Function is more reliable than quantity to follow up the humoral response to the Receptor Binding Domain of SARS-CoV-2 Spike protein after natural infection or COVID-19 vaccination.

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Abstract

On this work we report that despite of a decline in the total anti-Spike antibodies the neutralizing antibodies remains at a similar level for an average of 98 days in a longitudinal cohort of 59 Hispanic/Latino exposed to SARS-CoV-2. We are also reporting that the percentage of neutralization correlates with the IgG titers and that in the first collected samples, IgG1 was the predominant isotype (62.71%), followed by IgG4 (15.25%), IgG3 (13.56%), and IgG2 (8.47%) during the tested period. The IgA was detectable in 28.81% of subjects. Only 62.71% of all subjects have detectable IgM in the first sample despite of confirmed infection by a molecular method. Our data suggests that 100% that seroconvert make detectable neutralizing antibody responses measured by a surrogate viral neutralization test. We also found that the IgG titers and neutralizing activity were higher after the first dose in 10 vaccinated subjects out of the 59 with prior infection compare to a subgroup of 21 subjects naïve to SARS-CoV-2. One dose was enough but two were necessary to reach the maximum percentage of neutralization in subjects with previous natural infection or naïve to SARS-CoV-2 respectively. Like the pattern seen after the natural infection, after the second vaccine dose, the total anti-S antibodies and titers declined but not the neutralizing activity which remains at same levels for more than 80 days after the first vaccine dose. That decline, however, was significantly lower in pre-exposed individuals which denotes the contribution of the natural infection priming a more robust immune response to the vaccine. Also, our data indicates that the natural infection induces a more robust humoral immune response than the first vaccine dose in unexposed subjects. However, the difference was significant only when the neutralization was measured but not by assessing the total anti-S antibodies or the IgG titers. This work is an important contribution to understand the natural immune response to the novel coronavirus in a population severely hit by the virus. Also provide an invaluable data by comparing the dynamic of the immune response after the natural infection vs. the vaccination and suggesting that a functional test is a better marker than the presence or not of antibodies. On this context our results are also highly relevant to consider standardizing methods that in addition to serve as a tool to follow up the immune response to the vaccines may also provide a correlate of protection.
Introduction

COVID-19 pandemic possesses an unprecedented challenge to the scientific community. At the same time, it is adding new knowledge on the molecular biology, epidemiology, and immunology at an accelerated speed. One of the crucial questions still under scrutiny is the robustness of the immune response to the natural infection with SARS-CoV-2, given the fact that virus specific ab response is relatively short following SARS CoV-1 and other common cold coronaviruses (CCC). https://doi.org/10.1016/j.immuni.2020.05.002. Particularly interesting is the new immunological scenario with the availability of few vaccine formulations.

To contribute to this field, we followed up a cohort of 59 individuals (volunteers or convalescent plasma donors) at different time points following infection. In addition, we choose a set of 7 of those individuals plus 3 additional subjects (n=10) to compare their vaccine’s response with 21 uninfected-vaccinated subjects. Serum samples for both vaccinated groups were collected between 12 and 28 days after each of the two doses and a third sample was collected between 19 and 83 days after the second dose. Because the limited period of SARS-CoV-2 circulation the quantity, quality and extent of long-term memory responses is still under study. Recent works on the durability of the humoral immune response after the natural infection with SARS-CoV-2 showed the presence of neutralizing antibodies for several months (1-5) or the persistence of IgG responses over the first few months after infection which highly correlates with neutralizing antibodies (3, 6). From the beginning of COVID-19 pandemic, the analysis of the functional properties of the antibodies was very limited because the need of biosafety level 3 (BSL-3) facilities to growth the virus. However, in a relatively short period of time, several surrogate’s neutralization assays became available with an excellent performance profile when compared to the classical focus reduction neutralization test (FRNT) (3, 7-11). For this work we choose the cPass SARS-CoV-2 Surrogate Virus Neutralization Test Kit (GenScript, USA) which measures the interaction of purified SARS CoV-2 spike protein receptor binding domain (RBD) with the extracellular domain of the human ACE2 receptor (11). This assay showed the best sensitivity and the lower false negative rate compared to other five assays (10) and was granted an Emergency Use Authorization (EUA) by the Federal Drug Administration (FDA). Interesting we detected few cases (n=6) where the neutralization activity was still present even when the IgG was undetectable by our method (OD <.312).

