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Serological anti-SARS-CoV-2 neutralizing antibodies association to live virus neutralizing test titers in COVID-19 paucisymptomatic/symptomatic patients and vaccinated subjects

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ABSTRACT

A large number of immunoassays have been developed to detect specific anti-SARS-CoV-2 antibodies; however, not always they are functional to neutralize the virus. The reference test for the anti-spike neutralizing antibodies (nAbs) ability to counteract the viral infection is the virus neutralization test (VNT). Great interest is developing on reliable serological assays allowing antibodies concentration and antibody protective titer correlation. The aim of our study was to detect nAbs serum levels in paucisymptomatic, symptomatic and vaccinated subjects, to find a cut-off value able to protect from virus infection.

nAbs serum levels were detected by a competitive automated immunoassay, in association to VNT with the SARS-CoV-2 original and British variant strains.

The median nAbs concentrations were: 281.3 BAU/ml for paucisymptomatics; 769.4 BAU/ml for symptomatics; 351.65 BAU/ml for the vaccinated cohort; 983 BAU/ml considering only the second dose vaccinated individuals. The original strain VNT analysis showed 1:80 median neutralization titers in paucisymptomatic and vaccinated subjects; 1:160 in symptomatic patients; 1:160 in the second dose groups. The British variant VNT analysis showed lower neutralization titers in paucisymptomatic and vaccinated groups (1:40); the same titer in symptomatic patients (1:160); the second dose group confirmed the original strain titer (1:160).

In conclusion, our data showed optimal correlations with a proportional increase between neutralizing activity and antibody concentration, making nAbs detection a good alternative to virus neutralization assays, difficult to carry out in routine laboratories. Finally, ROC curve analysis established a cut-off of 408.6 BAU/ml to identify subjects with a low risk of infection.

1. Introduction

Understanding the immune memory to SARS-CoV-2 can improve diagnostics and vaccines as well as assessing the COVID-19 pandemic course for the future. The analysis of the anti-SARS-CoV-2 antibody responses evolution during infection may provide important insight into therapeutic approaches and COVID-19 vaccination [1].

In the past months, a large number of commercial immune-assays have been evaluated for the detection of specific anti-SARS-CoV-2 antibodies [2–8]. However, their presence not always indicates whether the antibodies are functional to neutralize the virus.

The virus neutralization test (VNT) is assumed to be the reference for assessing the antibodies ability to block the virus from entering in the human cells [9]. Nevertheless, such an assay requires virus manipulation in a biosafety level 3 laboratories (BSL3), with trained staff and specific equipment.

Among the serological tests, the most important role is played by anti-spike protein assays, which include antibodies against the receptor

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binding domain (RBD), the subunit 1 (S1) or the full spike (S). The spike glycoprotein is critical because it is able to mediate virus entry into human cells by binding to the angiotensin converting enzyme 2 (ACE2) receptor [10].

Moreover, clinical trials using monoclonal antibodies (mAbs) against S-RBD have shown reduced viral load in patients with mild or moderate COVID-19 [11–13].

Furthermore, it has been described that anti-spike neutralizing antibodies (nAbs) produced by COVID-19 patients can block the human cells viral infection *in vitro* and counteract viral replication *in vivo* [14–16].

However, the impact of nAbs on COVID-19 course is still controversial as some studies did not find nAbs variations among hospitalized patients with different disease outcomes [14].

It has also been observed that the commercial tests performances are higher in patients with severe COVID-19 due to the strong immune response. At this purpose, the reinfection occurrence has been also evaluated, highlighting that subjects who had neutralizing antibodies did not encounter a second infection but the risk may be higher in previously mild COVID-19 patients [1,17].

On the vaccination side, the role of anti-SARS-CoV-2 and neutralizing antibodies has not yet been fully validated as it is not known how long the antibodies persist and which is the value that confers protection. Reliable assays for quantitative serological detection are therefore pivotal to assess vaccines immunological responses and they could become essential if a correlation between antibodies concentration and antibody protective titer could be identified [18].

SARS-CoV-2 live VNT assay represents the most sensitive method for monitoring nAbs titers in infected patients and/or vaccinated subjects, however it requires a team of experts, long and complicated procedures and the availability of a BSL-3 laboratory which limits the screening of patient and vaccine samples. For this reason, in order to simplify the monitoring of neutralizing antibodies, it is necessary to develop a standard specific and sensitive assay.

To this end, there is a great deal of interest in serological testing studies showing an association with VNT. Since according to the food and drug administration (FDA) recommendations the required titer for plasma donors is $\geq$160, it would be desirable to find the corresponding serum concentration value of anti-SARS-CoV-2 neutralizing antibodies to prevent infection in vaccinated people and reinfection in COVID-19 patients, avoiding the time-consuming, expensive and dangerous VNT procedure.

The aim of our study was to detect nAbs serum levels in paucisymptomatic not hospitalized patients and in symptomatic hospitalized patients, as well as in vaccinated healthcare workers, by a competitive automated immunoassay in association with their parallel VNT analysis, in order to find a cut-off serum value able to hamper virus infection.

2. Patients and methods

2.1. Patients' cohort

The study was conducted at "Tor Vergata" University COVID-Hospital of Rome and it was approved by the local ethical committee (protocols no. R.S.44.20).

A total of 98 patients were enrolled between March 2021 and May 2021 providing informed consent prior to collection of samples.

