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Evaluation of serological anti-SARS-CoV-2 chemiluminescent immunoassays correlated to live virus neutralization test, for the detection of anti-RBD antibodies as a relevant alternative in COVID-19 large-scale neutralizing activity monitoring

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ABSTRACT

The Spike-Receptor Binding Domain (S-RBD) is considered the most antigenic protein in SARS-CoV-2 and probably the key player in SARS-CoV-2 immune response. Quantitative immunoassays may help establish an anti-RBD Abs threshold as an indication of protective immunity. Since different immunoassays are commercial, the standard reference method for the neutralizing activity is the live Virus Neutralization Test (VNT).

In this study, anti-RBD IgG levels were detected with two chemiluminescent immunoassays in paucisymptomatic, symptomatic and vaccinated subjects, and their neutralizing activity was correlated to VNT titer, using SARS-CoV-2 original and British variant strains.

Both immunoassays confirmed higher anti-RBD Abs levels in vaccinated subjects. Furthermore, despite different anti-RBD Abs median concentrations between the immunoassays, a strong positive correlation with VNT was observed.

In conclusion, although the SARS-CoV-2 immune response heterogeneity, the use of immunoassays can help in large-scale monitoring of COVID-19 samples, becoming a valid alternative to VNT test for diagnostic routine laboratories.

1. Introduction

The novel human coronavirus 2 associated to Severe Acute Respiratory Syndrome (SARS-CoV-2), discovered in Wuhan, China, as the causative agent of the 2019 Coronavirus Disease (COVID-19) [1], belongs to a distinct class of β-coronaviruses and its genome shows a 79% gene sequence homology to SARS-CoV (Severe Acute Respiratory Syndrome CoronaVirus) and about 50% homology to MERS-CoV (Middle East Respiratory Syndrome CoronaVirus) [2].

SARS-CoV-2 is an enveloped single stranded, positive sense RNA virus and its genome encodes several non-structural proteins (NSP) and 4 structural proteins: Spike protein (S); Membrane protein (M); Envelope protein (E) and Nucleocapsid protein (N) [3]. Among these 4 structural proteins, the Spike and Nucleocapsid proteins are the most immunogenic antigens, as previous studies for MERS-CoV and SARS CoV have shown [4].

The Spike protein is a very large transmembrane protein consisting of two subunits: N-terminal S1, responsible for virus binding to Angiotensin Converting Enzyme 2 (ACE2) receptor of different cell types, and C-terminal S2, responsible for virus fusion to human cell membranes presenting the ACE2 receptor. The S1 subunit is in turn divided in two domains: NTD (N-Terminal Domain) and RBD (Receptor Binding Domain), who directly interacts with the ACE2 receptor of host cells [5–7].

Structural studies performed on the SARS-CoV-2 RBD/ACE2 complex showed that 6 amino acids are essential for ACE2 receptors binding and

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virus entry in human cells and the higher spread rate of SARS-CoV-2 in human population could be explained by the higher affinity of RBD domain to ACE2 receptor described in SARS-CoV-2 as compared to SARS-CoV [8,9]. After SARS-CoV-2 infection, the host usually develops an immune response with production of IgA and IgM antibodies (Abs) in 7–14 days from symptoms onset, followed by IgG response after two weeks [10].

In this context, the RBD domain is considered the most antigenic protein and the primary specific target for active neutralizing antibodies (NAbs). The multiple conformational epitopes of the RBD are also responsible of the strong immune response and the anti-RBD Abs are considered the key player in viral response.

Important differences in antibodies concentrations have been also reported in COVID-19 patients. [11]. Although SARS-CoV-2 antibodies level in human serum and/or plasma correlates with protective immune response and decline of viral load, high Abs titers and early seroconversion were associated to disease severity. In this line, Wu et al. have recently demonstrated the presence of a higher antibody titer in elderly than in young patients, hypothesizing a possible connection with the clinical status of these two patients' categories [12].

Anti-RBD Abs are considered as the most clinically relevant antibodies against SARS-CoV-2 not only for their neutralizing activity, but also for the affinity to ACE2 receptors in human cells. These anti-RBD Abs, in fact, induce a competitive mechanism able to block the binding of the viral RBD to the ACE2 and the subsequent virus infection [13].

These observations demonstrate that SARS-CoV-2 RBD domain could be an important immunogenic target of antiviral drugs and COVID-19 vaccines. However, data on kinetics and duration of anti-RBD Abs responses of SARS-CoV-2 infected patients and vaccinated subjects are necessary to understand the mechanisms of protective immunity and the duration immunity against COVID-19, representing a key correlate of protection from possible reinfection.

Many studies have reported that anti-RBD antibodies levels significantly decrease with time, remaining detectable in most individuals, but there are few data regarding virus sera neutralization tests in vitro [14].

The clinical utility of serological testing is controversial and a reliable and high-throughput assay to measure anti-RBD Abs is urgently needed for COVID-19 serodiagnosis, convalescent plasma therapy and vaccine development and monitoring.

Serological enzyme-linked immunosorbent assays (ELISA) to detect anti-RBD Abs are providing promising results in terms of accuracy and reproducibility, but many different commercial serological anti-RBD Abs assays have been introduced into the international market. As a consequence, it is often difficult to compare results and a standardization of the different tests is necessary to improve the comparability of data over time. In addition, such immunoassays do not provide any information on the functionality of the antibodies detected and on the Abs titers able to guarantee immunity from SARS-CoV-2 infection.

Actually, the standard reference methods for the neutralizing activity to assess the immunization status, detect functional antibodies using SARS-CoV-2 replicating virus, such as the live Virus Neutralization Test (VNT). These tests are not routinely performed in clinical laboratories, as they require cell cultures and viral procedures in Biosafety Level 3 (BSL3) laboratories; VNT tests are also lengthy procedures demanding great expertise from the laboratory staff.

Several commercial Surrogate Virus Neutralization Tests (sVNTs) detecting anti-SARS-CoV-2 antibodies with neutralizing activity have been developed in the last year, designed to be easy to perform on automated platforms. The detection method is based on the principle of competitive immunoassay using either the ACE-2 receptor or SARS-CoV-2 anti-S1 therapeutic monoclonal antibodies [15].

Unfortunately, the minimal neutralizing antibody titer correlating with protective immunity has not yet been established and remains unclear.

The standardization of NAbs and anti-RBD Abs quantification is also important in the application of hyperimmune plasma for the treatment of critically ill COVID-19 patients [16]. The Food and Drugs Administration (FDA) recommended the use of plasma with a neutralizing titer of at least 1:160, but the correlation between neutralizing titer and quantification of antibodies by immunoassay has yet to be clarified.

