***In vitro neutralizing activity of BNT162b2 mRNA-induced antibodies against full B.1.351 SARS-CoV-2 variant***

***Short Running Title: Neutralizing activity of antibodies against B.1.351 SARS-CoV-2***

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**Abstract**

**Background**: SARS-CoV-2 variation represents a serious challenge to current COVID-19 vaccines. Recent reports suggest that B.1.351 and other variants may escape the neutralization activity of the antibodies generated by current vaccines.

**Methods**: Ninety-nine healthcare workers undertaking BNT162b2 mRNA vaccination were sampled at baseline, on the day of the second dose, and 14 days after the latter. Neutralization activity against SARS-CoV-2 B.1, B.1.1.7 and B.1.351 was investigated using a Vero-E6 model.

**Results**: Eleven of the study participants had prior infection with SARS-CoV-2. Neutralization titers against the B.1 and the B.1.1.7 variants were not statistically different and were significantly higher than titers against the B.1.351 variant across pre-exposed and non-pre-exposed vaccinated individuals (*p*<0.01). While all vaccinated individuals presented neutralizing antibodies against B.1 and B 1.1.7 after the second dose, 14% were negative against B.1.351, and 76% had low titers (1/20-1/80). Pre-exposed vaccinated individuals showed higher titers than non-pre-exposed after the first (median titers of 1/387 versus 1/28, respectively) and the second doses (1/995 versus 1/703, respectively). As high as 72% of the pre-exposed vaccinees presented titers >1/80 after a single dose, while only 11% of non-exposed vaccinated individuals had titers >1/80.

**Conclusions**: BNT162b2 mRNA-induced antibodies show a lower in vitro neutralizing activity against B.1.351 variant compared to neutralization against B.1.1.7 or B.1 variants. Interestingly, for individuals pre-exposed to SARS-CoV-2, one dose of BNT162b2 mRNA may be adequate to produce neutralizing antibodies against B.1.1.7 and B.1, while two doses of BNT162b2 mRNA provide optimal neutralizing antibody response against B.1.351 too.

**INTRODUCTION**

In December 2019 a new coronavirus, named SARS-CoV-2 appeared in China producing an acute respiratory disease known as COVID-19. Due to the vast spread worldwide the World Health Organization has declared COVID-19 a pandemic.1,2 Vaccines against SARS-CoV-2 are available since December 2020. SARS-CoV-2 spike (S) protein is the focus of vaccines and the major target of neutralizing antibodies.

Antibodies against the S protein provide protection against COVID-19 and correlate with neutralizing (NT) antibodies appearing after symptoms and can last for several months.3,4 Neutralizing antibodies are important in hospitalized people but mild infected individuals could develop moderate NT titers.5 Other authors consider that most infected individuals with mild or moderate illness develop an important IgG response against the S protein.6 Neutralization of SARS-CoV-2 after recovering from the infection or after vaccination by high titers of neutralizing antibodies is of utmost importance to protect individuals from future exposures.7

SARS-CoV-2 has evolved into several lineages and different variants have been emerging and circulating during the COVID-19 pandemic. SARS-CoV-2 variants are important because they may present with specific genetic markers that have been associated with differences in receptor binding, lower neutralization by antibodies generated against previous infection or vaccination, different efficacy of treatments, potential diagnostic impact, or differences in transmissibility or disease severity.8

RNA messenger vaccines have proved to induce high levels of neutralizing antibodies6 that persist in serum six month after vaccination.9 However, it is unclear whether natural variation and evolution of SARS-CoV-2 may evade the immune response, and recent reports along the last months suggest that SARS-CoV-2 has the potential to escape antibody mediated immunity.10,11 Several variants of concern (VOC) are currently under evaluation because of their ability to evade the neutralizing effect of the antibodies induced by currently available vaccines. The spread of virus in immune competent population due to natural infection or vaccination has the potential to generate new SARS-CoV2 variants in a way to escape from herd immunity.12

The aim of this study was to assess the ability of the antibodies generated after vaccination with BNT162b2 mRNA to in vitro neutralize SARS-CoV-2 B.1, B.1.1.7 and B.1.351 lineages using a full virus neutralization assay.

**MATERIALS AND METHODS**

**Participants**

Ninety-nine healthcare workers that received two doses of BNT162b2 mRNA vaccine, 76 women (mean age 46, range 25-66) and 23 men (mean age 44, range 27-60) participated in the study. Eleven participants have had SARS-CoV-2 infection before the vaccination (pre-exposed group). Three serum samples were collected from every participant at three time points: on the day of first dose, on the day of the second dose and 14 days after the second dose. All the serum samples were inactivated at 56ºC for 30 min before use.