We included: (i) 31 samples [median age 55 years (range 22–81); 16 M/15F] from patients with RT-PCR-confirmed SARS-CoV-2 infection who have been managed in outpatient settings and exhibited mild to moderate symptoms. These patients were not hospitalized and samples were collected after six months from SARS-CoV-2 infection (paucisymptomatic patients); (ii) 37 samples [median age 54 years (range 26–78); 22M/15F] from RT-PCR-confirmed patients who showed severe symptoms and were admitted to the intensive care (ICU) or respiratory system department of "Tor Vergata" University Covid-Hospital. Samples were collected after six months from SARS-CoV-2 infection (symptomatic patients); (iii) 30 samples [median age 44 years (range 28–64); 7M/23F] from vaccinated healthcare workers who have received at least the first dose of Pfizer vaccine. Total median time: 23.5 days (range 10–51 days); first dose (n = 15) and second dose samples (n = 15) median times: 20 days (range 10–21 days) and 45 days (range 26–51 days), respectively (vaccinated subjects).

Unfortunately, according to hospital data access policy, we cannot access to further clinical data, except for those concerning laboratory medicine department examinations.

The samples were centrifuged at 2500 g for 10 min, within 1 h from collection and frozen at −80 °C until analysis; a part of them was transported to the DIESSE Diagnostica Senese laboratory (Siena, Italy) to carry out the serum virus neutralization test.

The study was in accordance with the Helsinki Declaration, as revised in 2013.

2.2. Anti-SARS-CoV-2 neutralizing antibodies assay

The "Chorus SARS-CoV-2 NEUTRALIZING Ab" (DIESSE Diagnostica Senese, Siena, Italy) is a kit for the quantitative determination of total (IgG/IgA/IgM) anti-S1 SARS-CoV-2 antibodies performed on the automated Chorus TRIO instrument (DIESSE Diagnostica Senese, Siena, Italy). The detection method is based on the principle of competitive test using SARS-CoV-2 anti-S1 therapeutic monoclonal antibodies.

The SARS-CoV-2 anti-S1 antibodies present in the serum test samples compete with the peroxidase-conjugated SARS-CoV-2 anti-S1 therapeutic monoclonal antibodies to bind the spike protein RBD antigen of the S1 subunit fixed on the solid phase support. The higher the concentration of antibodies present in the serum, the lower the possibility of peroxidase-conjugated SARS-CoV-2 anti-S1 therapeutic monoclonal antibody binding to the fixed antigen, and *vice-versa*. After loading 100 μl of the samples into the specific well, automated washings eliminate the unbound components and the TMB (tetramethylbenzidine) chromogenic substrate is added to trigger a colorimetric reaction by the enzyme-labeled monoclonal antibodies. The intensity of the colorimetric reaction is inversely proportional to the concentration of anti-S1 antibodies in the test sample.

The results are expressed in Binding Antibody Units (BAU/ml), according to the first World Health Organization international standard. The samples are considered positive for values >50 BAU/ml; negative for values <20 BAU/ml and equivocal for all the values between 20 and 50 BAU/ml.

The linearity range is 20–1500 BAU/ml. Samples >1500 BAU/ml can be diluted as declared by manufacturer. This test is CE approved.

2.3. Live virus neutralization test (VNT)

The live virus neutralization test (VNT) is a specialized type of immunoassay to detect antibodies able to inhibit virus replication *in vitro*. The live VNT was performed to establish the lower serum cut-off value to protect against SARS-CoV-2 infection and is considered the gold standard method for the assessment of nAbs.

The VNT assays were performed by the DIESSE laboratory (Diagnostica Senese, Siena, Italy) using either the SARS-CoV-2 virus 2019-nCov/Italy-INMI1-strain (SARS-CoV-2 original strain) and the HUMAN NCOV19 ISOLATE/ENGLAND/MIG457/2020 LINEAGE B.1.1.7 (SARS-CoV-2 British variant strain).

The neutralizing antibodies titer was determined using a 4 step-protocol: epithelial cell line VEROE6 colture, SARS-CoV-2 virus titration, viral growth in cell colture and micro-neutralization assay with subsequent cytopathic effect (CPE)-read out.

2.3.1. Cell colture

Epithelial cell line VEROE6, derived from the kidney of African green

monkey Cercopithecus aethiops, were acquired from the American Type Culture Collection (ATCC-CRL 1586). Adherent sub-confluent VERO E6 cells monolayers were prepared in DMEM medium containing 10% FBS.

2.3.2. SARS-CoV-2 virus titration

SARS-CoV-2 virus 2019-nCov/Italy-INMI1-strain (original strain) was purchased from Spallanzani Institute (Rome, Italy) via the European Virus Archive Global (EVAg), whereas the HUMAN NCOV19 ISOLATE/ENGLAND/MIG457/2020 LINEAGE B.1.1.7 (British variant strain) was purchased from the Department of Health: Public Health England-Virology & Pathogenesis group (London, United Kingdom), via the European Virus Archive Global (EVAg).

The viruses were titrated in serial 1log dilutions to obtain a 50% tissue culture infective dose (TCID50) on 96-well culture plates of VERO E6 cells. The plates were daily observed at inverted optical microscope for 3 days, to evaluate the presence of cytopathic effect.

The end-point titers were calculated according to the Reed & Muench method based on eight replicates for each titration.

2.3.3. Viral growth in cell culture

Vero E6 cells were seeded in T175 flasks at a density of 40,000/cm2 and propagated using DMEM, supplemented with 10% FBS and 100 IU/ml penicillin–streptomycin. After 4–7 days, the cells were infected with 12–14 ml of DMEM with 2% FBS containing the virus at a multiplicity of infection of 0,01. After 1 h of incubation at 37 °C in a humidified atmosphere with 5% CO_2, 70 ml of DMEM containing 2% FBS were added. The flasks were daily observed, and the virus was harvested until a CPE of 80%-90% was observed. Then it was aliquoted and stored a −80 °C.

