



# Treatment with integrase inhibitors alters SARS-CoV-2 neutralization levels measured with HIV-based pseudotypes in people living with HIV

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## Abstract

The presence of neutralizing antibodies (NAbs) is a major correlate of protection for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Thus, different in vitro pseudoviruses-based assays have been described to detect NAbs against SARS-CoV-2. However, the determination of NAbs against SARS-CoV-2 in people living with HIV (PLWH) through HIV-based pseudoparticles could be influenced by cross-neutralization activity or treatment, impeding accurate titration of NAbs. Two assays were compared using replication-defective HIV or VSV-based particles pseudotyped with SARS-CoV-2 spike to measure NAbs in COVID-19-recovered and COVID-19-naïve PLWH. The assay based on HIV-pseudoparticles displayed neutralization activity in all COVID-19-recovered PLWH with a median neutralizing titer 50 (NT50) of 1417.0 (interquartile range [IQR]: 450.3–3284.0), but also in 67% of COVID-19-naïve PLWH (NT50: 631.5, IQR: 16.0–1535.0). Regarding VSV-pseudoparticles system, no neutralization was observed in COVID-19-naïve PLWH as expected, whereas in comparison with HIV-pseudoparticles assay lower neutralization titers were measured in 75% COVID-19-recovered PLWH (NT50: 100.5; IQR: 20.5–1353.0). Treatment with integrase inhibitors was associated with inaccurate increase in neutralization titers when HIV-based pseudoparticles were used. IgG purification and consequent elimination of drugs from samples avoided the interference with retroviral cycle and corrected the lack of specificity observed in HIV-pseudotyped assay. This study shows methodological alternatives based on pseudoviruses systems to determine specific SARS-CoV-2 neutralization titers in PLWH.

## KEYWORDS

HIV-1, neutralization, pseudoviruses, SARS-CoV-2

Erick De La Torre-Tarazona and Alba González-Robles contributed equally to this work and are considered as first authors.

Hospital Clinic HIV Investigators are listed in the Supporting Information: appendix.

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## 1 | INTRODUCTION

The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in China in late 2019 and its subsequent spread to the rest of the world has created a pandemic situation unprecedented in the last decades. At the end of 2022 more than 620 million infections with SARS-CoV-2 and more than 6.5 million deaths have been reported.<sup>1</sup> During a SARS-CoV-2 infection or after SARS-CoV-2 vaccination, patients develop adaptive immune responses against the virus, which generates neutralizing antibodies (NAbs) predominantly directed against spike (S) protein of SARS-CoV-2, interfering with the viral entry into target cells.<sup>2,3</sup>

One of the research efforts are directed toward the measurement of NAbs in COVID-19 convalescent and vaccinated individuals, and establish the kinetics of their persistence in serum. Thus, the determination of NAbs titers should be a significant tool in clinical strategies as predictive of immune protection, transfusion of hyperimmune sera for therapeutic intervention, or correlation with vaccines effectiveness to new emerging SARS-CoV-2 variants.<sup>4-6</sup>

Due to the highly transmissible and pathogenic nature of SARS-CoV-2, handling of live virus requires a biosafety level 3 (BSL3) containment. Pseudovirus-based systems have been extensively used to evaluate the neutralizing capacity of plasma/serum, because it is a robust and accurate safe system that can be used at BSL-2 laboratories. For this purpose, different viral particles pseudotyped with S protein of SARS-CoV-2 have been developed, which can be easily produced and successfully employed to screen NAbs in serum/plasma from patients.<sup>7,8</sup>

On the other hand, people living with HIV (PLWH) could be considered as a high risk population for SARS-CoV-2 infection, because they have higher risk to develop respiratory failure or death, in particular in those patients with an immunocompromised status.<sup>9,10</sup> In this context, it is important to assess in a precise manner the titers of NAbs against the different SARS-CoV-2 variants because represent the main surrogate parameter of protection against infection and severe disease. Few works include HIV-infected patients in the assessment of NAbs using pseudotyped viruses. It has been described that anti-HIV antibodies in sera/plasma from HIV-infected people could cross-react with SARS-CoV-2 antigens or HIV-based SARS-CoV-2 pseudoparticles.<sup>11</sup> Moreover, the effect of specific drugs in altering HIV-based pseudoviruses life cycle is unknown and could bias neutralization results in PLWH on antiretroviral therapy (ART), overestimating the neutralizing titers against SARS-CoV-2. Therefore, the estimation of NAbs against SARS-CoV-2 could be not correctly determined in these individuals.