In this context, we decided to analyze and compare anti-RBD IgG levels measured by two indirect chemiluminescent immunoassays, using samples collected from three different study populations: COVID-19 symptomatic patients, COVID-19 paucisymptomatic patients and vaccinated subjects.

Furthermore, to better characterize our results and their clinical significance, we performed live Virus Neutralization tests to evaluate the neutralizing activity of our cohorts' serum samples, trying to correlate VNT titers to anti-RBD Abs levels. In this line, ROC curve analysis were performed in order to establish a possible cut-off of anti-RBD Abs levels able of guaranteeing immunity against SARS-CoV-2 infection or reinfection and to better understand the development and the distribution of anti-RBD Abs.

2. Patients and methods

2.1. Patients' characteristics and specimens

The present study included a total of 98 subjects, enrolled between March 2021 and May 2021. Serum Samples were recovered, in accordance with local ethical approvals (protocols no. R.S.44.20), from "Tor Vergata" University Covid-Hospital of Rome, as follows:

- 31 Covid-19 paucisymptomatic patients (median age 55 years (range 22–81); 16 M/15F) collected from "Tor Vergata" University Covid-Hospital of Rome. These subjects were tested positive for SARS-CoV-2 by reverse transcription polymerase chain reaction (RT-PCR) and all manifested clinical symptoms strongly related to SARS-CoV-2 infection, but hospitalization was not required.
- 37 Covid-19 symptomatic patients (median age 54 years (range 26–78); 22 M/15F) collected from first access to Emergency Department or after admission to respiratory system department and from first positive nasopharyngeal swab. These subjects were tested positive for SARS-CoV-2 by RT-PCR and manifested a more severe disease course, requiring hospitalization.
- 30 vaccinated subjects (median age 44 years (range 28–60 years) 7 M/23F) collected from "Tor Vergata" vaccination center (range 10–51 days, starting from the day of the Pfizer vaccine first dose); first dose ($n = 15$) and second dose groups (n = 15) median times: 20 days (range 10–21 days) and 45 days (range 26–51 days), respectively.

Informed consent was obtained from all subjects enrolled in the study and according to hospital data policy, we cannot access to further clinical data, except for those concerning laboratory medicine department examinations.

Serum samples were collected and were separated by centrifugation at 2500*g* for 10 min, within 1 h from collection. A part of samples was used to carry out the serum virus neutralization test by DIESSE laboratories (DIESSE Diagnostica Senese, Siena, Italy).

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

2.2. MAGLUMI® SARS-CoV-2 S-RBD IgG (CLIA)

MAGLUMI® SARS-CoV-2 S-RBD IgG by SNIBE Diagnostic (Shenzhen, China), is an indirect chemiluminescent immunoassay designed for the quantitative detection of IgG anti-RBD levels in serum or plasma samples, using the fully automated MAGLUMI 800 analyzer. It can be used to characterize immune response of COVID-19 patients and individuals who have been vaccinated against the virus, representing an important tool for assessment of the efficacy of COVID-19 vaccines.

Serum samples were used in combination with sample buffer and magnetic particles coated with RBD antigen, and subsequently were incubated in order to promote immune complexes. After sedimentation in a magnetic field, the supernatant was removed, and anti-Human IgG antibodies labeled with ABEI (amino-butyl-ethyl-isoluminol) were added to develop a chemiluminescent reaction.

The emitted light is measured as relative light units (RLUs) and RLUs are directly proportional to the corresponding IgG anti-RBD concentration in samples. Results were reported in AU/ml and manufacturer's recommended cut-off value is > 1,00 AU/ml.

To standardize data and to convert the results in Binding Antibody Units (BAU/ml), SNIBE Diagnostic provided the following unit conversion relationships: 1 AU/mL is equivalent to 4,33 BAU/ml (BAU = AU x 4,33). Therefore, the cut-off value is > 4,33 BAU/ml.

All data in the present study are reported in BAU/ml.

2.3. IgG S-RBD anti-SARS-CoV-2 (CLIA)

IgG S-RBD anti-SARS-CoV-2 (CLIA) by MINDRAY (High-tech Industrial Park, Nanshan, Shenzhen, China) is a two-step chemiluminescent immunoassay for the quantitative determination of IgG anti-RBD in human serum or plasma, analyzed on the fully automated MINDRAY CL-1200i platform.

In the first step, serum samples react with sample buffer and paramagnetic microparticles coated with RBD antigens of the SARS-CoV-2 Spike protein, used to capture corresponding IgG anti-RBD. In the second step, anti-human IgG antibodies conjugated with alkaline phosphatase were added to the reaction to form sandwich structure with antibodies captured by paramagnetic microparticles. After washing, the AMPPD substrate solution (3-(2′-spiroadamantly)-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane) was added to catalyze the alkaline phosphatase chemiluminescent reaction, measured as Relative Light Units (RLUs), that are directly proportional to the corresponding IgG anti-RBD concentration in samples. Results were calculated as AU/mL and the cut-off value is >10 AU/ml.

To standardize data and convert the results in Binding Antibody Units (BAU/ml), manufacturer's recommended conversion factor is 1 AU/ml = 0,8229 × BAU/ml (BAU = AU/0,8229). Therefore, the cut-off value is >12,15 BAU/ml.

All data in the present study are reported in BAU/ml.

2.4. SARS-CoV-2 neutralization test in vitro

The SARS-CoV-2 neutralization test has been considered the "gold standard" for the serological detection of neutralizing antibodies and its results show a strong correlation with the protective titer of the test sample. The outputs are generated as relative titers and are based on the cytopathic effect (CPE) induced by the virus on cultured cells.

The VNT analysis was conducted in a specialized biosafety level 3 (BSL3) by DIESSE Diagnostica Senese laboratories (Siena, Italy), using both SARS-CoV-2 virus 2019-nCov/Italy-INMI1-strain (Original strain), acquired from Spallanzani Institute (Rome, Italy) via the European Virus Archive Global (EVAg), and the HUMAN NCOV19 ISOLATE/ENGLAND/MIG457/2020 LINEAGE B.1.1.7 (British variant strain) purchased from the Department of Health: Public Health England-Virology & Pathogenesis group (London, United Kingdom), via the European Virus Archive Global (EVAg).

The VNT test was performed using cells from African green monkey (Cercopithecus aethiops) Vero E6 (ATCC® CRL-1586™) which naturally express high levels of ACE2. Adherent sub-confluent cell monolayers of Vero E6 cells were prepared in DMEM medium containing 10% FBS.