2.3.4. Micro-neutralization assay and CPE-read out

Serum test samples were heat-inactivated for 30 min at 56 °C; two-fold serial dilutions, from 1:10 to 1:1280, were then mixed with an equal volume of viral solution containing 100 TCID50 of SARS-CoV-2 virus. The serum-virus mixture was incubated 1 h at 37 °C in a humidified atmosphere with 5% CO_2. After incubation, 100 μl of each dilution mixture were added in duplicate to a cell plate containing a semi-confluent VERO E6 monolayer. The plates were then incubated for 3 days at 37 °C in a humidified atmosphere with 5% CO_2 and analyzed with an inverted optical microscope. The highest serum dilution able to protect from CPE more than 50% of the cells was considered as the neutralization titer.

2.4. Data analysis and statistics

Statistical analysis was performed with GraphPad Prism 8 Software (GraphPad Software, San Di-ego, California, USA).

D'Agostino and Pearson test, Shapiro–Wilk normality test, and Kolmogorov–Smirnov test were used to evaluate non-Gaussian distributions in all study populations.

The categorical data were displayed as numbers and/or percentages and continuous data as median and range.

Non-parametric results were analyzed with the Mann-Whitney test. Correlations were performed using the non-parametric Spearman's rank correlation.

For all results, $p < 0.05$ was considered statistically significant.

Specificity (false-positive rate), sensitivity (true-positive rate), Area Under Curve (AUC) and the optimal cut-off were calculated using receiver operating characteristic curves (ROC curves).

For each group were considered as negatives, patients with a neutralizing titer lower than the median neutralizing titer found using the live VNT (i.e. <1:80 for paucisymptomatic and vaccinated groups, <1:160 for symptomatic group, for the SARS-CoV-2 original strain; <1:40 for paucisymptomatic and vaccinated groups, <1:160 for symptomatic group, for the British variant strain).

3. Results

A total of 98 samples, divided in paucisymptomatic patients, symptomatic patients and vaccinated subjects, were analyzed with the "Chorus SARS-CoV-2 NEUTRALIZING Ab" automated assay and results were reported in Fig. 1A and Table 1.

Among the paucisymptomatic patients' group, the anti-S1 nAbs median concentration was: 281.3 BAU/ml; 27/31 (87%) samples were positive; 2/31; 6.5%) resulted as "equivocal" with values of 43.9 BAU/ml and 47,4 BAU/ml respectively; 2/31 (6.5%) resulted negative, both with values <20 BAU/ml.

The two "equivocal" samples were repeated, but results remained unchanged, showing a good repeatability of the measurements.

In the symptomatic study cohort, all samples were positive (37/37; 100%) and the median concentration was higher than paucisymptomatic patients: 769.4 BAU/ml. Moreover, the anti-S1 SARS-CoV-2 antibodies median concentrations showed a statistical significance between these groups (281.3 BAU/ml vs 769.4 BAU/ml; $p = 0.003$, respectively), Fig. 1A.

In the vaccinated cohort, the anti-S1 SARS-CoV-2 nAbs median concentration was 351.65 BAU/ml; 28/30 subjects resulted positive (93.3%); 2/30 (6.6%) "equivocal" with nAbs levels of 25.9 BAU/ml and 40.8 BAU/ml, respectively. Both the equivocal vaccinated patients were collected 10 days after first Pfizer vaccine dose.

When comparing vaccinated results to paucisymptomatic and symptomatic patients' groups, we found contradictory data with a no statistical difference between vaccinated and paucisymptomatic patients but a statistically significant difference between vaccinated subjects and symptomatic patients ($p = 0.65$ and $p = 0.03$, respectively), Fig. 1A.

We decided to repeat the analysis dividing the vaccinated cohort in first dose group ($n = 15$) and second dose group ($n = 15$). Data showed statistically significant difference in anti-S1 median concentrations between them: 162.4 BAU/ml vs 983 BAU/ml, p value <0.0001, respectively, Fig. 1B.

As a consequence, only the second dose vaccinated subjects were compared to paucisymptomatic and symptomatic patients' groups founding statistically significant difference with paucisymptomatic patients (281.3 BAU/ml vs 983 BAU/ml, $p = 0.0005$) and, as expected, no statistical difference with symptomatic patients: (769.4 BAU/ml vs 983 BAU/ml, $p = 0.22$, Fig. 1C).

The second aim of our study was to carry out live VNT analyses for comparing them to the serological results, in order to identify a nAbs cut-off value capable of guaranteeing immunity against SARS-CoV-2 reinfection (Table 1). Our results on the original strain showed a median neutralization titer of 1:80 in paucisymptomatic patients and in the total vaccinated subjects; in symptomatic patients, a higher median neutralization titer was found (1:160).

As for anti-S1 nAbs analysis, we subsequently divided the vaccinated in first and second dose groups. Results showed a significant statistical difference between the groups: 1:40 vs 1:160, $p < 0.0001$; respectively.

Spearman's test for SARS-CoV-2 neutralizing antibody titers and VNT results showed optimal correlations: $r = 0.85$ for paucisymptomatics; $r = 0.74$ for symptomatics and $r = 0.82$ for the vaccinated (Fig. 2A).

Furthermore, an additional live VNT was performed using the SARS-CoV-2 British variant strain (Table 1). Results showed a lower median neutralization titer in paucisymptomatic and vaccinated groups (1:40) compared to the VNT results from SARS-CoV-2 original strain; the symptomatic patients' cohort showed similar results (1:160). Also in this case, by dividing the vaccinated group between first and second dose, a significant statistically difference in median neutralization titers was found: 1:20 and 1:160, $p < 0.0001$; respectively.