This work propose methodological alternatives to test SARS-CoV-2 NAbs in samples from PLWH, with high specificity and avoiding nonspecific neutralization. We have compared two models of pseudotyped particles, HIV and VSV vectors genome encoding renilla or firefly luciferase, respectively, pseudotyped with the S glycoprotein of SARS-CoV-2.

## 2 | MATERIALS AND METHODS

### 2.1 | Samples

We analyzed 48 samples (serum or plasma) provided by HIV Unit at Hospital Clinic (Barcelona, Spain). Twenty-four samples were from PLWH who had not been infected with SARS-CoV-2 (COVID-19 naïve) and 24 samples were from PLWH who had been infected with SARS-CoV-2 (COVID-19 recovered). SARS-CoV-2 infection was detected by qPCR. Naïve patients were defined by the absence of symptoms compatible with COVID-19 and negative serology against SARS-CoV-2. All individuals were receiving ART. No differences in clinical characteristics, as time on ART and CD4 cells count, among naïve and recovered COVID-19 individuals were observed (Supporting Information: Table 1). The study was approved by the local ethics committee of Hospital Clinic and informed consent was obtained from all of the PLWH serum or plasma donors before blood sampling.

### 2.2 | Cell lines and generation of pseudotyped viruses

HEK-293T (National Institute for Biological Standards and Control) and VeroE6 (provided by Dr. A. Alcamí, CBMSO) cells were cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine and 100 units/mL penicillin and streptomycin (Lonza).

The spike of the SARS-CoV-2 virus was generated (GeneArt Gene Synthesis; Thermo Fisher Scientific) from the codon-optimized sequence previously reported<sup>12</sup> and inserted into pcDNA3.1D/V5-His-TOPO (pcDNA3.1-S-COV-2Δ19-G614). This plasmid contains D614G mutation and a deletion of the last 19 aa from the original spike.

HIV pseudoviruses carrying renilla-luciferase reporter were prepared by cotransfection of HEK-293T cells with pNL4-3ΔenvRen backbone<sup>13</sup> and pcDNA3.1-S-COV-2Δ19-G614 (HIV\_SARS-CoV-2) or pcDNA-VSV-G (HIV\_VSV-G), using the calcium phosphate method. Forty-eight hours posttransfection, cell culture supernatants were collected and p24 antigen was titrated by electrochemiluminescence Immunoassay (Roche Diagnostic).

Pseudovirus based on the VSV system was generated as previously described<sup>14</sup> with some modifications. Briefly, 293T cells were transfected with plasmid encoding the SARS-CoV-2 spike. Next day, cells were infected with a VSV-G-Luc virus (MOI = 1) (generated from a lentiviral backbone plasmid that uses a VSV promoter to express firefly-luciferase) for 2 h and gently washed with PBS1X. Cells were incubated overnight in DMEM supplemented with 10% FBS and 10% of the I1 hybridoma supernatant (ATCC CRL-2700) to neutralize contaminating VSV-G particles. Next day, the resulting viral particles (VSV\_SARS-CoV-2) were collected and titrated in VeroE6 cells by enzyme luminescence assay<sup>15</sup> (Supporting Information: Figure 1A).

## 2.3 | IgG purification

IgG were extracted from selected samples using a protein A affinity chromatography method following manufacturer's instructions (Protein A HP SpinTrap™; Cytiva). Briefly, 100 μL of heat-inactivated serum mixed with 500 μL of sterile binding buffer (20 mM sodium phosphate, pH 7.0) were added to the protein A Spin columns and incubated for 30 min at room temperature in agitation. Then, spin columns were washed three times with 600 μL of binding buffer, and finally were recovered with 200 μL of elution buffer (0.1 M glycine-HCl, pH 2.7). The IgG purification was confirmed and quantified using a Nanodrop One spectrophotometer (Thermo Fisher Scientific). A proportional volume to the starting samples were used in the neutralization assays.

