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Evaluation of S-RBD and high specificity ACE-2-binding antibodies on SARS-CoV-2 patients after six months from infection

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ABSTRACT

The antibody response to SARS-CoV-2 has not yet fully defined, but the availability of sensitive and specific serological assays is crucial to observe the presence of specific antibodies against the human receptor binding domain (S-RBD) and high specificity ACE-2-binding antibodies or neutralizing antibodies (NT) in response to vaccines. Indeed, these peculiar antibodies should prevent viral interaction between RBD and Angiotensin-Converting Enzyme 2 (ACE2) receptor, located on surface of host cells. In this study, 72 samples from 37 hospitalized COVID-19 patients and 35 not-hospitalized patients were analyzed longitudinally. The detection of S-RBD and NT antibodies was carried out using CLIA tests.

Hospitalized patients showed elevated serum levels of S-RBD (97.22%) and NT (77.78%) antibodies, differently, not-hospitalized, who were paucisymptomatic or asymptomatic patients, showed lower serum levels of S-RBD (65.71%) and NT (38.14%) antibodies.

The results suggest that the NT serum level is strongly related to disease severity ($p < 0.001$) and to the serum level of S-RBD antibodies ($p < 0.0001$).

1. Introduction

In the late December 2019, an outbreak of pneumonia caused by a novel human coronavirus (SARS-CoV-2) was reported in Wuhan, Hubei province, China [1]. The virus is highly pathogenic, and it is still spreading around the world, becoming a major public health concern. Specific and sensitive serological assays are urgently needed to understand the epidemiology of SARS-CoV-2, although a real-life laboratory validation is required [2].

As known, coronaviruses use the homotrimeric spike glycoprotein (comprising the S1 and S2 subunits in each spike monomer) present on the envelope, to bind to their cellular receptors; the interaction between SARS-CoV-2 spike protein and the angiotensin converting enzyme 2 (ACE2) cell receptor is the critical step for SARS-CoV-2 to enter into target cells. In particular, the virus binds to ACE2 receptor with a high affinity, through a specific S protein region called the Receptor Binding Domain (RBD) [3].

In this regard, human anti-S-RBD IgG are immunoglobulins directed against the RBD[4]; as a consequence, being RBD within subunit S1 the primary target of SARS-CoV-2 IgGs, these immunoglobulins can disrupt the interaction between S-RBD and the ACE2 receptor. Therefore, S-RBD antibodies could prevent the virus from entering and infecting a cell by

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neutralizing or inhibiting its biological effect. However, it is not known if these responses are associated with protection against subsequent infections [5].

Besides, the elicitation kinetics, the identification of the most immunogenic epitopes and the neutralization power of neutralizing (NT) antibodies, evaluated by competitive assays exploiting S-RBD-ACE2 interaction, are still to be determined. Furthermore, several studies reported that NT antibodies bind with high affinity ACE2 receptor, completely preventing the virus-host cell interaction [6]. Other studies on the performance of monoclonal antibodies (mAbs) describe the neutralization power of these antibodies, which could have therapeutic applications [7–9].

The aim of the present work was to longitudinally study the antibodies serum levels, in both not-hospitalized and hospitalized patients, detecting the presence of S-RBD antibodies and NT antibodies after six months from infection, using a chemiluminescent immunoassay (CLIA). Improving the knowledge of humoral immunity to SARS-CoV-2, could play a role in therapy design and evaluation of vaccine efficacy.

2. Materials & methods

2.1. Patients

Blood samples obtained from 72 patients were centrifuged at 2000g for 10 min and stored at −80 °C within one hour from collection. Patients were classified in two categories, hospitalized and not hospitalized, as follows: 37 hospitalized (9 female and 28 male) of which 2 out of 37 hospitalized in ICU at the University Hospital Tor Vergata (Rome, Italy) because of fever and acute respiratory symptoms, collected at one week after hospital admission and after six months from SARS-CoV-2 infection; 35 not-hospitalized patients (16 female and 19 male), quarantined at home, paucisymptomatic or asymptomatic, to which blood was withdrawn by the Lifebrain laboratory (Guidonia Montecelio, Rome, Italy) personnel, collected after six months from SARS-CoV-2 infection.