Spearman's rank correlation between nAbs immunoassay and the VNT analysis using the SARS-CoV-2 British variant strain, showed optimal coefficients similar to the results obtained from SARS-CoV-2

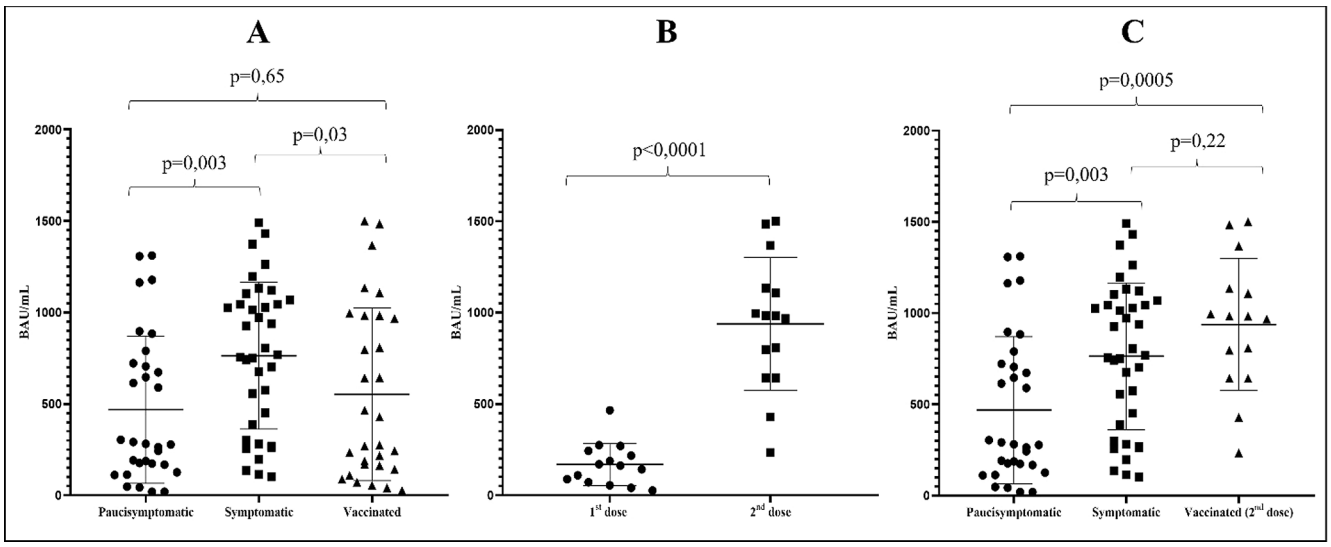

Fig. 1. Anti-S1 SARS-CoV-2 antibodies levels in paucisymptomatic, symptomatic and vaccinated subjects (A); Anti-S1 SARS-CoV-2 antibodies levels in the total vaccinated subjects (B); Anti-S1 SARS-CoV-2 antibodies levels in paucisymptomatic, symptomatic and vaccinated subjects after 2nd dose of Pfizer vaccine (C).

Table 1
Neutralizing antibody levels and live virus neutralization titers using both SARS-CoV-2 original strain and SARS-CoV-2 British variant strain, for each study cohort.

	Paucisymptomatic patients	Symptomatic patients	Vaccinated subjects		
	(n = 31)	(n = 37)	Total (n = 30)	1st dose (n = 15)	2nd dose (n = 15)
Median [nAbs]	281.3 BAU/ml	769.4 BAU/ml	351.65 BAU/ml	162.4 BAU/ml	983 BAU/ml
Range	(20–1311.7 BAU/ml)	(102.1–1491.3 BAU/ml)	(25.9–1500 BAU/ml)	(25.9–465.1 BAU/ml)	(233.9–1500 BAU/ml)
SARS-CoV-2	1:80	1:160	1:80	1:40	1:160
Original strain	(range 0–1:640)	(range 1:40–1:1280)	(range 1:10–1:1280)	(range 1:10–1:80)	(range 1:80–1:1280)
Median neutralization titer					
SARS-CoV-2	1:40	1:160	1:40	1:20	1:160
British variant strain	(range 0–1:1280)	(range 1:10–1:1280)	(range 0–1:1280)	(range 0–1:40)	(range 1:20–1:1280)
Median neutralization titer					

original strain: r = 0.88 for paucisymptomatic; r = 0.73 for symptomatic and r = 0.84 for vaccinated (Fig. 2B).

The best fit cut-offs, specificities, and sensitivities were calculated using the receiver operating characteristic curves (ROC curves).

In paucisymptomatics (1:80 median neutralization titer), an AUC value of 0.9580 was achieved, with a sensitivity of 94.1% and a specificity of 78.6% at a cut-off value of 217.5 BAU/ml; in symptomatic cohort (1:160 median neutralization titer), an AUC value of 0.9400 was detected, with a sensitivity of 92% and a specificity of 75% at a cut-off value of 503.5 BAU/ml; vaccinated subjects (1:80 median neutralization titer), showed an AUC value of 0.9732, with a sensitivity of 93.8% and a specificity of 85.7% at a cut-off value of 256.6 BAU/ml (Fig. 3A; Table 2).

