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Not all SARS-CoV-2 IgG and neutralizing antibody assays are created equal

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We read with interest the recent article of Malipiero et al. [1], who used a commercial anti-RBD (receptor binding domain) IgG quantitative chemiluminescent immunoassay and a surrogate virus neutralization test (sVNT) to investigate the kinetics of anti-SARS-CoV-2 (neutralizing) antibodies up to 6 months after administration of a primary cycle of Pfizer/BioNTech BNT162b2 vaccine in 57 local healthcare workers. Notably, these authors found that despite high neutralizing bioactivity was retained throughout the study period, the anti-RBD IgG antibodies markedly declined 6 months after primary vaccination. Such discrepancy is quite surprising and prompts us to make some biological and analytical considerations.

First, although it is conceivable that a standard primary vaccination cycle has been administered to the population of healthcare workers studied by Malipiero et al. (i.e., two 0.3 mL intramuscular injections of 30 µg of vaccine at 3-week interval), the specific protocol that was used is not comprehensively specified in their published work, so that the trajectory of anti-SARS-CoV-2 antibodies levels would not be readily interpretable, neither straightforwardly comparable with that found in other studies.

The use of a sVNT (ACE2-RBD Neutralization assay; Dia.Pro Diagnostic Bioprobes, Milano, Italy) is another important aspect that deserves further scrutiny. It is now widely acknowledged that the reference technique for studying the neutralizing potential of serum or plasma entails the use of live virus neutralization assays, which basically include focus-reduction neutralization tests, plaque reduction neutralization tests and live virus micro-neutralization assays [2]. In a recent study, Meyer et al. found a relative modest correlation between a sVNT and live virus or pseudovirus neutralization tests (correlations of 0.656 and 0.494, respectively), underpinning also that the sensitivity of the sVNT technique was only around 80% (e.g., only samples with ≥ 160 titre were found to be always positive with sVNT) [3]. In another preliminary report, Sholukh et al. reported that the correlation between a sVNT and a reference cell-based neutralization assay (50% neutralizing dilution; ND50) was only around 0.40 [4]. These analytical drawbacks would hence lead us to conclude that sVNTs are not suitable replacement of cell-based neutralization assays for being used as the reference technique for testing the performance of commercial anti-SARS-CoV-2 (neutralizing) antibodies. Importantly, several other reports, such as

that published by Bayart et al. for example [5], have clearly shown that neutralizing antibodies assessed with pseudo-virus neutralization test (pVNT), which is a more reliable technique for this purpose than the sVNT [2], undergo a dramatic decline over time (i.e., over 90%) in Pfizer/BioNTech BNT162b2 vaccine recipients, with nearly half of vaccinated subjects turning negative after 6 months.

As then concerns the commercial anti-S-RBD IgG antibodies method used in the study of Malipiero et al. (sCOVG, Siemens Healthineers, Erlangen, Germany), a recent clinical and analytical evaluation of this quantitative chemiluminescence immunoassay published by Irsara et al. has revealed excellent performance compared to VNTs assayed on Vero 76 clone E6 cells, displaying a correlation of 0.843, an overall qualitative agreement of 98.5% and diagnostic sensitivity as high as 91% [6].

We are hence persuaded that concluding that the value of anti-RBD IgG is a (relatively) poor marker of neutralizing bioactivity seems unwarranted at this point in time, since this could only be reliably defined by using live virus neutralization assays. It shall also be clearly acknowledged that the current performance of the many commercial immunoassays that measure neutralizing anti-SARS-CoV-2 antibodies vary significantly when compared with the gold standard technique, as recently shown in a study that compared five anti-SARS-CoV-2 IgG chemiluminescent techniques with a plaque reduction neutralization test (PRNT) (i.e., correlations between 0.799 and 0.872) [7]. Finally, the clinical significance of the many currently available anti-SARS-CoV-2 IgG immunoassays needs to be tested and validated in patients infected by highly mutated variants, such as the recently emerged Omicron (B.1.1.529) strain [8].

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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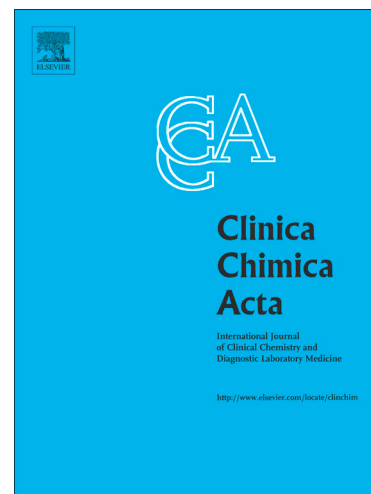
Answer to the letter of Lippi & Plebani entitled “Not all SARS-CoV-2 IgG and neutralizing antibody assays are created equal”

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Answer to the letter of Lippi & Plebani entitled "Not all SARS-CoV-2 IgG and neutralizing antibody assays are created equal".