## 2.4 | Neutralization assays

Neutralization assays were performed realizing fourfold serial dilutions of heat-inactivated sera or purified IgG (1:32-1:131072), which were preincubated with pseudoviruses (~10 ng p24 Gag/well of HIV\_SARS-CoV-2 or 0.1 MOI of VSV\_SARS-CoV-2) for 1 h at 37°C and subsequently VeroE6 cells were added. At 24–48 h postinfection (depending of VSV or HIV pseudovirus, respectively) cells were lysed and viral infectivity was assessed by measuring luciferase activity (Britelite plus kit; PerkinElmer or Renilla Luciferase Assay; Promega) using a 96-well plate luminometer (Supporting Information: Figure 1B). The titers of NABs were calculated as neutralizing titer 50 (NT50), expressed as the highest dilution of serum which resulted in a 50% reduction of luminescence activity compared to control without serum. Pseudoviruses expressing VSV-G protein instead of the SARS-CoV-2 spike were used as a specificity control virus in neutralization testing in both HIV- and VSV-based pseudovirus systems.

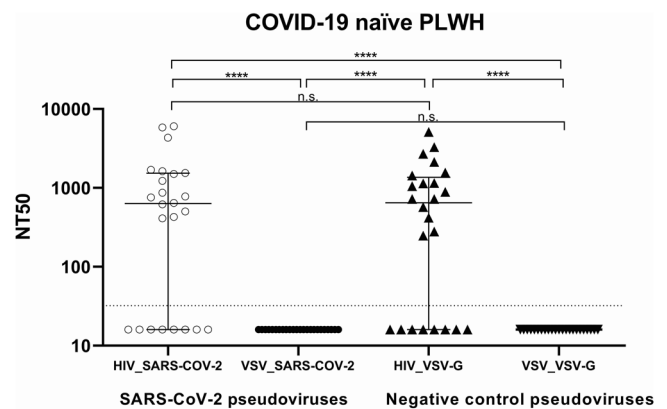
## 2.5 | Statistical analysis

Sigmoid curves and NT50s were calculated by nonlinear regression (GraphPad Software, Inc.). Comparative statistical analyses were performed with the Wilcoxon or Mann-Whitney tests (GraphPad Software, Inc.).

# 3 | RESULTS

## 3.1 | Neutralization activity of COVID-19 naïve HIV-positive individuals

We analyzed 24 HIV-positive patients (receiving ART), which had not been infected with SARS-CoV-2 (COVID-19 naïve). First, we analyzed neutralization activity using the HIV particles pseudotyped with the SARS-CoV-2 Spike (HIV\_SARS-CoV-2) or VSV-G envelope as negative control (HIV\_VSV-G). We observed a high proportion of



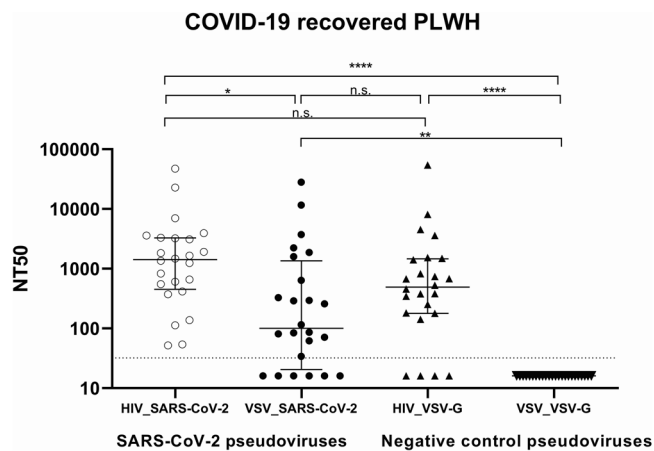
**FIGURE 1** Neutralization titers against SARS-CoV-2 spike and VSV-G envelope pseudotyped viruses in COVID-19 naïve HIV-infected individuals. Statistical analysis: Wilcoxon test. n.s., not significant; \*\*\*\* $p < 0.0001$ . Serum samples from COVID-19 naïve HIV-infected individuals were preincubated with pseudoviruses at 37°C for 1 h. Sera and virus mixture were then incubated with VeroE6 cells for 24–48 h. Luciferase was measured to assess infection. NT50 was summarized as median and interquartile range. NT50, neutralizing titer 50; PLWH, people living with HIV; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

these individuals (16 out of 24, 67%) displaying neutralizing activity against both HIV pseudoparticles. NT50 medians were 632 (interquartile range [IQR]: 16–1535) and 647 (IQR: 16–1365) for HIV\_SARS-CoV-2 and HIV\_VSV-G, respectively (Figure 1). After analyzing the same 24 samples with VSV-system, no neutralization activity (NT50 < 32) was detected against VSV-based pseudoparticles expressing the S protein of SARS-CoV-2 (VSV\_SARS-CoV-2) or the G envelope glycoprotein of VSV (HIV\_VSV-G) (Figure 1). Overall, these results point to nonspecific neutralization of pseudotyped particles based on HIV-core in a large proportion of sera from PLWH.