The same good ROC curve analysis results were found from live VNT using the SARS-CoV-2 British variant. Paucisymptomatic cohort (1:40 median neutralization titer) showed AUC value of 0.9430, with a sensitivity of 89.5% and a specificity of 75% at a cut-off value of 189.7 BAU/ml; in symptomatic patients group an AUC value of 0.9006 was observed, with a sensitivity of 82.6% and a specificity of 92.9% at a cut-off value of 753.3 BAU/ml; vaccinated subjects' analysis showed an AUC value of 0.9732 with a sensitivity of 93.8% and a specificity of 92.9% at a cut-off value of 272.2 BAU/ml (Fig. 3B; Table 2).

In addition, our previously published anti-SARS-CoV-2 S-RBD IgG antibody concentrations in paucisymptomatic and symptomatic groups, implemented with a new analysis on vaccinated anti-SARS-CoV-2 S-RBD IgG antibody concentration, were compared to VNT titer (data not shown) [19]. The Spearman's test values were higher in nAbs/VNT correlation respect to RBD/VNT correlation for both strains, in paucisymptomatics (r = 0.85 vs r = 0.84, original strain; r = 0.88 vs r = 0.85, British variant strain) and in symptomatics (r = 0.74 vs r = 0.62 original strain; r = 0.73 vs r = 0.65, British variant strain), except for vaccinated subjects (r = 0.82 vs r = 0.87, original strain; r = 0.84 vs r = 0.87, British variant strain).

Finally, all patients from the three groups were combined in order to provide a general nAbs cut-off value, creating a single population that was analyzed using a neutralizing titer cut-off of 1:160, based on the FDA recommendations [20].

Our results showed an AUC value of 0.8990, with a sensitivity of 88.6% and specificity of 70.4% at a cut-off value of 408.6 BAU/ml for SARS-CoV-2 original strain and an AUC value of 0.8966, with a sensitivity of 82% and specificity of 84.8% at a cut-off value of 713.6 BAU/ml for the British variant strain (Fig. 4A and B; Table 3). Lastly, Spearman's rank correlation comparing anti-SARS-CoV-2 neutralizing antibodies to VNT titer still kept optimal coefficients in both strains (r = 0.82 and r = 0.83) (data not shown), whilst the correlation was lower when comparing anti-SARS-CoV-2 S-RBD IgG antibodies to VNT titer in both strains (r = 0.60 and r = 0.56).

4. Discussion

The protective role of anti-spike neutralizing antibodies against SARS-CoV-2 has been studied by several research groups and they have been reasonably associated to antiviral immunity, blocking the RBD–ACE2 interaction and subsequent SARS-CoV-2 cell entry; nevertheless, increasing data on the kinetics of virus neutralizing antibody responses are needed [21–24].

SARS-CoV-2 nAbs response remain poorly documented and little is known about the durability of humoral responses. In particular, data

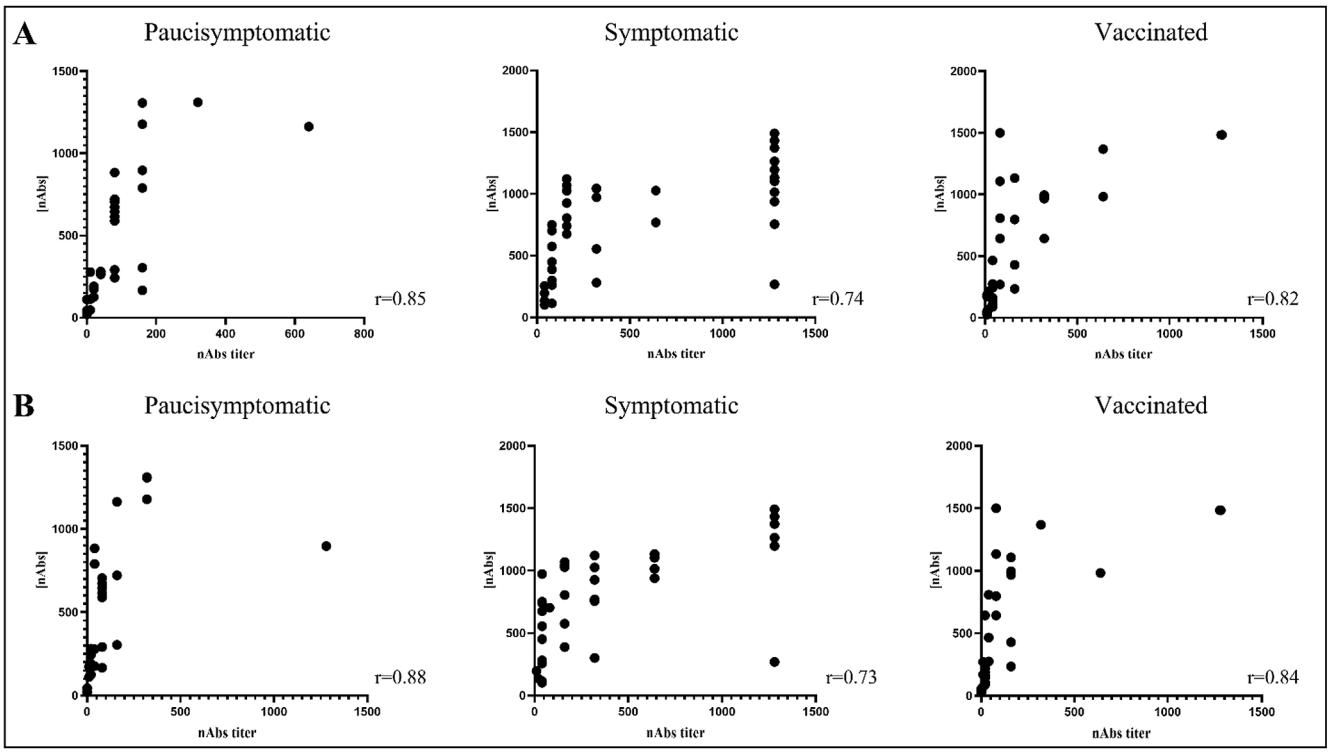

Fig. 2. Spearman's test correlation coefficients (r) between nAbs levels and live VNT titers in paucisymptomatic group; symptomatic group and total vaccinated subjects. (A) SARS-CoV-2 original strain; (B) SARS-CoV-2 British variant strain.

