/CO, min-max 0.8–11.2), however, 7 out of nine patients were seropositive after the infection and/or vaccination. The neutralization did not significantly change compared to the first time point (median 92.9%, min-max 23.2%–99.2%), the same was observed for avidity. Neutralization activity in two samples with low levels of anti-RBD IgG increased significantly to 85 and 89%, probably due to anti-RBD IgM production. (Fig. 1, *yellow dots*)

DISCUSSION

In COVID-19 patients, neutralizing antibody titers correlate with the severity of the infection [5, 6] and can be achieved even at low somatic hypermutation [7, 8]. The RBD region is found to be immunodominant and it is the target of approximately 90% of the neutralizing antibodies presented in the sera of SARS-CoV-2-infected people. Furthermore, it has been determined that

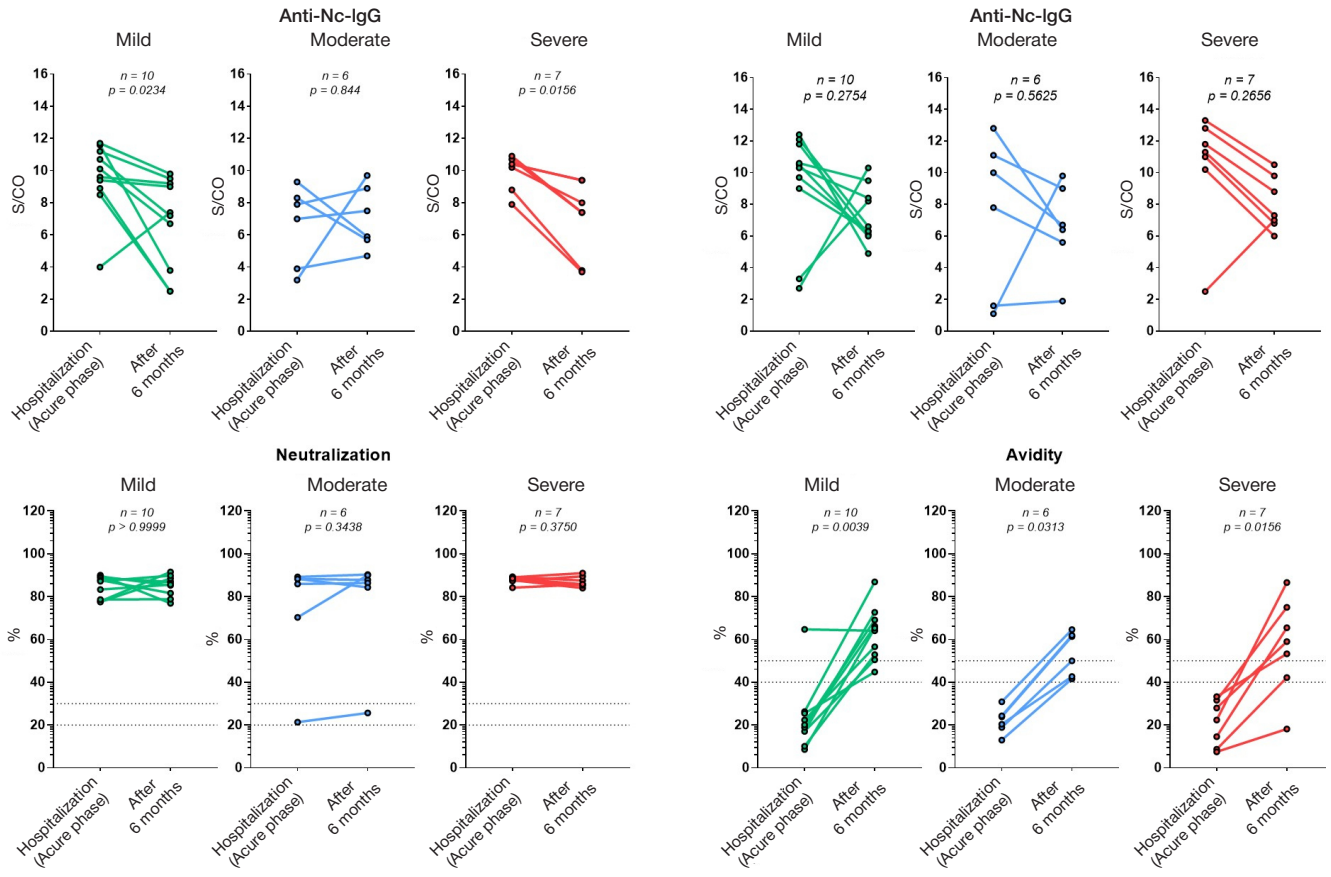

Fig. 3. Antibody levels in patients with different disease severity

anti-RBD IgG titers decrease with time post symptom onset, presenting a half-life of approximately 49 days. Importantly, antibody avidity increases over time due to increased maturation (somatic hypermutation, followed by selection in germinal centers). In the serum of hospitalized COVID-19 patients, there is a higher number of IgG against S protein and RBD, compared with that in non-serious and asymptomatic patients [9]. It was previously shown that antibody avidity increased over duration of infection and remained elevated [1]. In convalescent donors' plasma higher neutralizing titer had a stronger positive correlation with anti-spike IgG avidity than with anti-nucleocapsid IgG avidity proposing the anti-RBD IgG to be the main source of neutralizing activity.

Comparing samples of hospitalized patients and vaccinated volunteers 4–6 months after the infection or the vaccination, vaccinated volunteers had significantly lower levels of anti-RBD IgG and neutralization activity, but significantly higher anti-RBD IgG avidity (Fig. 4A, C). All hospitalized patients remained positive for anti-RBD IgG, while in one of the vaccinated volunteers antibody levels dropped below baseline (Fig. 4C).