Our work, which include a predominantly Latino population, confirms that the neutralizing antibodies remained detectable at high levels for 4 to 7 months following a natural infection. Currently there is very limited information regarding the magnitude of the immune response to the COVID-19 vaccine in naive vs. pre-exposed subjects and aspect was very limited addressed in recent clinical trials reports (12-15). Nevertheless, as we are showing here, few recent released reports suggest that the levels of the antibodies in previously infected persons trend or are significantly higher than in SARS-CoV-2 naive
persons (16-19). Those reports and our own results suggest that a single dose may be enough during the first vaccination stages leading to an optimal use of the vaccine availability worldwide. Recently the debate has centered in the efficacy of the natural immune response to SARS-CoV-2 vs. the response to the vaccine. We also addressed this in our cohort showing that the quantity and the quality of the antibodies induced by the natural infection are significantly higher and better, respectively, compared to the one induced by the vaccination.
Material and Methods

Cohorts
This research protocol will have two main sources of samples:
1- From adults’ volunteers (> 21 years old) participating in the IRB approved clinical protocol “Molecular Basis and Epidemiology of Viral infections circulating in Puerto Rico”, Pro0004333. Protocol was submitted to and ethical approval was given by Advarra IRB on April 21, 2020. This is a running 5 years protocol and encompass the collection of blood samples from adults exposed or suspected to be exposed to viral infections. An Informed Consent Form and a study questionnaire also approved by the IRB were administered to the volunteers. From March 2020 to April 2021, we were able to follow up for serial samples at least 59 subjects. From those 59, seven (5) subjects received the two doses of Pfizer’s vaccine and two the Moderna’s formulation. We also added three vaccinated subjects for a total of 10 (ID511, ID512 and ID297). From those three, two received Pfizer’s and one Moderna’s vaccine. In addition, a cohort of 21 volunteers that were followed from months, but were never exposed to the virus, were also vaccinated. Eighteen (18) received Pfizer’s vaccine and three (3) Moderna’s formulation.

2- De-identified blood samples received from local laboratories network and Blood Banks. These subjects were self-enrolled and were amply verbally informed about the relevance of their participation as plasma donors for the treatment of COVID-19 patients. They were also given the opportunity to ask questions. Furthermore, they were handled throughout the standard blood donors’ protocols signing the Blood Bank’s standard consent with ample information about the use of their samples for research purposes only. In addition, before receiving the samples from these sources the subjects’ data that has been stripped of all identifiers so that the information cannot be traced back to an individual.

Detection of SARS-CoV-2 IgM antibodies
CovIgM-Assay is an indirect ELISA for the determination of human IgM antibody class, which was optimized via checkerboard titration. This assay is a Laboratory Developed Test (LDT) with an Emergency Use Authorization (EUA) submitted to the U.S. Federal Drug Administration (FDA) (EUA202043). In summary microplates were coated overnight at 4°C with 2μg/mL of recombinant SARS-CoV-2 S1-RBD protein (GenScript No. Z03483-1) in carbonate-bicarbonate buffer. Plates were washed 3 times with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST) and blocked for 30 min at 37°C with 250μL/well of 3% Bovine Serum Albumin (BSA) in PBST. Diluted serum or plasma samples (1:100 in blocking buffer) were added in duplicates to the wells and incubated at 37°C for 30 min. The excess antibody was washed off with PBST. Horseradish peroxidase (HRP) labeled-mouse anti-human IgM-mu chain (Abcam) diluted 1:30,000 in PBST was added (100μL/well) and
incubated for 30 min at 37°C. After another washing step, TMB solution was added (100μL/well) followed by 15 min incubation. The reaction was stopped by the addition of 50μL/well 10% HCl and the absorbance was measured at 450nm (A450) using a Multiskan FC reader (Thermo Fisher Scientific). In every CovIgM-Assay determination, four wells in which samples were replaced by 100μL/well of PBST were included as background control. Moreover, two in-house controls, a high positive control (HPC) and negative control (NC) were included. HPC and NC were prepared by diluting an IgM anti-SARS-CoV-2 at a concentration of 80μg/mL and 0.070μg/mL, respectively, in PBST containing 10% glycerol. The IgM anti-SARS-CoV-2 was purified from the plasma of a convalescent patient using 5/5 HiTrap IgM columns (GE Healthcare, USA). When the OD value of a serum or plasma sample at the working dilution (1:100) is equal or less to the cut-point (OD450= 0.229), the CovIgM-Assay in the sample is inferred to be negative.