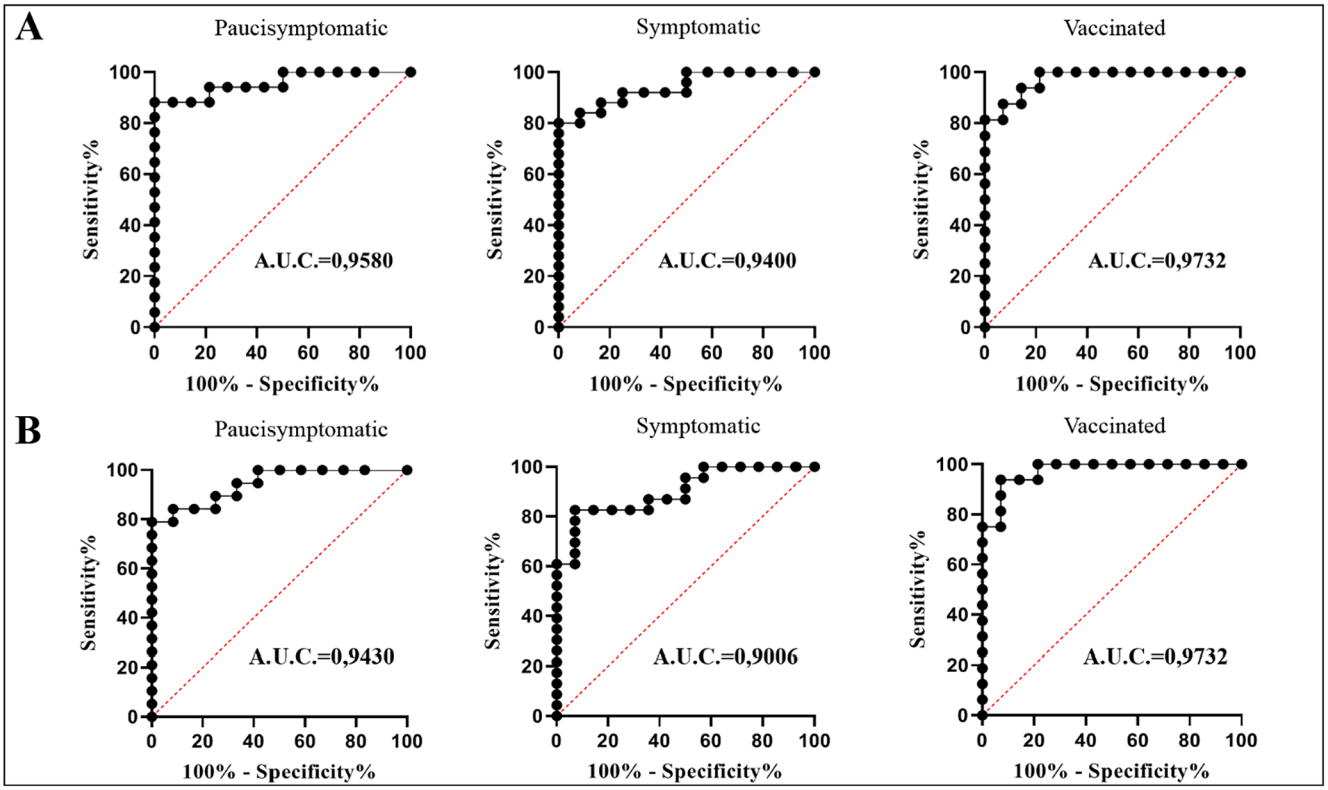

Fig. 3. SARS-CoV-2 original strain live VNT ROC curves in paucisymptomatic group, symptomatic group and total vaccinated subjects (A); SARS-CoV-2 British variant strain live VNT ROC curves in paucisymptomatic group, symptomatic group and total vaccinated subjects (B).

Table 2
ROC curve analysis parameters for each study cohort, using both SARS-CoV-2 original strain and SARS-CoV-2 British variant strain.

		Paucisymptomatic patients (n = 31)	Symptomatic patients (n = 37)	Vaccinated subjects (n = 30)
SARS-CoV-2 Original strain	Sensitivity	94.1%	92%	93.8%
	Specificity	78.6%	75%	85.7%
	Cut-off	217.5 BAU/ml	503.5 BAU/ml	256.6 BAU/ml
	Area under ROC curve (AUC)	0.9580	0.9400	0.9732
	95% confidence interval	0.8909 to 1.000	0.8692 to 1.000	0.9274–1.000
SARS-CoV-2 British variant strain	Sensitivity	89.5%	82.6%	93.8%
	Specificity	75%	92.9%	92.9%
	Cut-off	189.7 BAU/ml	753.3 BAU/ml	272.2 BAU/ml
	Area under ROC curve (AUC)	0.9430	0.9006	0.9732
	95% confidence interval	0.8688–1.000	0.8039 to 0.9974	0.9258–1.000

concerning the longevity of immunity, the persistence time of circulating nAbs and the standard method for antibodies quantification still needs to be further clarified [25–27].