The study was approved by Ethical Committee of Tor Vergata University Hospital of Rome, (protocol no. R.S.44.20). Informed consent was obtained from all the subject enrolled in the study. The study was in accordance with the Helsinki Declaration, as revised in 2013.

2.2. Anti-SARS-CoV-2 S-RBD IgG antibodies

The Maglumi SARS-CoV-2 S-RBD is an indirect Chemiluminescent Immunoassay (CLIA) for the in vitro semi-quantitative determination of IgG antibodies to the SARS-CoV-2 S-RBD protein, performed by fully automated Maglumi 800 analytical system (Snibe Diagnostic, Shenzhen, China).

Sample, buffer solution and magnetic beads coated with S-RBD recombinant antigen are mixed thoroughly. After settling in a magnetic field, the supernatant is decanted, and a wash cycle is performed. Then, anti-human IgG antibodies labelled with amino-butyl-ethyl-isoluminol (ABEI) are added and incubated to form immune-complexes. After a second precipitation in a magnetic field, the supernatant is decanted, and another wash cycle is performed. A starter reagent is added to initiate a chemiluminescent reaction, producing a light signal measured by a photomultiplier as relative light units (RLUs), which is proportional to the concentration of anti-SARS-CoV-2 S-RBD IgG present in the sample.

Cut-off value is 1.00 AU/mL and the conversion factors to transform AU/mL in BAU/mL is 4.33 as declared by manufacturer.

2.3. Anti-SARS-CoV-2 IgG/IgA/IgM neutralizing antibodies

Detection of anti-SARS-CoV-2 IgG/IgA/IgM Neutralizing Antibodies (NT) was performed by a competitive Chemiluminescent Immunoassay (CLIA), on the fully automated Maglumi analytical system (SNIBE Diagnostics, Shenzhen, China). The assay is designed by mimicking the virus-host interaction using the highly specific ACE2 protein-RBD protein interaction. The neutralizing antibodies will compete with the S-RBD antigens for the binding to ACE2 so that when neutralizing antibodies are present in the serum sample, they can prevent RBD-ACE2 bond.

Sample, buffer solution, magnetic beads coated with specific ACE2 antigens and anti-SARS-CoV-2 S-RBD recombinant antigens labelled with amino-butyl-ethyl-isoluminol (ABEI) are mixed thoroughly and incubated. Anti-SARS-CoV-2 NT antibodies present in the sample compete with ACE2 antigen immobilized on magnetic microbeads for the binding to recombinant SARS-CoV-2 S-RBD specific antigen labeled with ABEI. After precipitation in a magnetic field, the supernatant is removed, and a wash cycle is performed. Subsequently, the starter reagents are added to initiate a chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLUs), which are inversely proportional to the concentration of SARS-CoV-2 neutralizing antibodies present in the sample.

Cut-off value is 0.05 μg/mL and the conversion factor to transform μg/mL in IU/mL are 405, as declared by manufacturer.

2.4. Statistical analysis

We analyzed the data of gender and age (range: 22 to 81 years old) of all patients and divided by not hospitalized and hospitalized patients. The rate differences in age groups and gender were estimated by chi-square ($\chi 2$) test.

Descriptive analyses were performed, with measures of central tendency and dispersion for continuous variables and frequency distribution for qualitative ones. In the case of normally distributed data, they were represented by the mean ± standard deviation and Anova with Bonferroni post hoc test to determine the differences between groups. In the case of not normally distributed data, they were represented by the median and the percentiles; the variables were compared through the Kruskal-Wallis test.

Shapiro-Wilk test was applied to determine the distribution with a confidence interval (CI) of 95%.