**Detection of SARS-CoV-2 IgG antibodies**

The IgG antibodies were detected and quantified by using the CovIgG-Assay (20). This assay is a Laboratory Developed Test (LDT) with an Emergency Use Authorization (EUA) submitted to the U.S. Federal Drug Administration (FDA) (EUA201115). It is an indirect ELISA for quantitative determination of human IgG antibody class, which was optimized by checkerboard titration. In summary, disposable high bind flat-bottomed polystyrene 96-wells microtiter plates (Costar, Corning MA No. 3361) were coated overnight at 4°C with 2μg/ml of recombinant SARS-CoV-2 S1-RBD/S2 protein (GenScript No. Z03483-1) in carbonate-bicarbonate buffer (Sigma Aldrich No. 08058). Plates were washed 3 times with (PBST) and blocked for 30 min at 37°C with 250μl/well of 3% non-fat, skim milk in PBST. Samples (serum or plasma) were diluted 1:100 in PBST; 100μL/well was added in duplicates and incubated at 37°C for 30 min. The excess antibody was washed off with PBST. Horseradish peroxidase (HRP) labeled-mouse anti-human IgG-Fc specific (GenScript No. A01854) diluted 1:10,000 in PBST was added (100μl/well) and incubated for 30 min at 37°C. After another washing step, a substrate solution (Sigma Aldrich No. P4809) was added (100μl/well) followed by 15 min incubation. The reaction was stopped with 50μl/well 10% HCl and the absorbance was measured at 492nm (A492) using a Multiskan FC reader (Thermo Fisher Scientific). In every CovIgG-Assay determination two in-house controls, a high positive control (HPC) and negative control (NC) were included. HPC and NC were prepared by diluting an IgG anti-SARS-CoV-2 at a concentration of 30μg/ml and 0.070μg/ml, respectively in PBST containing 10% glycerol. The IgG anti-SARS-CoV-2 was purified from plasma of a convalescent patient using a 5/5 HiTrap rProtein-A column (GE Healthcare, USA). When the OD value of a serum or plasma sample at the working dilution (1:100) is equal or less to the cut-point (OD492= 0.312), the CovIgG-
Assay in the sample is inferred to be negative. However only samples with OD above of 0.499 were reported as having a titer within a range of 1:100 to ≥ 1:12,800. For the isotyping ELISA, the conjugate was changed for the specific isotype as follows: anti-IgA (alpha chain specific-HRP (Sigma), anti-IgG1, 2, 3 and 4 Fc-specific-HRP (Southern Biotech). All conjugates were used in a 1:3,000 dilution.

cPass SARS-CoV-2 neutralization antibody detection method
To determine the neutralizing activity of antibodies we used a surrogate viral neutralization test (C-Pass GenScript sVNT, Piscataway NJ) (10, 11). Briefly, serum or plasma samples were diluted according to manufacturer’s instructions and incubated with a soluble SARS-CoV2 receptor binding domain (RBD-HRP) antigen for 30minutes, mimicking a neutralization reaction. Following incubation, samples were added to a 96 well plate coated with human ACE-2 protein. RBD-HRP complexed with antibodies are removed in a wash step. The reaction is developed with tetramethylbenzidine (TMB) followed by a stop solution allowing the visualization of bound RBD-HRP to the ACE2. Since this is an inhibition assay, color intensity is inversely proportional to the amount of neutralizing antibodies present in samples. Data is interpreted by calculating the percent of inhibition of RBD-HRP binding. Samples with neutralization activity of ≥30% indicates the presence of SARS CoV-2 RBD-interacting antibodies capable of blocking the RBD-ACE2 interaction thus inhibiting the virus entrance to the cell. While this assay measures the blocking activity of those antibodies for consistency and clarity during the manuscript the activity is referred as percentage of neutralization.

Statistical Methods
Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, San Diego, CA, USA). The statistical significance between or within groups evaluated at different time points was determined using two-way analysis of variance (ANOVA) (Tukey’s, Sidak’s or Dunnett’s multiple comparisons test) or unpaired t-test to compare the means. The p values are expressed in relational terms with the alpha values. The significance threshold for all analyses was set at 0.05; p values less than 0.01 are expressed as P<0.01, while p values less than 0.001 are expressed as P<0.001. Similarly, values less than 0.005 are expressed as P<0.005. The Kappa values (κ) were considered as follows: poor agreement, κ<0.20; fair agreement, κ=0.21 to 0.4; moderate agreement, κ=0.41 to 0.6; substantial agreement, κ=0.61 to 0.8; very good agreement, κ=0.81 to 1.0.
Results

Sample’s collection
The samples for this study were collected longitudinally depending on the subject’s availability. No specific time was set for samples collection. However, the time between serial samples was very similar for all subjects. The average time between the time of the documented infection and the first samples (n=59) was 40.37 days (minimum 12 days, maximum 97 days and two extreme cases with 127 and 176 days for a median of 38 days). Once the subjects entered in the cohort, the average time between the first and the second samples (n=59) was 67.86 days (minimum 7 days, maximum 111 days, median 67.5 days). The average time between the second and the third samples (n=12) was 99.5 days (minimum 63 days, maximum 159 days, median 95 days) (Supplementary table S1).

From the two subgroups, exposed-vaccinated and unexposed-vaccinated, serum samples were collected between 15 to 20 days after each dose. In addition, a third sample from all the 21 unexposed and from 8 out of the 10 pre-exposed participants was collected between 19 and 83 days after the second dose (average of 40.1 and of 81.6 days for the unexposed and pre-exposed groups respectively) or an average of 60.3 and 100.5 days after the first vaccine dose for the unexposed and pre-exposed groups respectively (Supplementary table S2). Highly relevant for our findings is that the sample used as baseline in the pre-exposed before vaccination, was collected in average 142 days after the confirmed infection (minimum 67 days, maximum 310 days, median 126.4 days)(Supplementary table S3).

IgG titers and isotypes to SARS-CoV-2 trend to decline over the time.
In overall the values of the IgG titers from the first samples were significantly higher (geometric mean 1072) than in the second sample (geometric mean 618) (p< 0.0473) and the third sample (geometric mean 537) (p< 0.0474) while there were not significant differences between titers measured in the second and third sample (p < 0.3085) (Figure 1A). The OD results are showed in supplementary figure 1A and agree with the estimated titers (Supplementary table S4).