Currently, the protective nAbs titer has not been established. In this regard, the serological antibody monitoring is essential not only as a screening test to identify asymptomatic carriers, but also to assess the degree of immunization of infected patients, to evaluate the antibodies response to different vaccination strategies and to check the presence of a suitable antibody titer in COVID-19 convalescent plasma for the treatment of hospitalized SARS-CoV-2 infected patients [20,28–30]. Indeed, the quantification of nAbs is critical to indicate the best hyperimmune plasma donors, as well as being the gold standard to evaluate the different vaccine responses.

The nAbs monitoring can be performed using several immunoassays, which are in rapid evolution to contrast the growing needs of more sensitive and detailed information about neutralizing antibodies quantification [31]. The different immunoassays could provide important data on the nAbs titer quantization, but they could also have a variable correlation to virus neutralization test, considered the gold standard for the assessment of nAbs.

In our study, nAbs levels on the three different patients' cohorts were evaluated using a commercial automated immunoassay; subsequently a live VNT analysis was performed to identify a possible protective nAbs median titer.

The neutralizing serum samples Abs levels showed lower median concentrations in paucisymptomatic patients respect to symptomatic patients (281.3 BAU/ml vs 769.4 BAU/ml, p = 0.003). Our data are in line with several studies, such as Long et al., in which the authors pointed out that the anti-SARS-CoV-2 IgG levels in the asymptomatic group were significantly lower than in the symptomatic group [32], and by Liu et al., who demonstrated that ICU patients with severe clinical conditions developed a faster and higher level of nAbs response [33].

It can be thus hypothesized a possible association with a higher viral load which increases the concentration of SARS-CoV-2 antigens and the production of neutralizing antibodies, suggesting that nAb titers may play a role in the severity of COVID-19 disease correlating with an improved inflammatory state of symptomatic or ICU patients.

Interestingly, the two negative paucisymptomatic patients developed a second SARS-CoV-2 infection and the samples collected after the reinfection did not develop again detectable levels of anti-S1 nAbs. These data allow to hypothesize a possible involvement of genetic dynamics in the production of neutralizing antibodies and other different mechanisms in the immune response against SARS-CoV-2 infection, including B-cell and T-cell immunity.

The total vaccinated subjects showed a non-statistically different antibody titer compared to paucisymptomatic patients (351.65 BAU/ml vs 281.3 BAU/ml, p = 0.65, respectively) because the group included also samples after the first dose of Pfizer vaccine. Indeed, dividing vaccinated subjects in first and second dose groups, we found a

Table 3
ROC curve analysis parameters for the total collected samples (n = 98), according to the food and drug administration (FDA) recommended neutralization titer (1:160). Results from SARS-CoV-2 original strain and SARS-CoV-2 British variant strain VNT analysis are reported in different columns.

		SARS-CoV-2 Original strain	SARS-CoV-2 British variant strain
FDA neutralizzation titer (1:160)	Sensitivity	88.6%	82%
	Specificity	70.4%	84.8%
	Cut-off	408.6 BAU/ml	713.6 BAU/ml
	Area under ROC curve (AUC)	0.8990	0.8966
	95% confidence interval	0.8355–0.9625	0.8334–0.9598

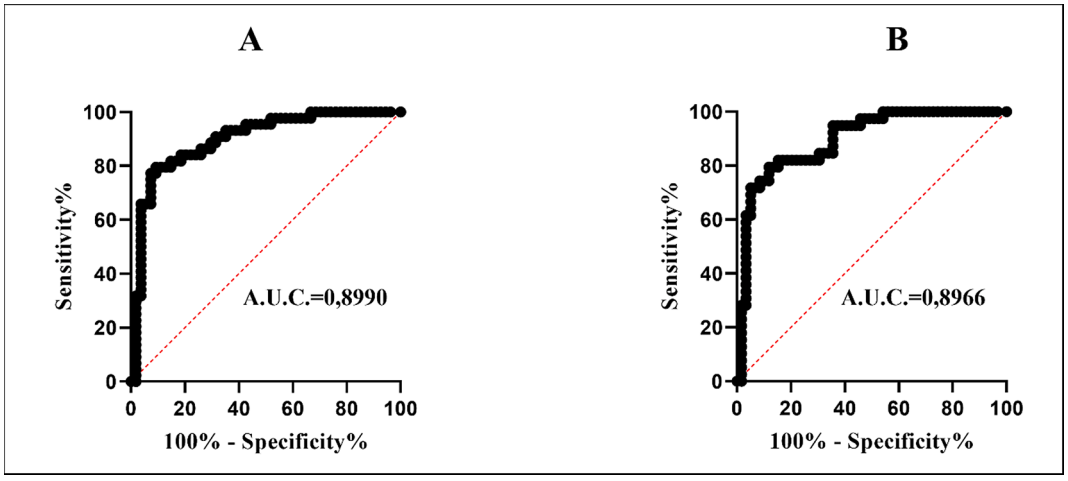

Fig. 4. ROC curves in the combined study cohort using the recommended FDA cut-off titer (1:160). (A) SARS-CoV-2 original strain; (B) SARS-CoV-2 British variant strain.

statistically significant difference between the second dose group and paucisymptomatic patients (983 BAU/ml vs 281.3 BAU/ml, $p = 0.0005$, respectively).

In this line, our results showed statistically significant differences between the whole vaccinated cohort and symptomatic patients (351.65 BAU/ml vs 769.4 BAU/ml, $p = 0.03$, respectively), but this difference is lost when comparing only vaccinated patients after the second dose of Pfizer vaccine to symptomatic cohort (983 BAU/ml vs 769.4 BAU/ml, $p = 0.22$, respectively). Our data demonstrates, as reported in literature, that peak of nAbs responses are markedly enhanced after the second Pfizer vaccine dose.