SARS-CoV-2 virus was 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 observed daily at inverted optical microscope for 3 days to evaluate the presence of CPE and the end-point titers were calculated according to the Reed & Muench method based on eight replicates for each titration.

Vero E6 cells were seeded in T175 flasks at a density of 40000/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% CO2, 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 under a microscope.

Serum test samples were heat-inactivated for 30 min at 56 °C and two-fold serial dilutions, starting from 1:10 to 1:1280, were performed in duplicate in DMEM on 96-well culture plates. Sera dilutions 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% CO2. After incubation, 100 μl of each dilution mixture was 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% CO2.

After 3 days of incubation, the plates were analyzed with an inverted optical microscope. The highest serum dilution able to protect from CPE more than 90% of cells was taken as the neutralization titer.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism Software 8.0.1 (San Diego, California, USA). The investigators were blinded to the group allocation during the experiment.

Non-parametric tests (Kruskal-Wallis and Mann Whitney tests) were performed in case of non-Gaussian distribution according to D'Agostino & Pearson omnibus normality test, Shapiro-Wilk normality test and Kolmogorov-Smirnov test.

Correlation was calculated by Spearman's rank correlation and statistical significance was defined as $p < 0.05$.

The categorical data were displayed as numbers and/or percentages and continuous data as median and range. Frequencies and percentages are reported for all categorical measures.

Specificity and sensitivity were calculated by Receiver Operating Characteristic Curves (ROC Curve), defining an optimal cut-off for each ROC curve analysis.

3. Results

To evaluate the IgG anti-RBD response and the specific immune profile of our study populations, the two commercial immunoassays targeting the structural RBD domain of SARS-CoV-2 were tested in parallel.

A total of 98 serum samples were analyzed, 31/98 (31,6%) COVID-19 paucisymptomatic patients, 37/98 (37,8%) COVID-19 symptomatic patients and 30/98 (30,6%) vaccinated subjects. Among the 98 individuals, 45 (45,9%) were males and 53 (54,1%) females; the median age was 55 years (range 22–81 years).

In a first step, we characterized the IgG anti-RBDs in paucisymptomatic patients' cohort (n = 31). All samples resulted positive using SNIBE assay with a cut-off value > 4,33 BAU/ml, and the median concentration was 67,33 BAU/ml (range 5,629–541,3 BAU/ml). Otherwise, the IgG anti-RBD results obtained by MINDRAY assay with a cut-off value > 12,15 BAU/ml, showed that 23/31 (74%) samples resulted positive and 8/31 (26%) resulted negative; the median concentration was 34,21 BAU/ml (range 2,310–244,2 BAU/ml); (Table 1; Fig. 1).

In a second step, we performed the IgG anti-RBD quantification in the symptomatic patients' cohort (n = 37). All samples resulted positive using the SNIBE assay and the median concentration was 194,3 BAU/ml (range 12,47–1365 BAU/ml). Also in this case, MINDRAY assay showed different results: 34/37 samples (92%) were positive, and 3/37 samples (8%) were negative; the median concentration was 97,10 BAU/ml (range 3,633–730,8 BAU/ml); (Table 1; Fig. 1).

Table 1
Median and range of IgG anti-RBD levels (SNIBE and MINDRAY assays) in the three cohorts.

	Paucisymptomatic patients ($n = 31$)	Symptomatic patients ($n = 37$)	Vaccinated subjects (n = 30)
SNIBE assay	67,33 BAU/ml (range 5,629- 541,3)	194,3 BAU/ml (range 12,47–1365)	655,1 BAU/ml (range 36,81–5387)
MINDRAY assay	34,21 BAU/ml (range 2,310-244,2)	97,10 BAU/ml (range 3,633-730,8)	907,4 BAU/ml (range 13,37–8382)

In the end, we analyzed the IgG anti-RBDs in vaccinated individuals' group ($n = 30$). All the samples resulted positive, using both immunoassays. The median concentrations were: 655,1 BAU/ml (range 36,81–5387 BAU/ml) using SNIBE assay and 907,4 BAU/ml (range 13,37–8382 BAU/ml) using MINDRAY assay; (Table 1; Fig. 1).

Overall, non-hospitalized COVID-19 subjects (paucisymptomatic patients) manifested a lower IgG anti-RBD levels as compared to hospitalized patients (symptomatic patients) for both the immunoenzymatic assays, in particular: 67,33 BAU/ml vs 194,3 BAU/ml, p value = 0,0001 (SNIBE assay) and 34,21 BAU/ml vs 97,10 BAU/ml, p value<0,0001 (MINDRAY assay). Vaccinated individuals showed a higher IgG anti-RBD levels as compared to paucisymptomatic and symptomatic patients using both SNIBE assay (655,1 BAU/ml vs 67,33 BAU/ml, $p <$ 0,0001 and 655,1 BAU/ml vs 194,3 BAU/ml, $p = 0{,}0004$, respectively) and MINDRAY assay (907,4 BAU/ml vs 34,21 BAU/ml, $p < 0{,}0001$ and 907,4 BAU/ml vs 97,10 BAU/ml, p = 0,0001, respectively); (Fig. 2).

Of note, our results confirmed an increase in antibody concentration between the first and second dose of Pfizer vaccine; the median values were: 214,4 BAU/ml (range 36,81–1021 BAU/ml) vs 1689 BAU/ml (range 604,5–5387 BAU/ml), p < 0,0001 (SNIBE assay) and 97,84 BAU/ml (range 13,37–1217 BAU/ml) vs 1781 BAU/ml (range 907,4–8382

Table 2
Median and range of IgG anti-RBD levels (SNIBE and MINDRAY assays) in vaccinated cohort divided in first and second dose of Pfizer vaccine.