**Neutralization (NT) assay**

**Viruses**: SARS-CoV-2 hCoV-19/Spain/MD-FISABIO-Vircell001/2020 lineage B.1, hCoV-19/Spain/AN-HUSC-24581802/2020 lineage B.1.1.7, and hCoV-19/Spain/GA-CHUVI-19118872/2020 lineage B.1.351 were used in this study.

**Cell line**: Vero C1008 [Vero 76, clone E6, Vero E6] (ECACC 85020206) cells were obtained from the European Collection of Authenticated Cell Cultures (ECACC) and cultured in MEM Eagle with Earle's BSS, with 25 mM HEPES, 2mM L-glutamine (LONZA, Verviers, Belgium), and 1x antibiotic-antimycotic mixture (GIGCO, NY, USA), supplemented with 10% fetal bovine serum (FBS) (BioWest, Nuaillé, France). Vero E6 cells were seeded 72 h before the infection in 96-well plates (Corning, Maine, USA).

**Assay**: Briefly, 70 µl of each serum was two-fold serially diluted in culture medium with 2% FBS, from 1:20 to 1:2560 dilution. A total of 70 µl of cell culture medium containing 100 median tissue culture infectious dose (TCDI50)of the virus was added to every well containing diluted serum. Plates were incubated at room temperature for 1 h and 100 µl of each mixture of serum dilution and virus were added to a Vero E6 plate well from which the culture medium had been previously removed. After 5-day incubation at 37ºC in 5% CO2, full cytopathic effect (CPE) was evaluated by microscopic examination. The highest serum dilution that completely inhibited the CPE was considered as the NT titer. Every serum sample was assayed in NT with the three strains: B.1, B.1.1.7 and B.1.351.

First WHO International Standard for anti-SARS-CoV-2 immunoglobulin (human), NIBSC code 20/136, was obtained from National Institute for Biological Standards and Control.

**Statistical analysis**

Normality of data was compared using a Shapiro-Wilk test. The non-parametric Wilcoxon test or the Wilcoxon test for unpaired samples were used to compare data sets because the data were not normally distributed. Analysis was conducted in R (R Core Team, 2020)13. For graphical representation, titers were transformed to log2(1/(titer/10)) with titers <1/20 been assigned to 0 and titers ≥1/2560 to 8.

**RESULTS**

High neutralization titers against the B.1 variant were attained after the second dose for all study participants: the geometric mean titer (GMT) was 1/38 after the first dose and rose to 1/731 two weeks after the second dose. Vaccinated individuals with a previous history of infection, as shown by a past positive PCR result for SARS-CoV-2, showed higher titers than non-pre-exposed individuals after the first (GMT of 1/387 versus 1/28, respectively, table 1) and the second doses (1/995 versus 1/703, respectively). As high as 72% of the pre-exposed vaccinees presented titers >1/80 with a single vaccine dose, while only 11% of non-exposed vaccinated individuals had analogous titers (table 1).

Titers against the three variants are represented in figure 1 for pre-exposed vaccinated patients and non-pre-exposed vaccinated individuals. NT titers against B.1 and the B.1.1.7 variant showed no statistically significant differences in the Wilcoxon test. NT titers against B.1 and B.1.1.7 were significantly higher than titers against the B.1.351 variant in all the studied groups (pre-exposed and non-pre-exposed vaccinated individuals, *p*<0.01). While all vaccinated individuals presented neutralizing antibodies against B.1 after the second dose (43% with titers >1/620), 14% were negative against the B.1.351 variant, and 76% only had titers 1/20-1/80.

The change between NT titers against B.1 and B.1.531 after the second dose compared to after the first dose was lower in the pre-exposed vaccinated individuals (about 3-titer) than in the non-pre-exposed vaccinees (almost 5-titer drop) as shown in table 1. This difference was statistically significant (*p*<0.01) in a Wilcoxon test for unpaired samples despite the low number of pre-exposed vaccinated individuals in the study. More than half of the pre-exposed vaccinated individuals had neutralizing titers >1/80 while only 3% of the non-exposed vaccinees had titers > 1/80 and only 13% were positive.

The WHO 1st International Standard for SARS-CoV-2 antibodies was assayed in the CPE-neutralization test used in the study for comparative purposes with other studies. Titers of 1/640, 1/1280 and 1/160 were obtained for the B.1, the B.1.1.7 and the B.1.351 variants, respectively.