Finally, to determine the S-RBD IgG levels related to the beginning of NT antibodies production, the results of S-RBD antibodies levels with NT antibodies positivity were compared to the results of S-RBD antibodies levels with NT antibodies negativity and analyzed by the Receiver Operating Characteristic (ROC) curve analysis. All data were examined using Med Calc Ver.18.2.18 (MedCalc Software Ltd, Ostend, Belgium).

The statistical significance level established for all tests performed was $p < 0.05$.

3. Results

The aim of this work was to determine if there was a significant difference in S-RBD and NT antibodies concentration between the hospitalized and not-hospitalized group, after six months from SARS-CoV-2 symptoms onset. The patients analyzed were mostly in an age range of 50–55 years old (age mean 53.61, SD 14.26) in both groups. There is an irrelevant difference (p greater than 0.05) in age group in hospitalized and not-hospitalized patients: the age mean of first ones was 53.36 (SD: 10.92), while the age mean of the other group was 53.88 (SD: 17.26). Conversely, a significant prevalence of morbidity was noted in both groups among male 66.67% (48 out of 72) despite of female rate 33.33% (34 out of 72), with $p < 0.01$.

The data were not normally distributed (verified by applying ShapiroWilk test) and were characterized by median and percentiles values for both S-RBD and NT antibodies (Table 1).

In Fig. 1 the data has been represented by a scatter-plot indicating on the vertical axis the observed antibody values and on the horizontal axis the study group. Fig. 1 A shows that the not-hospitalized patients' median value of S-RBD antibody levels (67.25 BAU/mL) is significantly

Table 1
S-RBD and NT antibodies statistical data for hospitalized and not-hospitalized patients.

	Sample size	Median	Interquartile Range (25%-75%)
S-RBD IgG not-hospitalized (BAU/mL)	N = 35	67.25	32.28 to 135.55
S-RBD IgG hospitalized (BAU/mL)	N = 37	224.16	150.01 to 339.40
NT Ab not-hospitalized (IU/mL)	N = 35	223.15	101.55 to 811.82
NT Ab hospitalized (IU/mL)	N = 37	958.23	475.47 to 2052.13

lower than median value of hospitalized patients (224.16 BAU/mL), with a $p < 0.001$. It can be also noticed that several hospitalized patients (7 out of 37: 18.92%) developed S-RBD antibodies levels greater than 400 BAU/mL, compared to not-hospitalized patients (3 out of 35: 8.57%). Fig. 1B shows data for NT antibody levels and even in this case the hospitalized patients show a median higher value (958.23 IU/mL) than not-hospitalized patients (223.15 IU/mL), with a $p < 0.001$. Likewise, S-RBD IgGs levels, NT antibodies values were higher than 2000 IU/mL in more hospitalized patients (9 out of 37: 24.32%) than in not-hospitalized patients (4 out of 35: 11.43%).

Overall, the data after six months from symptoms onset highlighted that in not-hospitalized group, S-RBD IgGs median serum levels were positive in 23 out of 35 patients (65.71%) and NT antibodies in only 13 out of 35 patients (37.14%). Conversely, the results in hospitalized group were considerably different; the S-RBD and NT antibodies were detected in 36 out of 37 patients (97.29%) and in 28 out of 37 patients (75.67%), respectively.

Moreover, an absolute S-RBD and NT antibodies absence in 8 out of 35 not-hospitalized patients (22.85%), compared to only 1 out of 37 hospitalized patients (2.70%), was found. Furthermore, 2 not-hospitalized patients were re-infected during the six months period; despite the double infection, the patients remained antibody negatives and they are still not-immune to SARS-CoV-2.

It was also observed that in hospitalized patients the results at one week after hospital admission showed high levels of S-RBD (25 out of 37: 67.56%) and NT antibodies (15 out of 37: 40.54%).