From 59 subjects naturally exposed to the virus, 40 (67.8%) experimented a decrease in the IgG titers (Figure 1B) while 19 (32.2%) showed an increase in the IgG titers from the first to the second sample (Figure 1C). We aimed to establish if the time elapse from diagnosis to the first sample collection had an impact in the rise or decline of IgG titers from the first to the second sample and we found no significant differences (Supplementary figure 2). From these results we can conclude that the differences in the IgG titers in those groups from the first to the second sample were not attributable to the time between collection. Also, no significant difference was observed in the time gap between first
and second sample for both groups (Supplementary figure 2). We identified three subjects (ID137, ID195 and ID367) showing a singular behavior of an increase in the IgG titers of 2.13, 8.65 and 52.1 times respectively in the third sample compared to the second one. Particularly, volunteer ID195 exhibited a fall of 8.65 times in the IgG titers after 50 days in the second sample followed by a similar increase in the third sample collected 75 days after the second sample.

**IgM titers to SARS-CoV-2 trend to decline over the time**

From 59 subjects, 37 (62.71%) and 18 (30.50%) had detectable IgM in the first and second samples respectively (Supplementary figure 1H). Five subjects out of the 12 where a third sample was collected, had still measurable IgM or develop IgM for first time (ID313 IgM was detected as early as 12 days after the presumptive diagnosis and persisted up to 192 days (approximately 6.4 months). Overall, IgM values showed a consistent pattern of decline in the second sample for most of the individuals (86.44%). Only one subject (265) showed a duplication of the values in the second sample collected 68 days apart and one subject (ID313), with negative IgM readings in the first and second samples and a positive value in the third sample collected 106 and 146 days after the first and the second samples respectively. We also found 3 subjects out of 59 (5.08%) with detectable IgM but without IgG in the second sample. Subject 312 showed detectable IgM but borderline IgG results in the third sample collected 57 and 69 days after the first and second dose respectively (86 and 98 days after the presumptive diagnosis). Subject 105 still had detectable IgM 192 days after the presumptive diagnosis was made. The earlier time that IgM was detected was 12 days after the presumptive infection in subject 166 followed by subjects 180 and 179 with 13 and 14 days after diagnosis respectively. In general IgM was detected in 37 subjects (77.97%) in the first sample and in 18 subjects (57.63%) in the first and second samples at an average of 43 days and 104 days after the presumptive infection respectively. In 18 subjects (30.51%) no IgM was detected in any of the serial samples collected.

**IgG titers but not IgM or IgA correlates with neutralizing activity.**

As we described before, the correlation of IgG estimated titers by the CovIgG-Assay correlates extremely well with the Focus Reduction Neutralization Test (20). For this work we performed same analysis between the titers and the neutralization results obtained with the surrogate assay cPass SARS-CoV-2 neutralization antibody detection method. By applying a Kappa analysis, we first aimed to determine if both techniques agree when classifying positive and negative samples using <100 and > 30% as cutoff for the IgG titers and percentage of neutralization respectively. Results indicated a Cohen’s Kappa value of 0.4304 for a moderate agreement (Supplementary figure 3A). Then, we aimed to determine whether both techniques agree when classifying samples with high IgG titers and high...
Neut Ab value resulting in a Cohen’s Kappa value of 0.5402 for a similar moderate agreement (Supplementary figure 3B). We completed same analysis for the IgM and IgA to explore the contribution of those antibodies subclass to the neutralization. We found that both techniques (IgM and cPass) have a fair agreement when classifying positive and negative samples (Cohen’s Kappa = 0.2391), while the IgA and the neutralization techniques showed only a slight agreement (Cohen’s Kappa = 0.0618) (Supplementary figure figures 3C-D).

Neutralizing activity remains at same level over time
The results shown that the levels of the neutralizing activity were not modified from the first (geometric mean 68.08%) to the second sample (geometric mean 63.89%) or from the second to the third samples (geometric mean 60.36%) (Figure 1D and supplementary table S4). However, like the IgG titers, we were able to identify two patterns in the way the percentage of neutralization behave. Compared to the first collected sample we found a decrease in the neutralizing activity in the second sample in 61.01% (36 out of 59) of the subjects while 38.98% of subjects (23 out of 59) showed a decrease in that activity (Figures 1E and F). While the percentage of subjects experimenting an increase or a decrease in their percentages of neutralization and IgG titers between samples was similar, the change at neutralizing level was less pronounced and not significant compared with significant changes in the IgG titers (Figure 1B). From here we conclude that the neutralizing capabilities, remains to the same level during the time we followed the subjects. Same situation apply by comparing the second and the third samples in the few subjects that we completed the collection of that third sample. We identify one subject (ID 313) showing a different pattern, with an increase of 3.34 times (by 68%) in the neutralizing activity from the second to the third sample. Other two subjects also showed an increase in their IgG titers, ID135 and ID195, have a very limited increase in their neutralizing levels of 1.2 and 0 times respectively. Despite the variability in the IgG titers, the percentage of neutralizations was kept over 50% in 90% of all three samples. The distinctive serological and neutralization pattern for subject 313 is strongly related to the clinical evolution (Supplementary figure 3).
Also, we identified 11 subjects without IgG titers and showing some degree of neutralization from 36% to 76%. Six of them out of those 11 subjects don’t even have detectable IgG. On the other hand, there were 3 subjects with detectable IgG but without or very limited neutralization activity (Supplementary table S4).