When comparing the groups of Sputnik V fully vaccinated patients and unvaccinated patients samples, collected during the acute phase of infection (2–3 weeks after symptoms onset) it was shown that, in vaccinated patients antibody levels to both RBD and Nc antigens were significantly lower, than in unvaccinated patients (Fig. 4B). Anti-RBD IgG avidity was significantly higher in vaccinated patients indicating that despite low antibody levels, vaccination did induce primary immune response and formation of memory B-cells and their antibody production during the infection demonstrates signs of secondary immune response. In the long-term perspective antibody affinity maturation rate is higher after vaccination than after natural infection. Although it is proposed that antibody maturation increases their neutralization potency [10], here we observe no significant correlation between these parameters.

Our study is under certain significant limitations. First of all, it is based on a small number of samples. Patients in this study had a limited spectrum of COVID-19 clinical manifestations. Particularly, our research does not include asymptomatic and outpatient individuals with a mild course of disease, which represent the most of COVID-19 cases in comparison with other disease manifestations. Second, we could not estimate the peaks of antibody production and maturation as only two timepoints were analyzed in each group.

CONCLUSIONS

We observed the anticipated dynamics of Ab levels in convalescent patients with the peak at acute phase followed by gradual decrease in the subsequent months. But despite the loss of anti-RBD antibodies concentrations, serum neutralization activity remained at high/sufficient level, probably due to improved Ab specificity and increased avidity. Here, we demonstrated the formation and persistence of high avidity IgG for at least 6 months after Sputnik V immunization. It indicates that protectivity markers remain at sufficient levels for at least 4-6 months after the infection or the vaccination. These results give rounds for the half-year period chosen for booster immunization with Sputnik V in Russia. Based on our data one can't estimate the proportion and characteristics of patients requiring earlier revaccination due to drop of vaccine-induced protection before the 6 months period. We propose that low anti-RBD IgG avidity two months after vaccination could be one of the potential markers for preliminary revaccination. Possibly this may indicate that estimation of RBD-specific antibodies avidity can serve not only as a prognostic marker of