## 3.2 | Neutralization activity of COVID-19 recovered HIV-positive individuals

We analyzed 24 HIV-positive individuals previously infected with SARS-CoV-2 (COVID-19 recovered). First, we observed neutralization activity against HIV\_SARS-CoV-2 in all samples. Also, nonspecific neutralization against negative control (HIV\_VSV-G) (NT50 > 32) was detected in 20 out of 24 sera (83.3%) (Figure 2). NT50 medians against HIV\_SARS-CoV-2 and negative control HIV\_VSV-G were 1417 (IQR: 450.25–3284.25) and 491 (IQR: 178.75–1465.5), respectively.

When neutralization assays were performed with VSV-based pseudoviruses, 18 individuals (75%) displayed neutralization activity against VSV\_SARS-CoV-2 pseudovirus and NT50 median was 100.5 (IQR: 20.5–1353.3). Also, none of these individuals showed neutralization activity against the negative control VSV\_VSV-G (NT50 < 32) (Figure 2). This last indicates that in contrast with HIV-based pseudotypes, VSV pseudoviruses system is able to determine in a



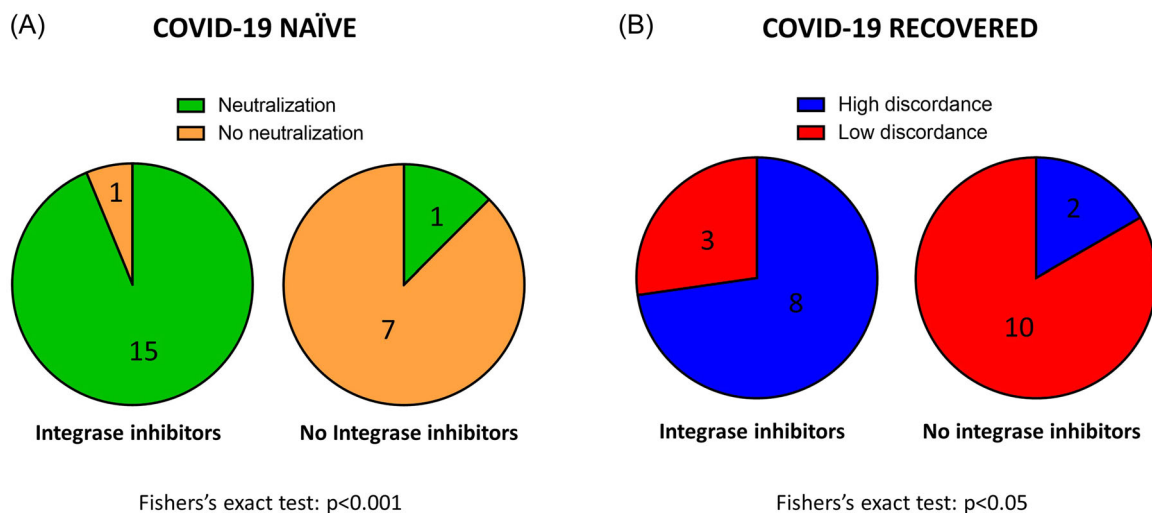
**FIGURE 2** Neutralization titers against SARS-CoV-2 spike and VSV-G envelope pseudotyped viruses in COVID-19 recovered HIV-infected individuals. Statistical analysis: Wilcoxon test. n.s., not significant; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ . Serum samples from COVID-19 recovered HIV-infected individuals were preincubated with pseudoviruses at 37°C for 1 h. Sera and virus mixture were then incubated with VeroE6 cells for 24–48 h. Luciferase was measured to assess infection. NT50 was summarized as median and interquartile range. NT50, neutralizing titer 50; PLWH, people living with HIV; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

highly specific manner anti-SARS-CoV-2 neutralizing activity in PLWH.