Natural infection induces high quality antibodies than one vaccine dose.
Then, we wanted to compare the magnitude of the humoral immune response to the natural infection versus the COVID-19 vaccine in unexposed subjects. For that, we choose samples from 25 participants
out of the 59 with the first sample collected between 12 and 39 days after the confirmed infection with SARS-CoV-2 (average 26.23 days) and from 20 unexposed participants that received the two dose of Pfizer vaccine. The samples for the unexposed subjects were collected in average 17.1 and 14.1 days after the first and the second dose respectively. As showed in figure 2A the mean time of the first sample collection after the infection was significantly higher than the first sample collected after the vaccination in the unexposed cohort (p<0.0001). Despite that, we found that the total anti-S antibodies and the IgG titers were similar after the infection or the first vaccine dose in the unexposed participants (Figures 2B and D). However, the quality of the antibodies measured by the surrogate assay showed a neutralizing activity significantly higher in the natural infected group compared with the unexposed-vaccinated group (p<0.0003) indicating a better quality of the natural-induced antibodies compared to the vaccine-induced (Figure 2E). As showed in figures 2B and C two vaccine doses were necessary to significantly increase the total antibodies and the IgG titers compared to the pre-exposed group (p<0.0004). The magnitude of neutralization was also significantly increased but more modestly than the quantity (p<0.0294) suggesting that the increase in quantity induced by the two vaccines dose was not matched with a similar increase in the quality (Figure 2D).

Neutralization is sustained in naïve and pre-exposed-vaccinated subjects

Samples were collected between 12 to 28 days after each dose with a mean of 19 days and of 14 days for the pre-exposed group and of 12 days and 26 days for the healthy groups after the first and second dose respectively. An additional third sample from all the 21 unexposed and from 8 out of the 10 pre-exposed participants was collected between 19 and 83 days after the second dose respectively (Supplementary table S2). There were not significant different in the time between samples for the first sample collected after each dose. However, there was a significant difference (p<0.0001) in the time of collection of the second samples after the second dose (third sample) between the two groups (Supplementary figure S4). The geometric mean baseline IgG titers in the pre-exposed population was 726 (in a range from 125 to 7191) and jumped to a geometric mean of 5239 (in a range from 3408 to 6586) after the first dose (Figure 3B and supplementary tables S5 and S6). After the second dose the range was from 2273 to 5847 with a mean of 3980 without significant difference with the titers after the first dose. On the other hand, the 21 healthy vaccinated persons were negative for IgG at baseline. After the first dose the IgG titers significantly increased to a geometric mean of 832 (in a range from 196 to 9365, p<0.0001) and after the second dose those values were still significantly increased (p<0.0001) to a geometric mean of 5446 (in a range from 3346 to 10,239) (Figure 3B).

In the second sample collected after the second dose (third sample) in the unexposed group the geometric mean of the titers was 1518 (range from 409 to 3278). In the pre-exposed group, the
The geometric mean of the titers was 1323 (range from 568 to 3536). In both group there was a significant drop from the values detected in the first samples after the second dose (p<0.0001 and p=0.0192 for the unexposed and pre-exposed groups respectively).

The total IgG values supported the reported IgG titers (Figure 3A). We look at the IgG1 isotype, the main contributor to the total IgG in the cohort of 59 individuals. The first dose induces a significant increase on this isotype for both groups (p<0.0018 and p<0.0001 for the naïve and pre-exposed vaccinated groups respectively). However, the boost was significantly higher in the pre-exposed group (p<0.0001) suggesting a role of the natural infection in this significant difference. Remarkable, the second dose boosted this particular isotype with a significant difference from the first dose in the naïve-vaccinated group only (p<0.0001). IgG1 values after the second dose in that group reached the values of the pre-exposed vaccinated group induced by the first dose. There were not significant differences in the levels of IgG1 between groups after the second dose (Supplementary figure 5A).

The geometric mean baseline of neutralization activity in the pre-exposed population was 69.46% (in a range from 39 to 97%) and jumped significantly (p<0.0001) to a geometric mean of 97.99% (in a range from 97 to 98%) after the first dose (Figure 3C and supplementary table S5). However, after the second dose the values remains similar in range with a mean of 97.19%. On the other hand, the 21 naïve-vaccinated persons were negative for neutralization at baseline (geometric mean 15%). After the first dose the neutralization significantly increased (p<0.0001) to a geometric mean of 57.34% (in a range from 28% to 76% with one subject with an out-layer value reaching 96%). The second dose produced an additional significant boost (p<0.0001) to a geometric mean of 96.85% (in a range from 95% to 98%) (Figure 3C). Contrary to the total antibodies and IgG titers (Figures 3A and B), the neutralizing activity was retained at very similar level in both groups in the third sample collected. The geometric mean was of 94.5% (in a range from 86% to 98%) and a geometric mean of 96.62% (in a range from 96% to 98%) for the unexposed and pre-exposed groups respectively. While there was not significant difference between groups nine (9) subjects in the unexposed group showed lower values than 0.5% of neutralization. This resulted in a drop of 1.02 times in the value of neutralization in the group while there were not changes in the pre-exposed cohort.