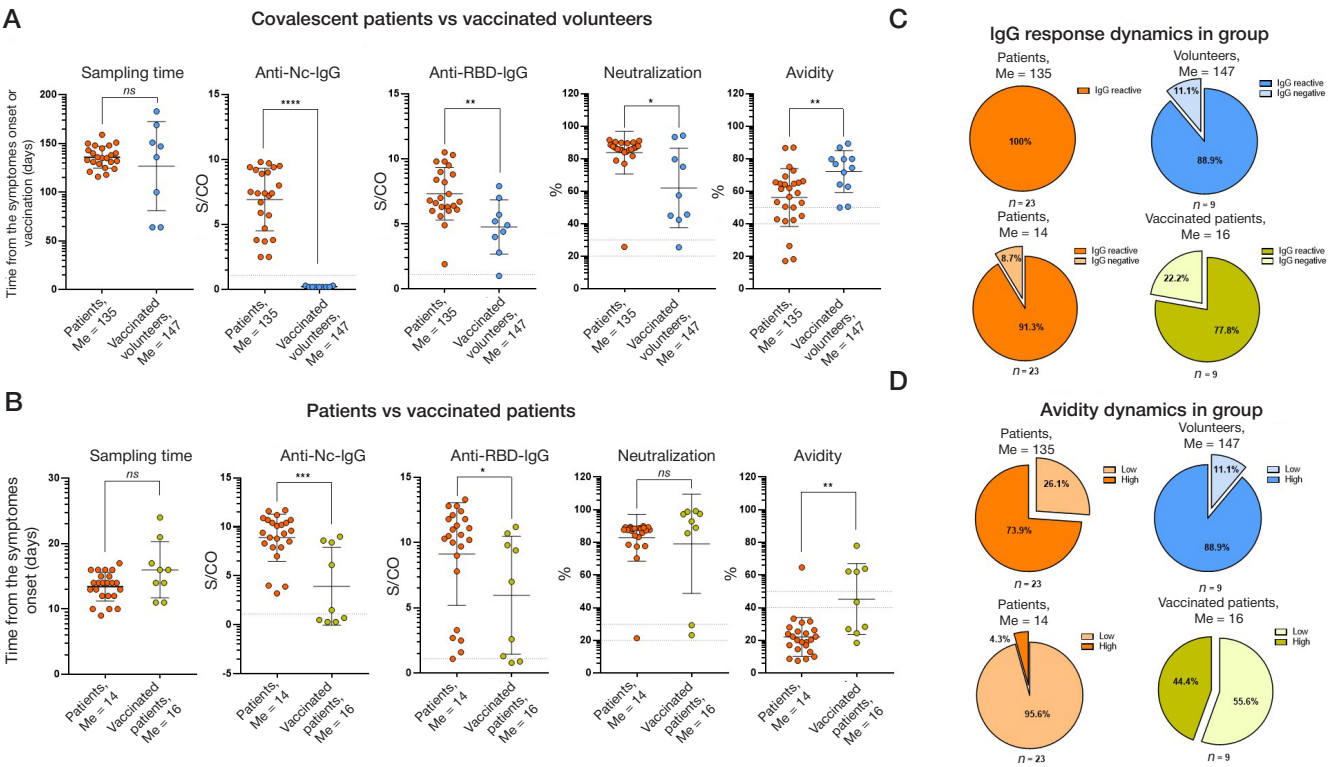

Fig. 4. Comparison of immune responses in different groups. (**A**) Comparison of unvaccinated convalescent patients and healthy vaccinated volunteers 4–6 months after the infection/vaccination. (**B**) Comparison of vaccinated and unvaccinated patients in the acute phase of infection (2–3 weeks from symptoms onset). (**C**) Comparison of anti-RBD IgG conversion in the studied groups. (**D**) Comparison of sera avidity dynamics in the studied groups. Significant differences are shown as asterisks (Mann–Whitney U test)

disease severity, but also to determine individuals, who require revaccination earlier than 6 months after receiving the initial dose. Further study on larger samples is needed to verify this hypothesis.

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