**DISCUSSION**

SARS-CoV-2 variation may represent a serious challenge to current COVID-19 vaccines. Recent reports suggest that B.1.351 and P1/P2 variants may escape the neutralizing activity of the antibodies generated by both BNT162b2 mRNA and mRNA-1273 vaccines. In our study, we recruited 99 participants undertaking two doses of BNT162b2 mRNA vaccination; participants were sampled at baseline, on the day they received the second dose, and 14 days after the second dose. Eleven participants had prior infection with SARS-CoV-2. Here we show that BNT162b2 mRNA-induced antibodies show a lower in vitro neutralizing activity against B.1.351 variant compared to neutralization against B.1.1.7 or B.1 variants. Interestingly, we also show that for individuals pre-exposed to SARS-CoV-2, one dose of BNT162b2 mRNA may be adequate to produce neutralizing antibodies for B.1.1.7 and B.1, while two doses of BNT162b2 mRNA provide optimal neutralizing antibody response for B.1.351 too.

The percentage of participants that achieved neutralizing antibodies titers 1/20-1/80 and also the mean titer was significantly higher at the time of receiving the second dose of BNT162b2 mRNA, across all the three SARS-CoV-2 B.1, B 1.1.7 and B. 1. 351 variants for the pre-exposed group. Our results are consistent with those found by Saadat et al,14 who showed that healthcare workers with previous COVID-19 infection had higher antibody titer responses to a single dose of mRNA vaccine than those who were not previously infected, and with those from Ebinger et al,15 who showed significant differences in spike-specific IgG antibody levels and ACE2 antibody binding inhibition responses elicited by a single vaccine dose in individuals with prior SARS-CoV-2 infection (n = 35) were similar to those found after two doses of vaccine in individuals without prior infection (n = 228). In addition to data provided in these studies, we provide data on neutralizing activity against B 1.1.7 and B.1.351 SARS-CoV-2 variants and, although providing a single dose of vaccine to individuals with a confirmed history of SARS-CoV-2 infection may be effective to neutralize B.1 and B1.1.7, this may not be enough for B.1.351 and, perhaps, for other new variants carrying also specific “vaccine escape” spike mutations, such as it has been proposed for E484K, presented in lineages B.1.351 and P1/P2.16,17. B.1.351 variant incorporates E484K in RBD of the S protein (Cheng et al18,Wang et al19). This mutation affects the binding of serum polyclonal neutralizing antibodies (Jangra et al20) due to the fact that it enhances the binding affinity between RBD and the ACE2 in human cells; it has also been shown that it reduces the neutralization activity and even may escape from the neutralizing antibodies in the convalescent plasma of COVID-19 patients, which may weaken the effectiveness of the vaccines and the efficacy of the neutralizing antibody therapeutics in development. This effect has also been proven for P1/P2 variants, and may be similar for all variants harboring this change.

Participants previously and non-previously infected by SARS-CoV-2 showed high titers of neutralizing antibodies against the B.1 and B 1.1.7 variants 14 days after receiving the second dose of BNT162b2 mRNA. However, significant differences in both the mean titer and the percentage of patients with neutralization titers were found compared to the activity against B.1.352 variant. In consistency with our results, recent studies have shown a lower activity of spike-based mRNA vaccines on the B 1.352 variant than on the other SARS-CoV-2 variants: Xie et al21 have shown that incorporating the E484K mutation into a B 1.1.7 N501Y+D614G background pseudovirus model resulted in a slightly lower neutralization capacity (0.81- to 1.41-fold). Wang et al22 using a SARS-CoV-2 pseudotype neutralization assay studied 20 volunteers who received either the Moderna (mRNA-1273) or Pfizer-BioNTech (BNT162b2) vaccines, finding that activity against SARS-CoV-2 variants encoding E484K or N501Y or the K417N:E484K:N501Y combination was reduced by a small but significant margin. WU et al,23 used the mRNA-1273 pseudoviruses bearing the spike protein from the original Wuhan-Hu-1 isolate, the D614G variant, the B.1.1.7 and B.1.351 variants, and found a decrease in titers of neutralizing antibodies against the B.1.351 variant and a subset of its mutations affecting the SARS-CoV-2 receptor binding domain (RBD). Tada et al24 also using pseudotyped viruses tested serum specimens from individuals vaccinated with the BNT162b2 mRNA vaccine, and found an average 3-fold reduction in titer for the B.1.351 spike, reduction that was attributable to the E484K mutation in the RBD. Liu et al25 engineered S mutations from pseudoviruses including all mutations in the B.1.1.7-spike, the P.1-spike, and the B.1.351-spike and studied the neutralizing activity of serum from participants vaccinated with BNT162b2. Neutralization of B.1.1.7-spike and P.1-spike viruses was roughly equivalent compared with neutralization of USA-WA1/2020, whereas neutralization of B.1.351-spike virus was lower. Garcia-Beltran et al11 evaluated the neutralization potency of 48 sera from BNT162b2 and mRNA-1273 vaccine recipients against pseudoviruses bearing spike proteins derived from 10 strains of SARSCoV-2. Again, B.1.351 together with four variants harboring receptor-binding domain mutations, including K417N/T, E484K, and N501Y, were highly resistant to neutralization. In contrast, Stamatos et al26 using pseudoviruses expressing either the full-length Wuhan Hu1 spike, or a spike containing the B.1.351-lineage S mutations D80A, D215G, K417N, E484K, N501Y and D614G, and the A701V, examined whether sera and monoclonal antibodies from convalescent donors, prior to and following a single immunization with the Pfizer or Moderna mRNA vaccines, neutralized the Wuhan-Hu-1 strain and a variant, B.1.351 from South Africa, found a 1000-fold increase in neutralizing antibody titers against both strains and SARSCoV-1.