In Fig. 2 are reported the linear correlations between S-RBD and NT antibodies in both groups. Not-hospitalized (Fig. 2A) and hospitalized (Fig. 2B) patients data have a linear relationship, indicating a directly proportional increase of S-RBD vs NT antibodies. To note, the Pearson coefficient R was higher in not-hospitalized patients vs hospitalized patients ($R = 0.95$; $p < 0.0001$ vs $R = 0.76$; $p < 0.0001$). However, this trend could be explained by the higher number of patients in the second group with antibodies concentration values greater than 400 BAU/mL and 2000 IU/mL. Indeed, those samples have been diluted and the discrepancies are probably due to the exceeding of the detection linearity limits of the assays.

Finally, the cut-off value of S-RBD IgG levels corresponding to the beginning of NT antibodies production has been calculated by a ROC curve, as illustrated in Fig. 3.

By comparing results from samples with detectable S-RBD and NT antibodies levels, to samples with detectable S-RBD and no NT

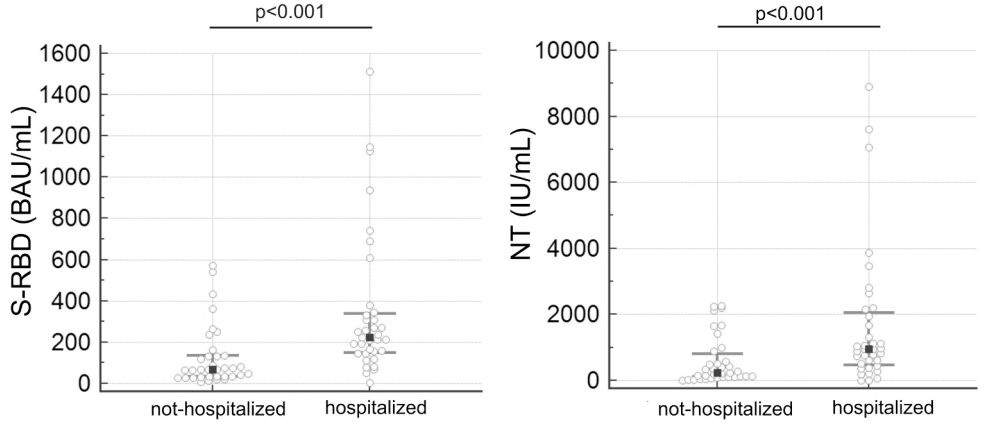

Fig. 1. A. Not-hospitalized and hospitalized patients median S-RBD IgG values distribution after six months from SARS-CoV-2 infection, expressed in BAU/mL. The observed p is<0.001. **B.** Not hospitalized and hospitalized patients median NT antibodies values distribution after six months from SARS-CoV-2 infection, expressed in IU/mL. The observed p is<0.001.

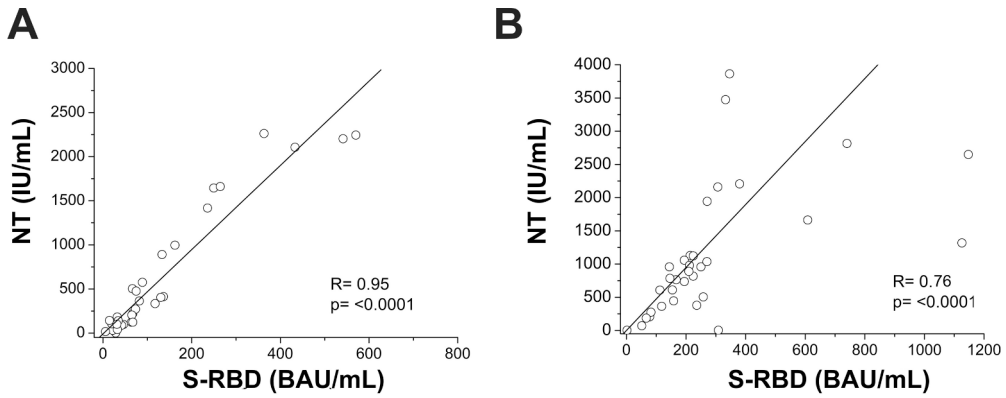

Fig. 2. A. Linear correlation between NT and S-RBD antibodies concentration in not hospitalized patients. $R = 0.95$ and $p < 0.0001$. **B.** Linear correlation between NT and S-RBD antibodies concentration in hospitalized patients. $R = 0.75$ and $p < 0.0001$.