### 3.3 | Mechanism of nonspecific neutralization of HIV-based SARS-CoV-2 pseudoviruses

We hypothesized that ART could be altering neutralization titers against HIV pseudoviruses, since drugs present in the sample of patients could inhibit the viral particles that contain the replicative machinery of HIV. First, we analyzed the association between ART regimen and neutralization of HIV pseudoparticles in COVID-19 naïve individuals. We observed that ART containing any integrase inhibitor was associated with nonspecific neutralization of HIV pseudoparticles (Fisher's exact test,  $p < 0.001$ , Figure 3A). Similarly, we observed that the high discordances between neutralization titers against both SARS-CoV-2 pseudoviruses systems (NT50 HIV\_SARS-CoV-2:NT50 VSV\_SARS-CoV-2 ratio  $>5$ ) were more frequently observed in those COVID-19 recovered individuals receiving integrase inhibitors (Fisher's exact test,  $p < 0.05$ , Figure 3B; Supporting Information: Table 2).

To counteract the effect of ART against HIV pseudoparticles, we performed IgG purification from PLWH samples. In those COVID-19

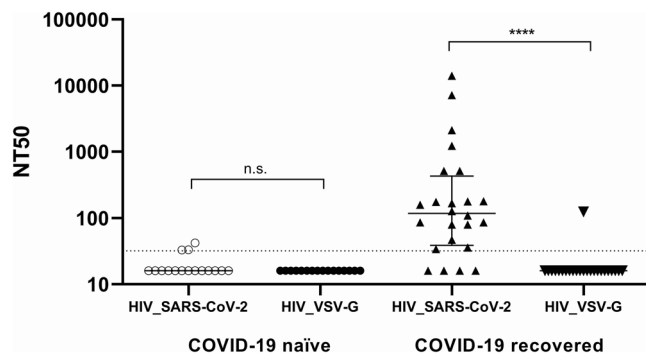


**FIGURE 3** Association between neutralization activity against SARS-CoV-2 pseudoviruses and ART regimen in PLWH. (A) COVID-19 naïve individuals. Neutralization is considered when NT50  $> 32$  against HIV\_SARS-CoV-2 and HIV\_VSV-G pseudoviruses. Fisher's exact test indicates an association between ART containing integrase inhibitors (INTI) with neutralization activity against HIV pseudoviruses ( $p < 0.001$ ). (B) COVID-19 recovered individuals. High discordance indicates a NT50 HIV\_SARS-CoV-2: NT50 VSV\_SARS-CoV-2 ratio  $>5$ . Fisher's exact test indicates an association between ART containing INTI with higher discordances between NT50 values to HIV\_SARS-CoV-2 and VSV\_SARS-CoV-2 pseudoviruses ( $p < 0.05$ ). ART regimens included nucleotide/nucleoside reverse transcriptase inhibitors in all patients. ART-containing INTI included one of the following compounds: Dolutegravir, Bictegravir, or Elvitegravir. In COVID-19 naïve individuals with ART not including INTI, 88% (7 of 8) individuals were treated with ritonavir/cobicistat-boosted-protease inhibitors regimens (PI/r/c) and 12% (1 of 8) with non-nucleoside reverse transcriptase inhibitors. In COVID-19 recovered individuals not treated with INTI, 33% (4 of 12) were treated with PI/r/c and 67% (8 of 12) with NNRTIs. ART, antiretroviral therapy; NT50, neutralizing titer 50; PLWH, people living with HIV; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

naïve individuals that displayed neutralization activity against HIV-based pseudoviruses, we observed NT50 values lower than 45 (close to the threshold detection level of 32) against HIV\_SARS-CoV-2 in all samples (no neutralization in 13 of 16 samples) and absence of neutralization against the negative control HIV\_VSV-G in all samples (Figure 4). Likewise, in COVID-19 recovered individuals, we observed a decrease of NT50 against HIV\_SARS-CoV-2 pseudovirus (median of 118, IQR: 39–428). These results were very similar and approach results obtained previously using the VSV\_SARS-CoV-2 system (median 100.5; IQR: 20–1353) ( $p = 0.88$ ). Also, we observed that almost all samples (23/24) did not display nonspecific neutralization

activity against HIV\_VSV-G (Figure 4). Therefore, previous IgG purification provides more reliable neutralization values of a specific response against SARS-CoV-2 when a pseudotyped system based on HIV particles was used to evaluate samples from PLWH.