		SNIBE assay	MINDRAY assay
Vaccinated subjects (n =30)	1st dose (n = 15)	214,4 BAU/ml (range 36,81–1021)	97,84 BAU/ml (range 13,37–1217)
	2nd dose (n = 15)	1689 BAU/ml (range 604,5–5387)	1781 BAU/ml (range 907,4–8382)

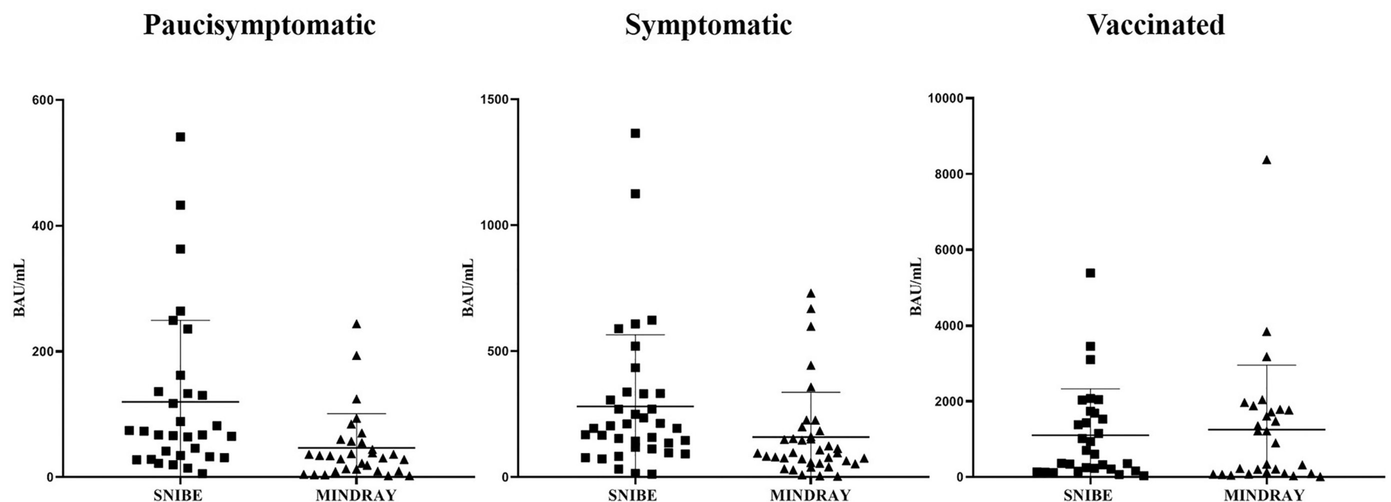

Fig. 1. IgG anti-RBD antibodies concentrations detected by SNIBE and MINDRAY assays in the study cohorts.

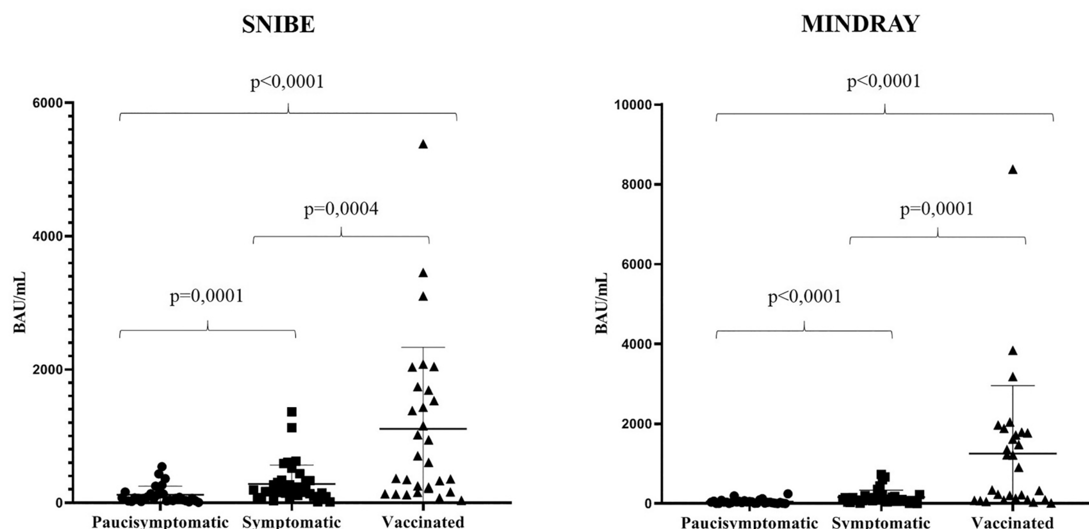

Fig. 2. Distribution of IgG anti-RBD antibodies concentrations in the three groups.

BAU/ml), $p < 0,0001$ (MINDRAY assay); (Table 2; Supplementary Fig. S1).

We performed additional analysis by comparing separately, first and second dose vaccinated subjects to paucisymptomatic and symptomatic patients (Supplementary Fig. S2). Differences were statistically significant between paucisymptomatic and both first and second dose vaccinated subjects, for SNIBE assay (67,33 BAU/ml vs 214,4 BAU/ml, $p = 0,023$ and 67,33 BAU/ml vs 1689 BAU/ml, $p < 0,0001$, respectively) and MINDRAY assay (34,21 BAU/ml vs 97,84 BAU/ml, p < 0,0001 and 34,21 BAU/ml vs 1781 BAU/ml, p < 0,0001, respectively), as well as for symptomatic patients vs second dose vaccinated subjects (194,3 BAU/ml vs 1689 BAU/ml, p < 0,0001 for SNIBE assay, and 97,10 BAU/ml vs 1781 BAU/ml, p < 0,0001 for MINDRAY assay). In contrast, comparing the symptomatic patients and first dose vaccinated subjects, results showed no statistically differences for both assays (194,3 BAU/ml vs 214,4 BAU/ml, $p = 0,8888$ for SNIBE and 97,10 BAU/ml vs 97,84 BAU/ml, p = 0,5222 for MINDRAY).

In addition, to better characterize the correlation between the different immunoassays, the Spearman's rank test was performed, finding very strong coefficients for each study cohort (paucisymptomatics: $r = 0.9439$; symptomatics: $r = 0.8542$; vaccinated subjects: $r = 0.9325$); (Fig. 3).

Moreover, in order to evaluate the neutralizing activity against SARS-CoV-2, all samples were examined with live virus neutralization test using both the SARS-CoV-2 original strain and the British variant strain (Supplementary Table S1). In paucisymptomatic cohort, VNT results from SARS-CoV-2 original strain showed a median neutralizing antibody titer of 1:80 (range 0–1:640), and a lower median neutralization titer of 1:40 (range 0–1:1280) from the British variant strain. In symptomatic patients' group, we found a higher median neutralizing titer of 1:160 (range 1:40–1:1280) with the original strain and similar results with the British variant strain (1:160, range 1:10–1:1280), unlike to paucisymptomatic patients. Finally, in vaccinated cohort a median neutralizing titer of 1:80 (range 1:10–1:1280) was found with the original strain and a lower median titer was observed with the British variant strain (1:40, range 0–1:1280). However, analyzing exclusively vaccinated subjects after the second dose, the median VNT titer increased to 1:160 using either the SARS-CoV-2 original strain (range 1:80–1:1280) or the British variant strain (range 1:20–1:1280) (Supplementary Table S1).