From all previously exposed subjects, 5 out of 10 (60%) still had detectable IgM at baseline. IgM was not boosted by the first dose and values decreased after the second dose. On the other hand, the first dose induced a significant increase (p<0.0001) in the IgM values in the naïve subjects. Those values were boosted only in two subjects, but as expected, were not modified in any of the other of 19 subjects. (Supplementary figure S5B). Eight (8) out of the 21 naïve patients (38.09%) didn’t show IgM after the first dose. Only one patient didn’t develop IgM antibodies after any of the two doses.
Finally, we look at the IgA contribution to the immune response after the vaccination. Interestingly we found that this isotype was significantly boosted in both groups, pre-exposed (p<0.0187) and in the naïve-vaccinated groups (p<0.0010) after the first dose. In addition, that increase in the IgA levels was significantly higher in the pre-exposed (p<0.0176) compared to the naïve-vaccinated group. The second boost resulted in an additional significant expansion in the naïve-vaccinated population but no in the pre-exposed group (Supplementary figure S5C).
Discussion

One limitation of our work is the limited number of subjects followed up after natural infection or after vaccination. However, due to the still limited data available on immune response to SARS-CoV-2 infection, previous reports have made great contribution with a similar number of subjects as on this study (16, 18, 19, 21). Our results contrast with a report describing a short persistence of the Nabs in plasma donors (22) but are in agreement with recent work indicating that neutralizing antibodies may persist longer (1, 5, 23). We were able to show a similar trend in our cohort with sustained neutralizing activity during the frame time of this study and highly relevant, despite of the significant decline in the IgG titers. In addition, we found some subjects with undetectable IgG (n=6) and IgG titers (n=11) while retain measurable percentage of neutralization, from 32 to 76 %, with the surrogate assay. This scenario has been described before suggesting that SARS-CoV-2 serologic assays may be less well suited for surveillance versus prediction of serum neutralization potency, which at the same time, facilitate the establishment of appropriate serologic correlates of protection against SARS-CoV-2 (24). Our results suggest that this approach should be also implemented in the context of vaccine efficacy evaluation at population level. From a technical point of view the discrepancies between samples without detectable antibodies but with neutralizing capabilities may be explained by differences in assays’ sensitivity. In our case we sue the same source of recombinant proteins for the antibodies and surrogate neutralization assays. However, the serological assays include the full S1 and S2 regions of the Spike protein encompassing the RBD coated to the plate, while the neutralization assay includes only the S1/RBD in suspension. It has been well discussed that the binding of the protein to the plate results in altered antigenic presentation with a consequent presentation of different antigenic sites compared to native stages of the proteins (11, 25-27). Nevertheless, we showed a 93.7% correlation between the IgG titers and the neutralization measured with cPass SARS-CoV-2 Neutralization Antibody Detection kit.

There are few published data on the antibody’s isotypes in response to this novel coronavirus. Before, spike- and RBD-specific IgM, IgG1, and IgA were detected in most of the subjects early after infection with all samples displaying neutralizing activity with the IgM and IgG1 contributing most to neutralization (23). Other work reported that early SARS-CoV-2-specific humoral responses were dominated by IgA antibodies and even the virus-specific antibody responses included IgG, IgM, and IgA, this last isotype contributed to virus neutralization to a greater extent compared with IgG (28). In our work we found that IgG1 was the predominant isotype while the IgA response was more limited. However, considering the non-significant changes in the IgA levels from the first to the second sample, its role in the sustained neutralization activity cannot be rule out. On the other hand, IgM showed an expected trend to decline
in the second collected sample in most of the subjects. Two out of four subjects (ID265 and ID382) which are IgG-/IgM+ also have detectable neutralizing activity with detectable IgM two and four months after the first samples was collected. These cases strongly suggest a contribution of the IgM to the neutralization, at least in some cases, as it has been described before (23). This result corresponds with a Kappa analysis suggesting a fair Cohen’s Kappa agreement between the IgM and the neutralization values. Additional isotypes-specific depletion experiments are needed to determine the specific role of these antibodies in SARS-CoV-2 neutralization. Using previous experience from our group (29, 30) those experiments are underway using a larger number of well characterized individuals.

While the number of subjects in our vaccinated cohort (naïve and previously exposed subjects) is limited, we are showing that the vaccine induces a higher boost in the magnitude of the humoral immune response, both at IgG and Nab level in the pre-exposed individuals compared to the naïve group. Our finding also shown that the second vaccine dose did not expand the anti-S antibodies, the IgG titers or the blocking antibodies beyond the peak reached after the first dose in the pre-exposed cohort. One subject receiving Moderna formulation (ID112), without know exposition to the virus, reach similar values in all three determinations as the pre-exposed subjects. However that volunteer was working in high-risk enviroment during the first months of the pandemic and an asymptomatic exposition cannot be rule out.