Additionally, we observed high discordances of NT50 values to both SARS-CoV-2 pseudoviruses in COVID-19 recovered individuals receiving integrase inhibitors before IgG purification (Figure 5A; Supporting Information: Table 2). However, after IgG purification no differences were observed among individuals receiving both ART regimens (Figure 5B; Supporting Information: Table 3). Likewise, a better correlation between NT50 values against VSV\_SARS-CoV-2 and HIV\_SARS-CoV-2 pseudovirus was noted after IgG purification (Figure 6).

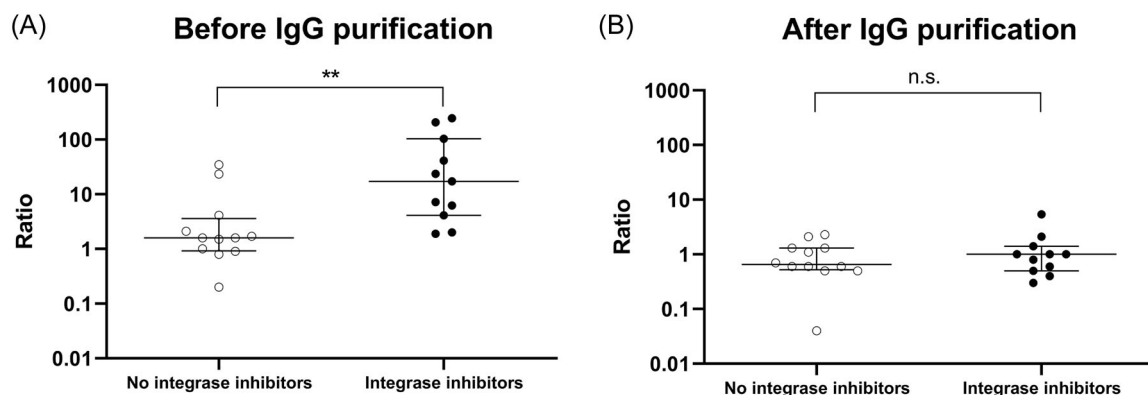


**FIGURE 4** Neutralization titers against HIV pseudoviruses on PLWH after IgG purification. Serum samples from PLWH were preincubated with pseudoviruses at 37°C for 1 h. Sera and virus mixture were then incubated with VeroE6 cells for 48 h. Luciferase was measured to assess infection. Statistical analysis: Wilcoxon test. n.s., not significant; \*\*\*\* $p < 0.0001$ . NT50 was summarized as median and interquartile range. LW, people living with HIV; NT50, neutralizing titer 50; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