The distributions of anti-RBD Abs levels correlated to neutralization titers have been reported for SNIBE and MINDRAY assays, using the SARS-CoV-2 original strain (Fig. 4A and Fig. 4B, respectively) and the SARS-CoV-2 British variant strain (Fig. 4C and Fig. 4D, respectively). The median antibodies levels increased with increasing live neutralizing activity in all the groups, for both strains. Samples with negative VNT titers (VNT titer <1:10), were observed, as expected, only in the paucisymptomatic group for the original strain and in the paucisymptomatic group and vaccinated subjects for the British variant strain. To note all samples with VNT titer <1:10 were characterized by lower anti-RBD Abs levels.

Based on these results, the best fit cut-off values for both immunoassays and for all the study cohorts were determined using receiver operating characteristic (ROC) analysis. For this analysis, serum samples that showed a VNT titer greater than or equal to the median neutralizing titer for the specific patients' cohort were considered as "positive", whereas all samples with a lower VNT titer were considered as "negative". SNIBE immunoassay in paucisymptomatic group determined an area under curve (AUC) value of 0,9412, with a sensitivity of 82,3% and a specificity of 100% at a cut-off value of 74,0 BAU/ml; in symptomatic cohort, an AUC value of 0,8933, with a sensitivity of 80% and specificity of 100% at a cut-off value of 181,4 BAU/ml and in vaccinated subjects, an AUC value of 0,9509, with a sensitivity of 93,7% and specificity of 92,7% at a cut-off value of 485 BAU/ml; (Table 3; Fig. 5A). On the other hand, MINDRAY immunoassay determined in paucisymptomatics, an AUC value of 0,9107, with a sensitivity of 81,2% and a specificity of 92,8% at a cut-off value of 35,55 BAU/ml; in symptomatic cohort an AUC value of 0,9000, with a sensitivity of 80% and specificity of 83,3% at a cut-off value of 79,41 BAU/ml and in vaccinated subjects, an AUC value of 0,8839, with a sensitivity of 87.5% and specificity of 92,8% at a cut-off value of 624,8 BAU/ml; (Table 3; Fig. 5B). Good ROC curve analysis results were found also from live VNT using the SARS-CoV-2 British variant, showing a general trend to slightly lower specificity and AUC values; (Table 3; Supplementary Fig. S3).

Finally, SNIBE and MINDRAY results were plotted with the corresponding neutralizing titer to evaluate the correlation between the two parameters. Spearman's rank coefficients calculated for the SARS-CoV-2 original strain showed the strongest correlation in vaccinated group both for SNIBE and MINDRAY assays. Differently, symptomatic patients showed the least correlation; (Fig. 6A; Fig. 6B). These results were also confirmed using the VNT results from the British variant strain (Fig. 6C; Fig. 6D).

4. Discussion

SARS-CoV-2 is responsible for the ongoing COVID-19 pandemic and the main clinical questions are the role of antibodies in modulating disease severity, the duration of individuals' serological responses after infection and/or vaccine strategy, and the extent to which patient antibody responses may be protective against reinfection.

Considering the heterogeneous immune response, it is not yet well known if anti-RBD Abs quantification could be a good prognostic factor

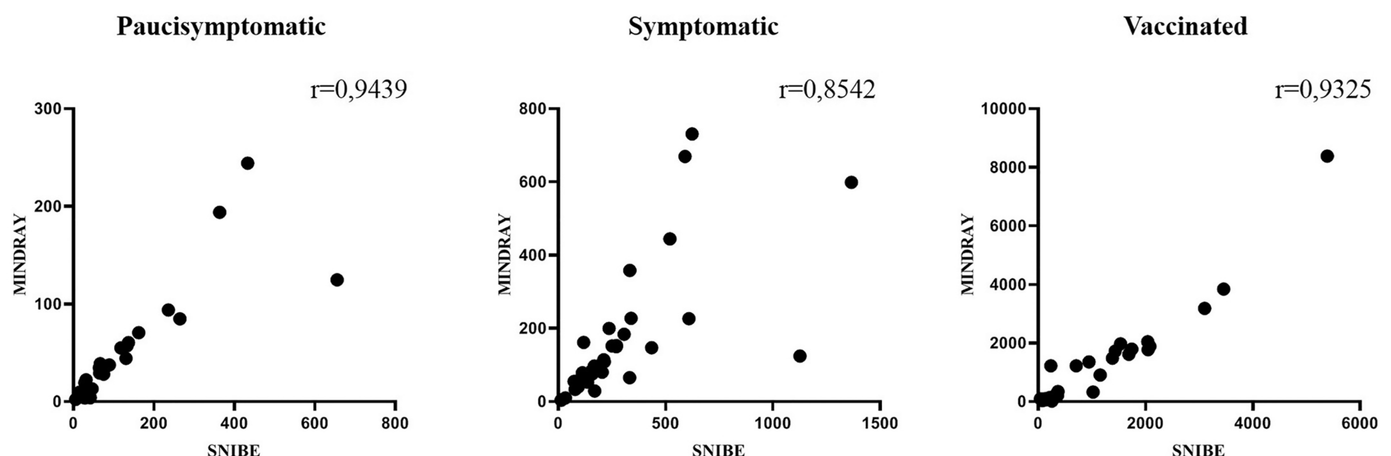

Fig. 3. Spearman's rank correlation between SNIBE and MINDRAY results.