Two relevant findings are the fast decline of the anti-S antibodies and titers just 40 or 80 days (for unexposed or pre-exposed cohorts, respectively) after the boost, and second, a sustained level of neutralization values in the same period. This pattern is the same as the one reported above seen by following the immune response to the natural infection in 59 subjects. In addition, we observe that while in both groups the decline of the total anti-S antibodies and IgG titers were significant, the drop in those values was deeper in the uninfected group. Highly significant, considering that he time after the natural infection and the sample use as baseline before the vaccination was more than 4.7 months in average for all 10 pre-exposed subjects, is that the baseline neutralizing activity (but not the total antibodies or their titers) was significantly higher than the one induced by the first vaccine dose in the unexposed group. Our results also confirms that the antibodies generated after the natural infection or vaccination, in a similar time frame, while similar in quantity, are significantly better in their function after the natural infection. Those results suggest that a natural infection with SARS-CoV-2 may be contributing to expand a population of memory B cells producing more specific anti-S antibodies after the vaccination. Together these findings highlight the value of measuring the function over the quantity to follow up the humoral immune response to the vaccines. Our results agree with a recent work where a predictive
model of immune protection from COVID-19 found that the level of neutralizing antibodies is highly predictive of immune protection from symptomatic SARS-CoV-2 infection (17). Of interest is the role of previous natural infection changing the magnitude of the antibody’s subtypes switching. Particularly for IgA, our results shown that a previous exposition led to a faster increase in the IgA after the first dose of vaccination while naïve subjects need a second shot of the vaccine to reach same levels of those pre-exposed to the novel coronavirus.

Another critical aspect to be considered is the timing between the natural infection and a potential vaccination against COVID-19. As others, we highlighted the relevance of the time lapse between infections or immunizations to induce an optimal immune response (29, 31, 32). Taking in to account the results presented here and those from previous works (16, 19, 33), and considering the limited vaccine availability worldwide, the vaccination of people with previous history of confirmed COVID-19 may be scheduled to receive as a single shot and deferred to the final phases of the vaccination campaigns and/or to be executed not before than 6 months after the documented infection. Because the limited number of samples, we unable to identify any significant differences or trend in the humoral immune response to the Pfizer or Moderna vaccines.

We are aware of the several limitations of this work in term of limited number of participants and limited clinical data mainly. Also we understand that this work will benefice adding T cells data particularly taking into account recent results pointing out that simple serological tests for SARS-CoV-2 antibodies do not reflect the richness and durability of immune memory to SARS-CoV-2 (5). Because of that, experiments to characterize the T cells immune response from our cohorts are underway.

Nevertheless, this work provides new and additional understandings to the still limited available data on COVID-19 immune phenomenon and, to the immune response to the vaccine, comparing naïve versus pre-exposed individuals, outside of the data provided by the vaccine’s manufactures. From our results and others (16-19), it is not conclusive of the usefulness of a second vaccine dose in pre-exposed subjects as the immune response need to be evaluated including the T cells immune response as a critical component in the response to SARS-CoV-2 virus (5, 19, 34, 35). It is undoubtable that the natural infection confers a strong and high quality humoral and T cells immune response (5, 34, 36). That fact have been recently underscore by a work showing that variants of concern partially escape humoral but not T-cell responses in COVID-19 convalescent donors and vaccinees (21). As the CDC’s guidelines on the impact of the vaccination in our lifestyle (travel quarantine and testing, maskless outside and indoors) continue to be swapping it is not clear why the immunity conferred by the natural infection is not taken in to account to support those guidelines or it is counted in the target herd-immunity to go back to the new social normality. On this context our results are also highly relevant to consider
standardizing methods that in addition to serve as a tool to follow up the immune response to the vaccines may also provide a correlate of protection.

Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Authors Contribution
CAS and AME conceptualized the work and supervised the studies. PP supervised the work and performed the serologic, neutralization test and supported the figures design. CSC, TRA, AA execute the serological work. CC and GL selected samples from blood donors. JDB, AKP, CC, GL, PP contribute to the results discussion and analysis. DA, CPC, PP coordinate and supervise the cohort's management and follow up. PP and TRA organized the data for future analysis. TA provided administrative and regulatory support. JDB and AKP designed and supervised the BSL3 work. ETS performed FRNT analysis. CAS wrote the initial draft, with the other authors providing editorial comments.

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FIGURES LEGEND

Figure 1: While total antibodies decline neutralization is retained over the time. The threshold for the total antibodies was 0.312. The threshold for IgG titers was 1:100 and for the blocking activity was 30%. Statistical significance was determined by 2way ANOVA multiple comparisons was used to test for increase or decrease among samples. P<0.05 was considered significant.
Figure 2: Natural immune response to SARS-CoV-2 is superior to a single COVID-19 vaccine dose.