Nevertheless, the use of specific immunoassays directed only against the spike protein RBD antigen could underestimate the protective SARS-CoV-2 neutralization titers. Liu et al. identified non-RBD binding neutralizing antibodies involved in other neutralization pathways. In particular, Authors found nine different antibodies: four against RBD, three against spike N-terminal domain (NTD) and two against nearby quaternary epitopes that overlap with the domains at the top of the spike protein [34]. These data underline the great variety of possible antibodies with neutralizing activity and the consequent need of specific neutralization test.

For this purpose, we decided to evaluate the protective SARS-CoV-2 neutralization titers using the live VNT, considering that it is the best sensitive nAbs quantification method.

In our study, two distinct VNT analysis were performed using either the SARS-CoV-2 original strain and the British variant strain. Data from the first VNT analysis showed a median neutralization titer of 1:80 in paucisymptomatic patients and vaccinated subjects, whereas in symptomatic patients a higher median neutralization titer of 1:160 was found; dividing the vaccinated subjects, a median neutralization titer of 1:40 in first dose group and of 1:160 in second dose group was observed.

Despite the great variety found in nAbs levels, our data showed an increase in neutralizing activity with an increasing antibody concentration. The VNT results could be correctly associated to the nAbs concentrations found with the automated immunoassay tested, in which symptomatic patients and second dose vaccinated subjects had shown higher median neutralizing antibodies levels. This allows to hypothesize that the assessment of neutralizing antibodies levels by a serological specific immunoassay may be a good alternative to be used for the immunization status evaluation. In this regard, the nAbs immunoenzymatic tests could represent an alternative to serum neutralization assays that are usually not feasible in routine laboratories.

COVID-19 patients who developed nAbs against a specific SARS-CoV-2 strain, may be unprotected against a subsequent reinfection with the SARS-CoV-2 variants. Indeed, British variant and other SARS-CoV-2 strains, such as the Delta variant, are characterized by several different mutations in the nucleotide sequence encoding for the SARS-CoV-2 immunogenic surface S1-proteins [35].

For this reason, the live VNT analysis was performed also with the SARS-CoV-2 British variant strain. In line with the previous hypothesis, a lower median neutralization titer in paucisymptomatic and vaccinated groups was found (1:40). However, the same median neutralization titer for symptomatic patients was found, with a slight different range (1:160; range 1:10–1:1280 vs 1:160; range 1:40–1:1280).

Interestingly, for the vaccinated subjects a lower neutralizing titer was found only in the first dose group (1:20); in the second dose group the median neutralizing titer previously identified for the SARS-CoV-2 original strain was confirmed (1:160), pointing out a lower immune protection against the British variant strain in subjects who have received only the first dose of the vaccine.

ROC curve analysis established the best-fit nAbs cut-off values capable to confer immunity from a SARS-CoV-2 reinfection; they showed good AUC, sensitivity and specificity values for all the groups in the study and with both the SARS-CoV-2 strains allowing to combine the group populations to identify a general best cut-off. As expected, considering the 1:160 FDA titer recommended for hyperimmune plasma therapy, the British variant strain cut-off was higher than the original strain (713.6 BAU/ml vs 408.6 BAU/ml). The application of these cut-offs could help in identifying patients and/or vaccinated people with a good degree of immunization from subjects exposed to a new risk of SARS-CoV-2 infection.

Furthermore, our data showed higher nAbs/VNT correlation respect to RBD/VNT correlation for the paucisymptomatic and symptomatic groups, except for vaccinated subjects. Since in the latter cohort the antibody response is specific against the spike protein, whilst in the COVID-19 patients it could be related to all the SARS-CoV-2 antigenic proteins, the higher nAbs/VNT correlation can be thus explained.

Unfortunately, this study is conditioned by a small sample size and certainly the cut-off values should be confirmed on a larger cohort of patients, taking into account the great inter-individual immune response heterogeneity.

In conclusion, monitoring serological nAbs levels may help to establish a neutralization titer in order to assess the degree of immunization and the protection status against a possible reinfection or a new infection by SARS-CoV-2, even after vaccination. Despite the SARS-CoV-2 serological response is characterized by great inter-individual variability, this study shows the neutralizing antibodies persistence after 6 months from infection in almost all patients, indicating a longer duration than initially assumed.

Lastly, the "Chorus SARS-CoV-2 NEUTRALIZING Ab" immunoassay showed good repeatability and sensitivity. It could be considered a useful tool in the assessment of nAbs levels to help characterize not only the immunization status but also the effectiveness of various vaccination strategies, the right neutralizing antibody titer for the use of hyperimmune plasma as a passive immunotherapy in critically ill COVID-19 patients and the screening of the suitable donors to increase COVID-19 convalescent plasma collection quality.

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6. Ethics approval

The study was performed according to "Tor Vergata" University Covid-Hospital of Rome local ethical approvals (protocols no. R. S.44.20). Informed consent was obtained from all subjects enrolled in the study. The study was in accordance with the Helsinki Declaration, as revised in 2013.

CRediT authorship contribution statement

Antonio Cristiano: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Marzia Nuccetelli:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Massimo Pieri:** Data curation, Formal analysis, Writing – review & editing. **Serena Sarubbi:** Data curation, Formal analysis. **Martina Pelagalli:** Data curation, Formal analysis. **Graziella Calugi:** Supervision. **Flaminia Tomassetti:** Data curation, Formal analysis. **Sergio Bernardini:** Conceptualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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