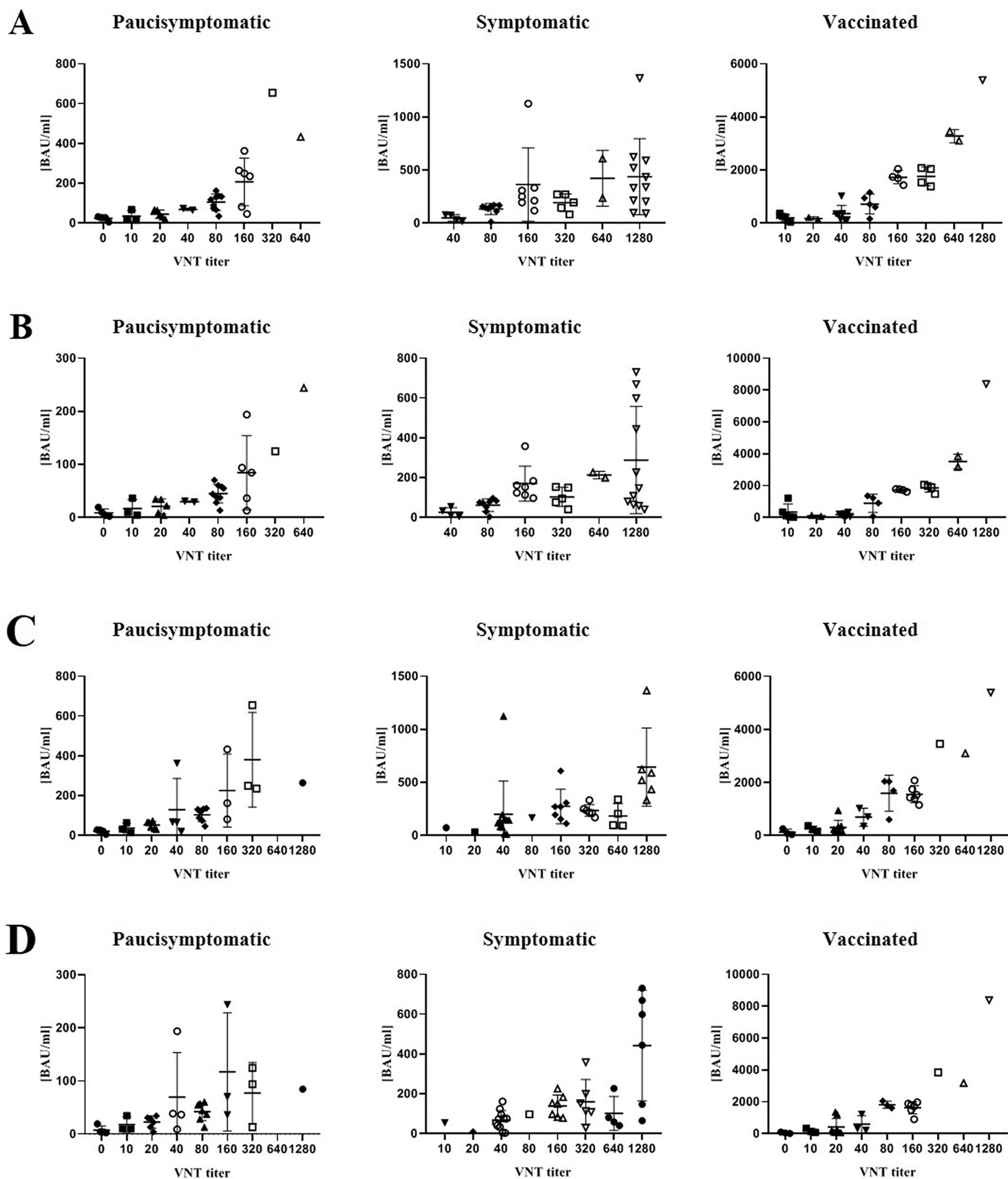

Fig. 4. Distribution of anti-RBD Abs levels correlated to neutralization titers. A) SNIBE results from original strain; B) MINDRAY results from original strain; C) SNIBE results from the British variant strain; D) MINDRAY results from the British variant strain.
VNT titers are expressed as the serum dilution reciprocals.

Table 3
ROC curve characteristics using both the SARS-CoV-2 original and British variant strains.

	SNIBE assay			MINDRAY assay		
	Paucisymptomatic (n = 31)	Symptomatic (n = 37)	Vaccinated (n = 30)	Paucisymptomatic (n = 31)	Symptomatic (n = 37)	Vaccinated (n = 30)
SARS-CoV-2 original strain						
Median VNT titer	1:80	1:160	1:80	1:80	1:160	1:80
Sensitivity	82,3%	80%	93,7%	81,2%	80%	87,5%
Specificity	100%	100%	92,7%	92,8%	83,3%	92,8%
Cut-off	74 BAU/ml	181,4 BAU/ml	485 BAU/ml	35,55 BAU/ml	79,41 BAU/ml	624,8 BAU/ml
Area under ROC curve (AUC)	0,9412	0,8933	0,9509	0,9107	0,9000	0,8839
95% confidence interval	0,8638 to 1,000	0,7913 to 0,9953	0,8731 to 1,000	0,8073 to 1,000	0,8043 to 0,9957	0,7369 to 1,000
SARS-CoV-2 British variant strain						
Median VNT titer	1:40	1:160	1:40	1:40	1:160	1:40
Sensitivity	83,3%	78,2%	100%	82,3%	82,6%	93,7%
Specificity	92,3%	92,8%	78,5%	100%	71,4%	71,4%
Cut-off	67,29 BAU/ml	194,1 BAU/ml	388,4 BAU/ml	35,50 BAU/ml	77,57 BAU/ml	211,1 BAU/ml
Area under ROC curve (AUC)	0,9231	0,8540	0,9777	0,9095	0,8230	0,8929
95% confidence interval	0,8221 to 1,000	0,7094 to 0,9987	0,9364 to 1,000	0,8011 to 1,000	0,6890 to 0,9570	0,7633 to 1,000