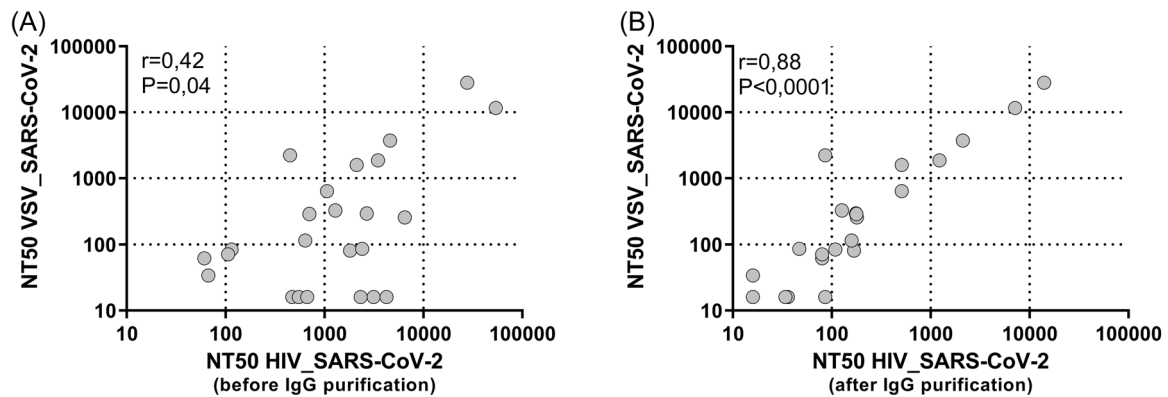
## 4 | DISCUSSION

We have compared assays using replication defective VSV or HIV particles pseudotyped with spike protein of SARS-CoV-2 to measure NABs in HIV-infected individuals. Previously, systems based on SARS-CoV-2 spike/lentiviral pseudovirions have been assessed to measure neutralizing activity in serum or plasma from SARS-CoV-2 infected patients.<sup>16–18</sup> Likewise, VSV with SARS-CoV-2 spike protein expressing luciferase has shown high specificity and sensitivity for evaluating the occurrence of NABs against SARS-CoV-2.<sup>19</sup> A previous work that compared VSV and LV systems using GFP as a reporter, observed that they present a high correlation and reproducibility evaluating the same samples from SARS-CoV-2 convalescent donors. Also, NABs titers obtained with these systems correlated well with immunoassays for binding antibodies (IgA and IgG).<sup>20</sup> However, samples from PLWH are not included usually in these assays. In this work we have compared both HIV and VSV pseudoparticles-based

## COVID-19 RECOVERED INDIVIDUALS



**FIGURE 5** Comparison of discordances among both SARS-CoV-2 pseudoviruses depending of ART regimen in COVID-19 recovered HIV-infected individuals, before (A) and after (B) IgG purification. Ratio among SARS-CoV-2 pseudoviruses: NT50 HIV\_SARS-CoV-2/NT50 VSV\_SARS-CoV-2. ART-containing integrase inhibitors, included one of the following compounds: Dolutegravir, Bictegravir, or Elvitegravir, provided in certain anti-HIV cocktail drugs. Mann–Whitney test: \*\* $p < 0.01$ ; n.s., not significant. Ratio was summarized as median and interquartile range. ART, antiretroviral therapy; NT50, neutralizing titer 50; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.



**FIGURE 6** Correlation between neutralizing titers against SARS-CoV-2 spike pseudotyped viruses in COVID-19 recovered HIV infected individuals. (A) NT50 values against HIV\_SARS-COV-2 before IgG purification. (B) NT50 values against HIV\_SARS-CoV-2 after IgG purification. Correlation analysis: Spearman's rank correlation coefficient. NT50, neutralizing titer 50; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

systems in samples from PLWH, analyzing also the factors involved in nonspecific neutralization against lentiviral pseudoparticles displayed by these individuals.

Neutralization activity observed in COVID-19 naïve individuals tested by the HIV-based pseudoviruses assay (Figure 1) would represent an artifact, due that these individuals have not been reported as SARS-CoV-2 positive and therefore they would not have developed antibodies against this virus. In general population, only a very low proportion (<0.5%) of COVID-19 recovered or vaccinated individuals neutralize unspecifically HIV\_VSV-G pseudovirus assay in previous analysis by our group,<sup>21,22</sup> indicating that the estimation of specific NABs against SARS-CoV-2 could be affected in PLWH by nonspecific neutralization of HIV\_SARS-CoV-2 pseudovirus.

Although anti-HIV antibodies (directed to *gag* or *env*-encoding proteins) generated during HIV infection are present in serum/plasma from PLWH, it is unlikely that they could influence the estimation of anti-SARS-CoV-2 NABs, because they would not interfere between SARS-CoV-2 and ACE-2 binding. However, in some cases the cross-neutralizing activity against SARS-CoV-2 can be observed in ART-naïve chronically HIV-1 infected people, possibly by the presence of HIV broadly neutralizing antibodies (bNABs) that cross-react with heavily glycosylated S protein of SARS-CoV-2.<sup>11</sup> In our work, the samples from COVID-19 naïve PLWH do not display this effect mediated by bNABs, because none of them was able to inhibit VSV\_SARS-CoV-2-based pseudovirus (Figure 1). Moreover, the median of NT50 values against VSV-SARS-CoV-2 observed in COVID-19 recovered PLWH appear to truly indicate the specific SARS-CoV-2 neutralization in contrast to the overestimated values observed with the HIV-based system (around 14-fold difference). The reason explaining this overestimation is the lack of specificity associated to the HIV-based method in samples from PLWH on ART, as shown by the capacity to neutralize HIV pseudoviruses expressing the G protein of VSV that are the negative control of the technique (Figures 1 and 2).