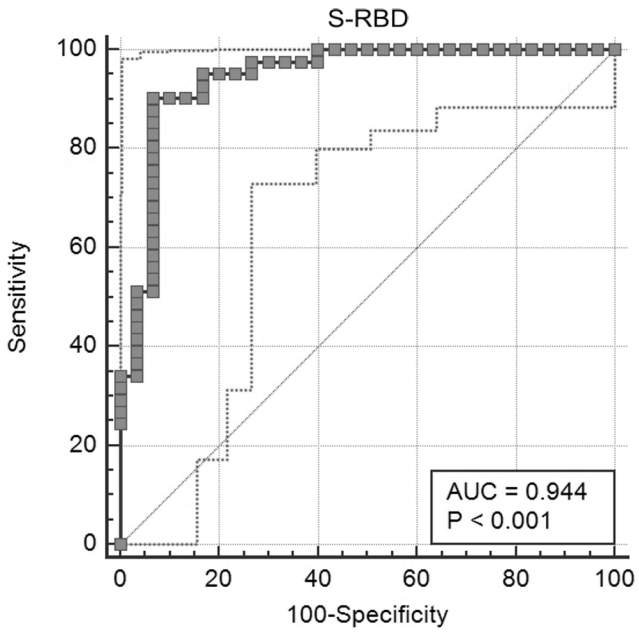

Fig. 3. S-RBD IgG levels related to the beginning of NT antibodies production ROC curve. Confidence Interval (CI) 95%: 0.862 to 0.985.

antibodies levels, a cut-off > 130.38 BAU/mL has been assessed with an AUC (area under curve) value of 0.944 and a sensitivity and specificity of 90.24% and 93.33%, respectively.

4. Discussion

Currently, our understanding of antibody responses to SARS-CoV-2 infection is still to be determined. It is certain that the infection spread mainly in pre-elderly men (50–65 years), as emerged, at the beginning of the pandemic [1]. Although, it is barely known the immunity after infection, and which is the protective role of S-RBD and NT antibodies. Moreover, as the administration of vaccines is proceeding, serological tests and a comprehensive antibodies characterization would be necessary to determine its effectiveness.

The persistence of antibodies in current analysis suggests that anti-S-RBD IgGs remain at high levels for at least 6 months after the onset of symptoms in both male and female. The results are in line with the emerging evidence on the seroprevalence, persistence and decay of antibody responses after SARS-CoV-2 infection [8,10,11]. Differently, antibodies absence after six months from SARS-CoV-2 infection, was observed in a small percentage (2.70%) of hospitalized individuals, compared to 22.85% of not-hospitalized patients.

It was also noticed a wide distribution of antibodies serum levels, which were proportionally related to disease severity in the groups analyzed: hospitalized patients had higher S-RBD IgGs levels than not hospitalized, as also shown in Roltgen's study [11]. Moreover, in addition to anti-S-RBD IgGs, it was also found that NT antibodies levels, after 6 months period, were extremely higher in hospitalized patients, than in not-hospitalized ones. Reasonably, these discrepancies could be due to various factors, such as immune status, age, co-morbidities, or antigenic load. Furthermore, the NT antibodies levels in not-hospitalized and hospitalized patients correlate proportionally to the S-RBD IgG levels.