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Dear Editor,

we really appreciate the interest of Lippi and Plebani for our recent paper, which offers us the opportunity to provide some additional clues. The primary aim of our study was to collect data on the long-term kinetics of binding and functional anti-SARS-CoV-2 antibodies in a cohort of BNT162b2-vaccinated health care workers (HCW) over an observation period of 6 months post-vaccination. The main findings were the not unexpected decline of binding anti-RBD antibodies and the lack of a consistent and time-independent correlation between anti-RBD IgG levels and neutralizing bioactivity, which settled at high level from T2 (50 days post 1st vaccine administration) thereafter with a non significant declining trend between T2 and T3 (6 months post 1st dose administration). Lippi and Plebani pointed out that a systematic lack of accuracy in surrogate virus neutralization assay (sVNT) platforms compared to conventional ones should prevent us from concluding that the absolute anti-RBD level is not a reliable proxy of neutralizing bioactivity in BNT126b2 vaccinated-individuals.

We certainly agree that sVNT platforms should not replace live virus neutralization assays as the gold standard for identifying and titering neutralizing antibodies to SARS-CoV-2 wild-type and variant strains, as we also admitted as a limitation of our study. However, we hint that the accuracy of these tests and their correlations relies on the clinical setting to which they are applied. Indeed, despite the existence of a WHO International Standard for both binding and neutralizing assays [2], it is not yet clear how the clinical setting (COVID-19 vaccinated vs. experienced patients) and the emergence of variants of concern could undermine the harmonization process between these different assay platforms under real-world conditions [3-4]. sVNT platforms, by investigating the wild-type RBD-induced neutralizing activity only, are likely to underestimate the neutralizing potency of serum from COVID-19 experienced individuals, especially when VOC are involved [5-6]. On the other hand, as repeatedly shown by comparative analyses in vaccinated cohorts [7-9], the diagnostic reliability of sVNT seems to be retained when assessing vaccine-induced immune-responses, provided that the mRNA sequence of the vaccine RBD matches that of the recombinant RBD in the binding and competition assays. The cross-sectional studies cited by Lippi and Plebani [5-6] quantified humoral immunogenicity markers in two cohorts of convalescent COVID-19 patients while the cohort studied by Bayart et al. [10] is as well affected by a large number (31%) of

individuals with a positive serological scrutiny for SARS-CoV-2 infection before vaccination. In fact, the primary aim of the study by Meyer et al. [5] was the diagnostic value and comparability of different immune assay platforms for a diagnosis of SARS-CoV-2 infection rather than the long-term monitoring of the immune response to a spike-based vaccine. Notably, the very same study of Sholukh et al. [6] showed that the correlations between the sVNT, on the one side, and 4 live virus/pseudovirus neutralization assays, on the other, are much higher ($r=0.73-0.8$) when the percentage of neutralization (ND_{50} in the original manuscript) is considered instead of the ND_{50} titer reported by Lippi and Plebani. Further on, all cited studies did not assess whether patients were infected by the Wuhan original strain or a variant strain, which, for the above reasons, could affect inter-assays harmonization. As to the study by Bayart et al. [10], which showed a much faster decline in neutralizing antibody titers using a live VNT than we assessed with our sVNT, available literature has so far provided contrasting results about long-term decay of circulating antibodies after mRNA vaccination, with some studies providing evidence for persistent and sustained neutralizing bioactivity of serum up to 6 months from vaccination [11-12]. It's our opinion that such discrepancies are resulting from multiple factors, including but not limited to inter-assays inconsistencies, such as previous exposure to cross-reacting common cold coronaviruses, age, history of previous COVID-19, sex, and other comorbidities in vaccinated cohorts.

We would also like to remark that in our study we did not disclaim the existence of a correlation between anti-RBD IgG levels and neutralizing bioactivity in vaccinated individuals nor that the high neutralizing activity we assessed at 6 months is necessarily protective, since the 1 month-apart swab-based monitoring protocol we use might have missed asymptomatic carriers. Actually, we observed an increasing strength of correlations from T2 and T3 despite a declining titer of anti-RBD IgG, likely as a result of affinity maturation of IgG by somatic hypermutation of spike/RBD-specific B cells [13-15]. This temporal trend has already been tracked after SARS-CoV-2 vaccination [12] and implies that an absolute conversion factor between anti-RBD IgG levels and neutralizing titers would be unreliable. As opposite, the studies cited by Lippi and Plebani [16-17] were not designed to provide data on anti-RBD IgG and neutralizing activity and their correlation on the long-term, when divergent kinetics may become apparent. Should a neutralizing

antibodies absolute threshold of protection ever become available (hopefully using the WHO International Standard), implementation of the anti-RBD IgG level as a time-independent standalone correlate of protection may confuse risk stratification [14].

We are grateful to Lippi and Plebani for their appropriate remarks, which gave us the opportunity to better explain and contextualize our findings, but, for the above reasons, we believe the arguments and studies they brought about would not impair our findings. On the contrary, we believe that sVNT are user-friendly, high-throughput, standardized [18] immune-assays that may represent invaluable tools when implemented in the appropriate clinical and epidemiological context.

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