In consistency with the above, other studies have also shown a neutralization escape of B.1.351 to convalescent plasma. Wibmer et al27 found that nearly half (21 out of 44, 48%) of the samples assayed had no detectable neutralizing activity against the 501Y.V2 virus. Differences in the magnitude of the response against the new variants can be explained by the use of different type of viruses in the neutralization assays: pseudovirus, laboratory-created mutants or wild-type mutants. In that sense, wild type mutants may harbor additional mutations than those present in the spike accounting for such differences.11 While pseudovirus assays offer a better understanding of the role of each mutation in the differential responses against each variant, neutralization with wild type variants may reflect more closely the challenges that the new variants pose to the ongoing vaccination campaigns. Lack of homogeneity in neutralization assays is another factor that hampers comparison between various studies. We have therefore included values for the recently released international standard to facilitate comparison with future studies and promote the use of such standard.28

Interestingly the drop in the neutralizing antibody response was significantly lower in the small sub-group of vaccinees that had suffered from a previous infection confirmed by a PCR positive result. García-Beltran et al.11 reported that antibodies have a reduced but detectable binding to mutant RBD, suggesting that a larger response in the pre-exposed vaccinees explain this difference. In addition, a previous infection has confronted the immune system of the patient with an array of epitopes29 that have triggered a more diversified immune response upon vaccination. Finally, a more advanced maturation of the immune response of these pre-exposed individuals can also be a differential factor for this increased neutralizing response. As shown by Gaebler et al.30, antibodies expressed by memory B-cells displayed an increased resistance to RBD mutations six months after infection. In that sense, it would be interesting to study the immune responses of vaccinees after a longer period from the last immunization dose.

Our study may have some limitations, such as the low number of participants enrolled that had suffered from a previous SARS-CoV-2 infection, and the fact that we did not include other variants of concern, such as the most prevalent latest delta variant (B.1.617.2 and its sublineages), that harbors two gene mutations (L452R and P681R in spike) that are suspected to allow delta variant to be the most transmissible variant yet identified (Mlcochovaet al31).

Despite the above limitations, we could confirm the results of previous studies performed with engineered pseudoviruses with a higher number of participants using real life isolates of the B1, B 1.1.7 and B 1.351 SARS-CoV-2 variants. The clinical impact of neutralizing resistance remains uncertain; however, our results show the potential of B 1.351 variant to escape from neutralizing humoral immunity, and raises the concern on a possible recommendation to public health authorities to provide full vaccination, rather than just one dose, to COVID-19 pre-exposed patients infected with this variant that undergo vaccination with BNT162b2 mRNA.

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**Conflicts of interest:** none

**Conflicts of interest on metadata:** none

**Ethics:** The protocol was approved by the Ethics Committee of the Hospital Universitario Clínico San Cecilio (HUSC 0670-N-21). All participants provided informed consent.

**Transparency declarations:** none to declare

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Legend to Figure 1.

Neutralizing titers (log transformed as described in Methods) for each individual of the non-pre-exposed (A) and pre-exposed (B) groups. Titers against different strains are represented with different colors (blue for B.1, green for B.1.1.7, red for B.1.351). Samples are separated by collection time: samples collected before vaccination (basal, ●), samples collected on the same day that the second dose was administered corresponding to antibodies triggered by the first vaccine dose (dose 1, ▲), samples collected two weeks after administration of the second vaccine dose (dose 2, ■). Geometric mean titers for each subgroup are represented with large markers. Evolution between basal, dose 1 and dose 2 antibody geometric mean titers are represented by solid lines for each subgroup. Titers in each group were compared between each pair of strains by a Wilcoxon test. Significantly different paired groups are marked with \*\* (p < 0.01) while not significantly different paired groups are marked with \* (p > 0.05).