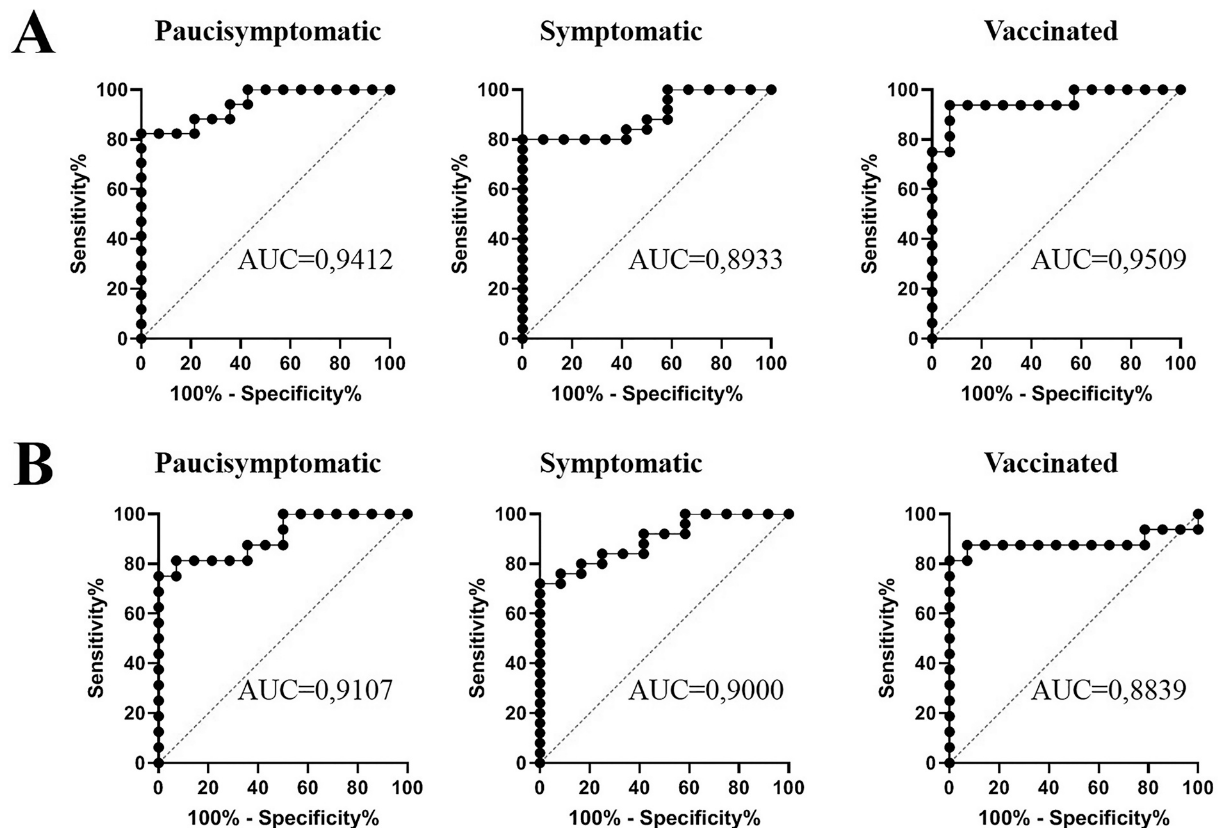

Fig. 5. SARS-CoV-2 original strain live VNT ROC curves. A) SNIBE assay; B) MINDRAY assay.

or indicator of protective immunity against SARS-CoV-2 infection. Nevertheless, the use of serological quantitative immunoassays could help to collect important information regarding population immunity. Validated international serologic assays for the detection of different anti-SARS-CoV-2 antibodies are essential to obtain information on the kinetics of the antibody response in infected and vaccinated subjects [17–19]. However, the serological tests available on the international market differ in the type of chemistry used in the assay, in the class of antibodies detected and in the targeted antigen. A further complication is the lack of a clear interpretation of the results and the various cut-offs established by the manufacturers. For this reason, a new international standard unit of measurement named “Binding Antibody Units” (BAU/ml) was introduced by the World Health Organization, with the aim of eliminating the discrepancies of the results and to improve the harmonization of data [20].

In this context, we focused our study on the RBD domain of the Spike protein, considered the most immunogenic and the primary specific target for active neutralizing antibodies, by analyzing results from two commercial immunoassays, comparing the IgG anti-RBD antibodies in samples collected from three different study cohorts and characterizing the immune response in each group.

A slightly dissimilar result between the anti-RBD antibodies assays was observed, probably due to the different cut-off values. Anti-RBD antibodies positivity was found in 100% of paucisymptomatic and

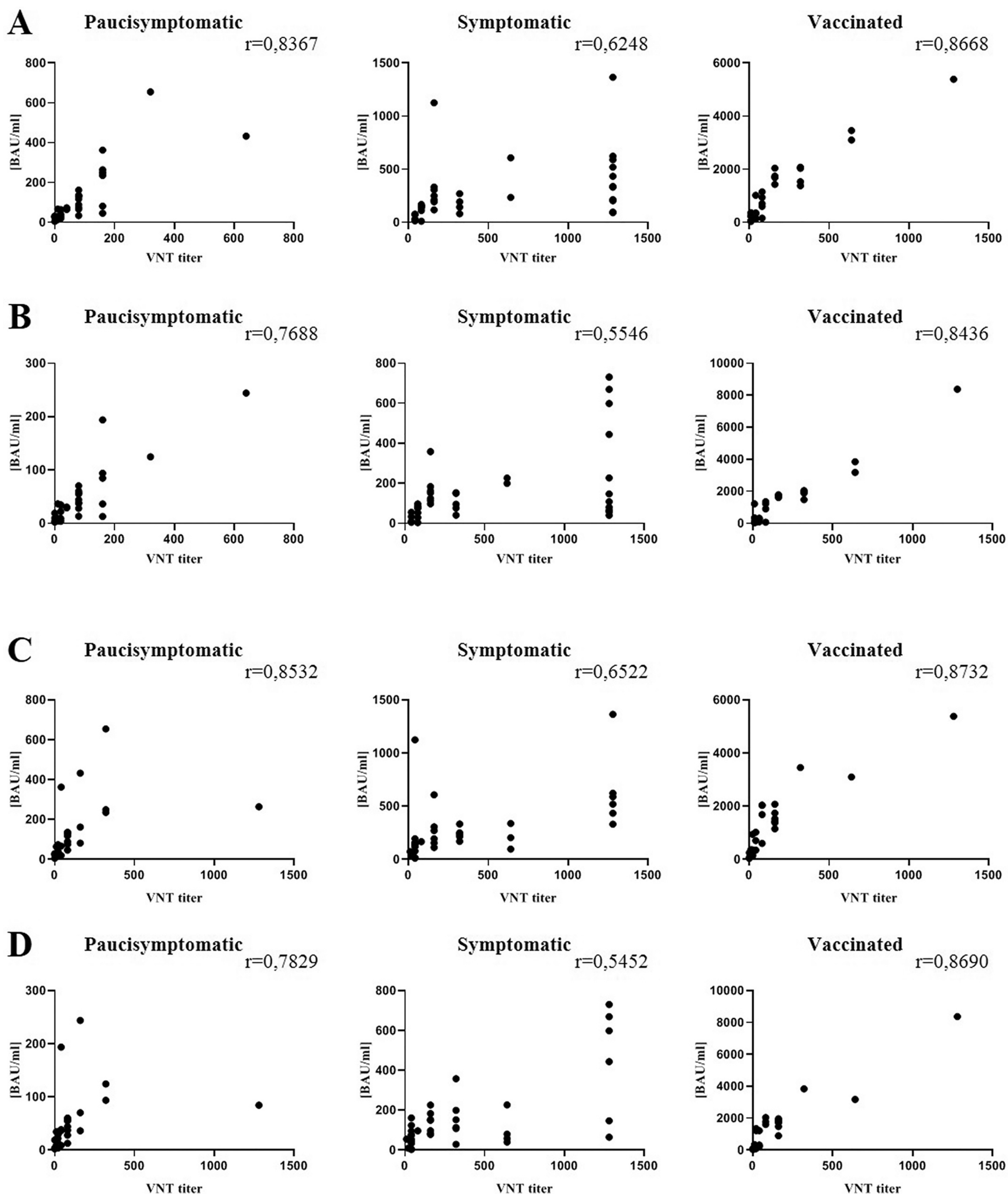

Fig. 6. Spearman's rank correlation of IgG anti-RBD concentrations to VNT titers. A) SNIBE results from original strain; B) MINDRAY results from original strain; C) SNIBE results from the British variant strain; D) MINDRAY results from the British variant strain.
Vaccinated subjects showed the strongest correlation for both SNIBE and MINDRAY assays ($r = 0,8668$ and r = 0,8436, respectively); symptomatic patients showed the least correlation (r = 0,6248 and r = 0,5546, respectively). Results were also confirmed using the British variant strain: r = 0,8732 (SNIBE) and r = 0,8690 (MINDRAY) in vaccinated group; r = 0,6522 (SNIBE) and r = 0,5452 (MINDRAY) in symptomatic patients' group.