We have observed that ART regimen was associated with a nonspecific neutralization of HIV pseudoviruses, since these drugs

could inhibit the viral particles that contain the replicative machinery of HIV (Supporting Information: Figure 2). This fact has been previously described by other authors in a work that measured the neutralization levels in COVID-19 patients using a lentiviral-based SARS-CoV-2 pseudovirus assay. Individuals taking ART or preexposure prophylaxis were excluded from this study because of the potent inhibition observed against pseudovirus infection in this model.<sup>17</sup> Our analysis shows that the inhibition of viral integration could be key to interfere with the luminescence activity of HIV pseudoviruses. The presence of nonspecific neutralization against HIV pseudoviruses in most COVID-19 naïve individuals (15/16) receiving integrase inhibitors on ART (Figure 3A) support our hypothesis, as well as the fact that higher discordances among NT50 of both SARS-CoV-2 pseudoviruses systems were more frequently observed in those COVID-19 recovered individuals treated with integrase inhibitors (Figure 3B). Nucleoside/nucleotide reverse transcriptase inhibitors, which are administered in the most of PLWH, are taken as prodrugs and must be taken into the host cell and phosphorylated before they become active.<sup>23</sup> Cell lines used in these neutralization systems present a high activation state (with higher dNTPs levels) and possibly the concentration of these drugs in plasma/sera or the time of incubation employed during the experiment are not enough to neutralize HIV pseudoviruses by themselves. However, integrase inhibitors are active drugs and could neutralize more efficiently HIV pseudoparticles, as shown by a lower (around fivefold) IC50 of Raltegravir (13.9 nM) compared to Zidovudine (70.2 nM) against HIV\_SARS-CoV-2 (Supporting Information: Figure 2).

To counteract nonspecific neutralization against HIV pseudoviruses, we performed IgG isolation from PLWH samples. A previous work purified IgG from sera of HIV-infected individuals to avoid the ART effect to measure neutralization against HIV-1 pseudoviral particles.<sup>24</sup> After IgG isolation from PLWH samples, we observed: absence of neutralization against HIV pseudoviruses in almost all COVID-19 naïve individuals which showed previous neutralizing activity (Figure 4); lower NT50 values against HIV\_SARS-CoV-2, similar to that obtained for

VSV\_SARS-CoV-2 (Figures 5 and 6B); and no neutralization against the negative control HIV\_VSV-G in COVID-19 recovered individuals (Figure 4; Supporting Information: Table 3).

Although it has been reported that humoral response is comparable between HIV-positive and negative subjects, some studies suggest that an inadequate immune reconstitution in PLWH on ART could hinder immune responses to SARS-CoV-2.<sup>25</sup> For this reason, it is mandatory to obtain a reliable method to measure the neutralizing activity against SARS-CoV-2 pseudoviruses in PLWH avoiding the nonspecific neutralization associated to lentiviral-based methods. Thus, in the present study we propose methodological alternatives that could be useful to follow-up the dynamic of NAbS after vaccination or COVID-19, as well as the evaluation of the immune response against the new emergent SARS-CoV-2 variants.

In summary, we recommend SARS-CoV-2 pseudovirus assays with VSV backbone to measure NAbS against SARS-CoV-2 in PLWH on ART, as in this system nonspecific-neutralization activity due to ART is not observed and is highly specific in the assessment of neutralizing activity in these patients. Alternatively, a lentiviral-based pseudovirus system could be used if IgG are purified from serum/plasma of these individuals. Finally, the inclusion of a negative control such as a lentiviral pseudovirus expressing the G envelope glycoprotein of the VSV is mandatory to confirm the specificity of the method.

## AUTHOR CONTRIBUTIONS

**Conceptualization:** José Alcamí and Javier García-Pérez. **Methodology:** Erick De La Torre-Tarazona, José Alcamí, Javier García-Pérez, María José Buzón, and Alba González-Robles. **Investigation:** Erick De La Torre-Tarazona, Almudena Cascajero, Paloma Jiménez, Javier García-Pérez, Alba González-Robles, María José Buzón, José María Miró, and Sonsoles Sánchez-Palomino. **Formal analysis:** Erick De La Torre-Tarazona, Javier García-Pérez, and José Alcamí. **Writing—original draft preparation:** Erick De La Torre-Tarazona. **Writing—review and editing:** Erick De La Torre-Tarazona, Javier García-Pérez, José Alcamí, and María José Buzón. All authors have read and agreed to the published version of the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

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