Panel A shows the meant time of sample collection after the natural infection (n=25) or after the first vaccine dose (n=20). In panel B and C results from the total anti-Spike protein and the IgG titer measured by Enzyme-linked immunosorbent assay and expressed as OD or titers respectively are presented. The threshold for the total antibodies was 0.312 and the threshold for IgG titers was 1:100. All participants, except one, with previous exposition to SARS-CoV-2 virus showed detectable antibodies and measurable titers at baseline. Because the threshold 1:100 of our titration assay, the IgG titers at baseline in the unexposed subjects, which had not detectable antibodies, were set arbitrarily to 50. Panel D blocking activity of the antibodies is expressed as percentage of neutralization by using a surrogate viral neutralization test (sVNT). The cutoff for this assay was 30%. As it is shown, the only sample in the pre-exposed group with antibodies below the threshold was reported as having more than 30% of neutralization. Also, while the antibodies and titers distribution cover the full Y axe values in both panel B and C, in panel D same samples are grouped on the top values area. Those two observations indicate a better reliability of the sVNT assay to follow up the immune response to natural infection and the vaccination. 2way ANOVA multiple comparisons was used to test for increase or decrease among samples. P<0.05 was considered significant.
Figure 3: Function is a better predictor of the humoral immune response to COVID-19 vaccine. Samples are described as 1st or 2nd samples after 1st or 2nd vaccine dose (1S-P1st-vd, 1S-P2nd-vd or 2S-P2nd-vd) and the mean time of samples collection is showed. In panel A and B we are showing the total antibodies and IgG titers respectively after full vaccination with two vaccine doses. Results shown that one vaccine dose in pre-exposed individuals is enough to reach similar immune response than in unexposed individuals after two doses. Antibodies levels and titers significantly decay in both groups in a second samples collected after the second vaccine (average of 60.3 and 100.5 days after the first vaccine dose for the unexposed and pre-exposed groups respectively). Despite the time difference in the samples collection between the two groups there were not significant differences in the levels of antibodies or titers between groups in the 2S-P2nd-vd. Panel C shows the antibodies’ blocking capabilities measured by a surrogate viral neutralization assay (sVNT). Highly relevant is the finding that the blocking baseline activity of the pre-exposed individuals is significantly higher than the one induced by the first dose in unexposed individuals. In addition, two vaccine doses were necessary in the naïve cohort to induce same percentage of neutralization induced by the first dose in the pre-exposed group. The magnitude of the neutralization remains at similar higher levels until the last time point evaluated in both groups confirming that the surrogate neutralization test is more suitable to determine the value of the immune response to the vaccine. The threshold for the total antibodies was 0.312. The threshold for IgG titers was 1:100 and for the blocking activity was 30%. Statistical significance was determined by 2way ANOVA multiple comparisons to test for increase or decrease among samples. \( p < 0.05 \) was considered significant. The black arrows indicate the moment of vaccine administration related to the samples.
Supplementary figure S1: Subclass and antibodies isotypes in a longitudinal cohort of 59 volunteers exposed to SARS-CoV-2. Panel A shows the significant decrease in the total anti-S antibodies in a second samples collected in average 67.8 days after the first sample (average 108 days after the confirmed infection). A third samples was collected from some of the participants (n=12) in average 99.5 days after the second sample (in average 207 days after the infection. Two different patterns in the evolution of the antibodies were identified with 74.5% of samples showing a decrease in the values from the first to the second samples (Panel B) and a 25.4% of samples with similar o increased values related to the first sample (Panel C). Panels D to G shows the result of different tested subclass with IgG1 being the predominant. Panels H and I shows the results for IgM and IgA subclasses. Statistical significance was determined by 2way ANOVA multiple comparisons to test for increase or decrease among samples. p<0.05 was considered significant.
Supplementary figure S2: Time of samples collection in groups with increased or decreased values. There were not significant differences in the time from diagnostic (Dx) to the first sample collection or between the first and the second samples collection in both groups. Statistical significance was determined by 2way ANOVA multiple comparisons was used. p<0.05 was considered significant.
Supplementary figure S3: IgG but not IgM or IgA correlates with neutralization. Panel A shows the correlation between the neutralization measured with the surrogate viral neutralization test (sVNT) and the IgG titers confirming a moderate agreement. Panel B also shown a moderate agreement between the sVNT and a Focus Reduction Neutralization Test (FRNT) using the whole virus. Panel C and D shows a fair and a slight agreement between the neutralization activity and the IgM and IgA respectively.
Supplementary figure S4: Time of samples collection after vaccination. The time between the first and second samples after the 1\textsuperscript{st} or the 2\textsuperscript{nd} vaccine dose (1S-P1st-vd, 1S-P2st-vd) were similar in both groups (pre-exposed and unexposed). However, the time of collection of the third sample (2S-P2nd-vd) was significant longer for the pore-exposed group compared with the unexposed cohort. Statistical significance was determined by 2way ANOVA multiple comparisons was used. $p<0.05$ was considered significant.
Figure 1
Figure 2
Figure 3
Supplementary figure 1
Supplementary figure 2
Supplementary figure 3
Supplementary figure S4