symptomatic patients by the SNIBE assay; in 74% of paucisymptomatic and 92% of symptomatic patients with the MINDRAY assay. Concordant results, with a 100% anti-RBD antibodies positivity, were instead observed in vaccinated subjects' group, where the median concentrations were considerably higher than in paucisymptomatic and symptomatic patients for both assays. To note, in all the negativity cases found with the MINDRAY assay, a low level of antibodies was detected also with the SNIBE assay, resulting in good correlation data.

However, despite the use of the international standard (BAU/ml), the median anti-RBD concentrations were different between SNIBE and MINDRAY assays, but among the different study populations, a significant increase in antibodies concentrations was observed. The results were approximately 3-fold increased in median anti-RBD concentration of symptomatic patients' group compared to paucisymptomatic patients using both assays, confirming data already present in literature which emphasize higher antibody levels in COVID-19 patients with severe conditions [21–24]. A different trend was observed in vaccinated subjects' group: SNIBE assay results showed approximately a 4-fold increase in median anti-RBD levels compared to symptomatic patients and approximately a 10-fold increase compared to paucisymptomatic patients; MINDRAY assay showed a distinct situation with approximately a 9-fold increase in median anti-RBD levels compared to symptomatic patients and approximately a 26-fold increase compared to paucisymptomatic patients. Nevertheless, in both cases, the vaccinated subjects showed a higher median concentration than the paucisymptomatic and symptomatic patients. Vaccinated subjects were also analyzed by separating samples between first and second dose of Pfizer vaccine, founding a significant anti-RBD median concentrations increase in patients after the second dose, confirming that the antibody peak is enhanced only after the second dose of the vaccine administration.

As previously mentioned, since the tested immunoassays probably may vary in the chemistry architecture of the assay, resulting in different cut-off values, this may partly explain the discrepancy obtained on the paired samples in all study cohorts. However, the Spearman's rank test showed strong correlation coefficients between SNIBE and MINDRAY results.

Interestingly, the median anti-RBD concentrations between the immunoassays in vaccinated subjects, had a smaller difference, with a 1.38-fold increase in the MINDRAY assay comparing the total group and only 1.05-fold increase comparing the second dose group. These data showed that differences in IgG anti-RBD can be due also to the antibodies' characteristics of the population groups. In fact, in paucisymptomatic and symptomatic patients the antibody response is related to all the SARS-CoV-2 antigenic proteins, whereas in vaccinated subjects the antibody response is more homogeneous because it is specific only against the Spike protein.

Moreover, several studies have been focused only on neutralizing antibodies concentration, however non-neutralizing antibodies have also been identified and they could mediate an additional effect in the host immune response [25,26]. These antibodies may currently not completely be detected as they are directed against an epitope other than the RBD domain, which is the target of most commercial immunoassays, including SNIBE and MINDRAY tests.

For this reason, in vitro viral serum neutralization tests were performed, using both SARS-CoV-2 original and SARS-CoV-2 British variant strains, characterized by different mutations of the SARS-CoV-2 immunogenic surface S1-proteins [27].

Since VNT assay offers the most informative evaluation of the SARS-CoV-2 antiviral activity, we tried to establish on our cohorts its correlation with anti-RBD antibodies levels detected by immunoassay. A median neutralization titer of 1:80 in paucisymptomatic and vaccinated groups, and a higher median neutralization titer in symptomatic cohort (1:160) was found using the SARS-CoV-2 original strain for both assays. As expected, a lower median neutralization titer was observed in paucisymptomatic and vaccinated subjects (1:40), using the British variant strain, whereas symptomatics have shown similar results (1:160), confirming a possible more heterogeneous immune response against different SARS-CoV-2 antigens in these patients. Since the vaccinated cohort median VNT titer was calculated combining first and second dose individuals, this group showed the same paucisymptomatic median VNT titer, despite the higher anti-RBD Abs levels. However, analyzing only vaccinated subjects after the second dose, the median VNT increased and was comparable to the median VNT of symptomatic patients. To note, the same trend and the same median neutralization titers were obtained on these cohorts of subjects in our recently published study with a surrogate assay [15].

Despite a strong heterogeneity of the antibody response, we observed a general trend of increasing anti-RBD antibody levels with increasing neutralizing titer but results from the two immunoassays showed a different median anti-RBD Abs concentrations, confirming the need for standardization in order to report data in a conventional format, as a useful worldwide analysis tool.

Finally, our results showed a strong positive correlation between the quantitative immunoassays and VNT analysis, suggesting that IgG anti-RBD antibodies could be the main promoters in conferring protection against SARS-CoV-2 infection and in the neutralizing activity of analyzed samples.

In conclusion, although the SARS-CoV-2 immune response remains heterogeneous and the neutralizing activity of different type of antibodies may differ, this study provides a comprehensive analysis of the good quantitative serological test values correlation with live neutralization activities. These findings support the importance of standardized monitoring of antibody levels and could provide important clinical information to guide the interpretation of anti-RBD levels, correlated to individual protection against a possible SARS-CoV-2 reinfection and to the population immunity. Indeed, the use of immunoassays can help in large-scale monitoring of COVID-19 samples compared to VNT analysis, becoming a valid alternative for diagnostic routine laboratories.

Lastly, these results should be confirmed on a larger cohort of patients, considering the great inter-individual immune response heterogeneity, and the collection of data from other international human epidemiological studies is crucial to find a threshold able to establish a level of antibodies necessary to prevent a possible SARS-CoV-2 infection and to allow the COVID-19 pandemic end.

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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.

Declaration of Competing Interest

The Authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2021.108918.

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