In addition, since some of not-hospitalized patients were S-RBD and NT antibodies negative after the six months period and they have experienced re-infection during these months, it would be suggested that the antibodies against the SARS-CoV-2 S-RBD could have inactivating power on the virus, confirming previous studies [8,10]. Unfortunately, it is not currently known the concentration threshold for immunization. In contrast, the hospitalized patients showed increased concentration of S-RBD and NT antibodies respect to not hospitalized, corroborating the relationship with symptoms severity, as reported in literature [11,12]. Moreover, in some of these patients, NT antibodies are produced since the first week from symptoms onset.

Overall, the observation that positive S-RBD IgGs and NT antibodies responses persist over time is encouraging and it suggests the development of a robust immune memory system in severe infections. The availability of NT antibodies against SARS-CoV-2 will offer advantages for the control of the current pandemic and the possible protection towards a re-appearance of the virus in the future.

Anyhow, the results denote that although most SARS-CoV-2 infected individuals may produce S-RBD IgGs, they may not be sufficient to effectively prevent the binding of RBD to ACE2 receptor to neutralize the entry of SARS-CoV-2 into the cells and, therefore, to make the virus harmless.

Finally, this study shows that the increase of S-RBD IgG levels correlates with increasing NT antibody levels. Further studies looking at the functionality of these antibodies could improve our understanding of SARS-CoV-2 acquired immunity.

The current study is unfortunately limited to a small number of samples; it will be interesting to verify these data in a larger cohort of individuals and perform more accurate in vitro studies will be necessary to confirm the effective role of these antibodies. Furthermore, i*n vitro* cross-neutralization studies could provide clues about their protective activity and could suggest a cut-off value from which immune memory is built. Likewise, by monitoring antibody levels, it will be possible to assess the immune response after vaccination and S-RBD and NT antibodies could lead a potential preventive approach to counter the current pandemic.

Lastly, more than 600 immunoassays are available for the anti-SARS-CoV-2 antibodies detection and most anti-SARS-CoV-2 S-RBD tests are not yet standardized either with the NIBSC (National Institute for Biological Standards and Control, UK), or the World Health Organization (WHO) [13] International Standard (IS) [14]. At this purpose, the data of this work was evaluated directly the binding antibody units (BAU) for S-RBD antibodies and the international units (IU) for NT antibodies using the WHO IS, as declared by the company. Once all tests will be standardized it will be easier to assess the serum protection and the validity of the vaccine, as well as compare the different serological specific-antibodies tests results.

5. Conclusion

This study highlights the value of serological tests to investigate antibody trends and immunity related to SARS-CoV-2 infections. Understanding the mechanisms of action of antibodies can provide valuable implications for the rapid development of antibody therapy and the SARS-CoV-2 vaccine. In fact, most of the vaccines produced so far are based on the viral protein S, which domain is detected by serological analysis. These tests will help in a close future to evaluate immune response and antibody levels in all individuals adhering to the vaccination campaign.

CRediT authorship contribution statement

Flaminia Tomassetti: Data curation, Formal analysis, Methodology, Conceptualization, Writing – original draft, Writing - review & editing. **Marzia Nuccetelli:** Conceptualization, Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing - review & editing. **Serena Sarubbi:** Data curation, Formal analysis, Methodology. **Francesca Gisone:** Data curation, Formal analysis, Methodology. **Marco Ciotti:** Conceptualization, Data curation, Formal analysis, Visualization, Methodology, Writing - review & editing. **Francesco Spinazzola:** Conceptualization. **Cristina Ricotta:** Conceptualization, Data curation, Formal analysis. **Monica Cagnoli:** Conceptualization, Data curation, Formal analysis. **Monica Borgatti:** Writing - review &

editing. **Marco Iannetta:** Conceptualization, Data curation, Formal analysis. **Massimo Andreoni:** Conceptualization, Visualization, Supervision. **Graziella Calugi:** Conceptualization, Visualization, Supervision. **Massimo Pieri:** Conceptualization, Data curation, Formal analysis, Methodology, Visualization, Supervision, Writing – original draft, Writing - review & editing. **Sergio Bernardini:** Conceptualization, Visualization, 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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