Antibody-mediated procoagulant platelets in SARS-CoV-2-vaccination associated immune thrombotic thrombocytopenia

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Supplemental material

Methods:

Testing for anti-PF4/heparin antibodies

A commercially available IgG-Enzym Immune assay (EIA) was used in accordance to manufacturer's instructions (Hyphen Biomed, Neuville-sur-Oise, France). Per manufacturer's recommendation, a sample was considered reactive if the optical density (OD) was greater than 0.500. The ability of sera to activate platelets was tested using the functional assay Heparin induced platelet aggregation assay (HIPA). In brief, serum was tested with washed platelets from four different healthy donors in the absence (buffer alone) or in the presence of heparin (0.2 IU/mL and 100 IU/mL). In addition, platelets were preincubated with PF4 (50µg/mL), Spike protein (50 µg/mL), a mixture of PF4 and Spike Receptor Binding Domain (RBD) (50µg/mL and 0.82 µg/mL, respectively) or vaccine (1:750). To verify the charge dependency, these conditions were also repeated in the presence of high concentration of heparin (100 IU/mL UFH). Reactions were placed in microtiter wells containing spherical stir bars and stirred at approximately 500 revolutions per minute (rpm). Wells were examined optically at five-minutes interval for loss of turbidity. A serum was considered reactive (positive) if a shift from turbidity to transparency occurred within 30 min in at least two platelet suspensions. Observation time was 45 min. Each test included a diluted serum from a patient with heparin induced thrombocytopenia (HIT) as a weak positive control, collagen (5µg/mL) as strong positive control and a serum from a healthy donor as a negative control.

Serological characterization of PF4-antibodies

Patient sera were tested for anti-PF4/RBD antibodies using an in-house PF4/RBD enzyme-linked immunosorbent assay (EIA). In brief, PF4 (25 µg/ml) was incubated with or without varying concentrations of RBD in carbonate coating buffer (0.05 M NaH2PO4, 0.1 % NaN3) at 4 °C, overnight in a 96 well microtitre plate (Nunc MaxiSorp™, Langenselbold, Germany). Then, plates were washed three times with PBS/Tween buffer (0.05 % Tween 20 in PBS) before blocking with 3% BSA for 2h, at RT or overnight at 4 °C. Plates were again washed three times and incubated (RT, 1 h) with patient sera (diluted 1:50 in PBS/Tween buffer). After further washing three times, plates were incubated (RT, 1 h) with 1:1000 diluted peroxidase-conjugated anti-human IgG (Jackson ImmunoResearch Laboratories Inc, USA). After five times washing, plates were incubated (RT, 6 minutes [min], dark) with substrate tetramethylbenzidine (TMB one, Kementec, Denmark). The reaction was stopped with H_2SO_4 and the absorbance was measured at 450 nm with 620 nm as a reference.

Assessment of antibody-mediated procoagulant platelets

To exclude unspecific effects like the activation of platelets via complement or non-specific immune complexes, all sera were heat-inactivated (56°C for 30 min*), followed by a sharp centrifugation step at 5,000g. The supernatant was collected. All experiments involving patients' sera were performed after incubation of 5 µL serum with 25 µL washed platelets ($7.5x10^6$) for 1.5 h* under rotating conditions at RT. When indicated, cell susspenions were preincubated with PF4 (25 µg/ml), Spike protein (0-100 µg/mL) or vaccine (1:75, V:V). Afterwards, samples were washed once (7 min*, 650g, RT, without brake) and gently resuspended in 75 µL of phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). Platelets were then stained with Annexin V-FITC and CD62-APC (Immunotools, Friesoythe Germany) and directly analyzed by flow cytometry (FC). As positive control, washed platelets were incubated with ionomycin (5µM, 15 min at RT) and TRAP-6 (10 µM, 30 min at RT). Test results were determined as fold increase of the percentage of double PS/CD62p positive

events in platelets upon incubation with patients' sera compared to cells incubated with healthy donors tested in parallel.

Results

Response to SARS-CoV-2 vaccination

We compared three groups with antibody response to COVID-19 vaccination or infection (Supplemental Figure 2 A-C). All VITT patients presented beginning immune response to the vaccine in IgG and IgA (Supplemental Figure 2A). Nevertheless case #2 was negative with all antigens. 6/8 patients presented antibodies against the Spike Trimer. For IgG only 4/8 patients presented antibodies against RBD and S1 domain. Most of the patients (6/8) presented antibodies against the S2 domain. No antibodies were detected against the nucleocapsid, so previous infection with SARS-CoV2 can be excluded. For IgA 5/8 patients presented antibodies against the Spike Trimer, 3/8 presented antibodies against the RBD and 4/8 against the S1 domain. Only case #4 presented IgA antibodies against the S2 domain and no antibodies were detected against the nucleocapsid structure. Immune response in IgG was not different compared to the vaccinated volunteers (Spike, p=0.478; RBD, p=0.215; S1, p=0.247; S2, p=0.639; Nucleocapsid, p=0.339). Compared to COVID-19 patients from ICU IgG antibodies against the Spike protein and S1 domain were not significantly different (Spike, p=0.0753; S1, p=0.0659). Significant difference was found in antibodies against RBD (p=0.139), S2 domain (p=0.0355) and IgG antibodies against the nucleocapsid (p=0.0036).

Supplemental figure legends:

Supplemental Figure 1: Serum anti-COVID IgG and IgA antibody levels and correlation with HIT-EIA

This figure shows the MFI of anti-COVID IgG and IgA antibodies values for Spike Trimer, RBD, S1, S2 and nucleocapsid quantified by using a bead-based Luminex assay in VITT patients (Panel A); vaccinated volunteers (Panel B) and COVID-19 patients (Panel C). Anti-PF4 antibody levels did not correlate with anti-COVID antibodies in VITT patients and vaccinated volunteers (Panel D). Each symbol shows an individual subject and numbers of tested subjects is indicated in the graphic.

Supplemental Figure 2: IgG binding to healthy PLTs

Panel A shows a representative flow cytometry histogram of AHG binding. The AHG-binding was measured in presence of buffer and high concentration of heparin (100 IU/ml heparin) after incubation of platelets from healthy donors (HC) with serum of VITT patients. The other panels show IgG binding to healthy PLTs (assessed by flow cytometry and expressed as fold increase normalized to controls) after incubation with sera from vaccinated volunteers (Panel B) and COVID-19 patients (Panel C). Where indicated, PLTs were treated with PF4, 0.2U/mL and 100U/mL Heparin, RBD and the vaccine ChAdOx1 nCoV-19. (Abbreviation: ns: not significant, and **p<0.01). IgG binding to SARS-CoV-2 RBD was tested for VITT patients and assessed by EIA (expressed as fold increase to buffer controls). An increased tendency of IgG binding to RBD was observed at increasing concentrations of RBD (Panel D). IgG binding to

SARS-CoV-2 S2 was not observed in VITT patients (Panel E). Each symbol represents individual subject and the number subjects tested is reported in each graphic.

Supplemental Figure 3: Heparin-induced platelet activation assay (HIPA)

This figure shows the results of the platelet activation assay (HIPA) with modifications in presence or absence of PF4, 0.2U/ml and 100U/ml Heparin, RBD and vaccine ChAdOx1 nCoV-19. None of the subjects showed any platelet activation under any conditions except one in presence of Spike RBD in vaccinated controls (Panel A). Similar results were seen for COVID-19 patients except one patient who showed enhanced activation in 2 out of 4 donors in the presence of 0.2U/ml heparin (*$p<0.05$, Panel B). Each symbol represents individual subject and the number of subjects tested is reported in each graphic.

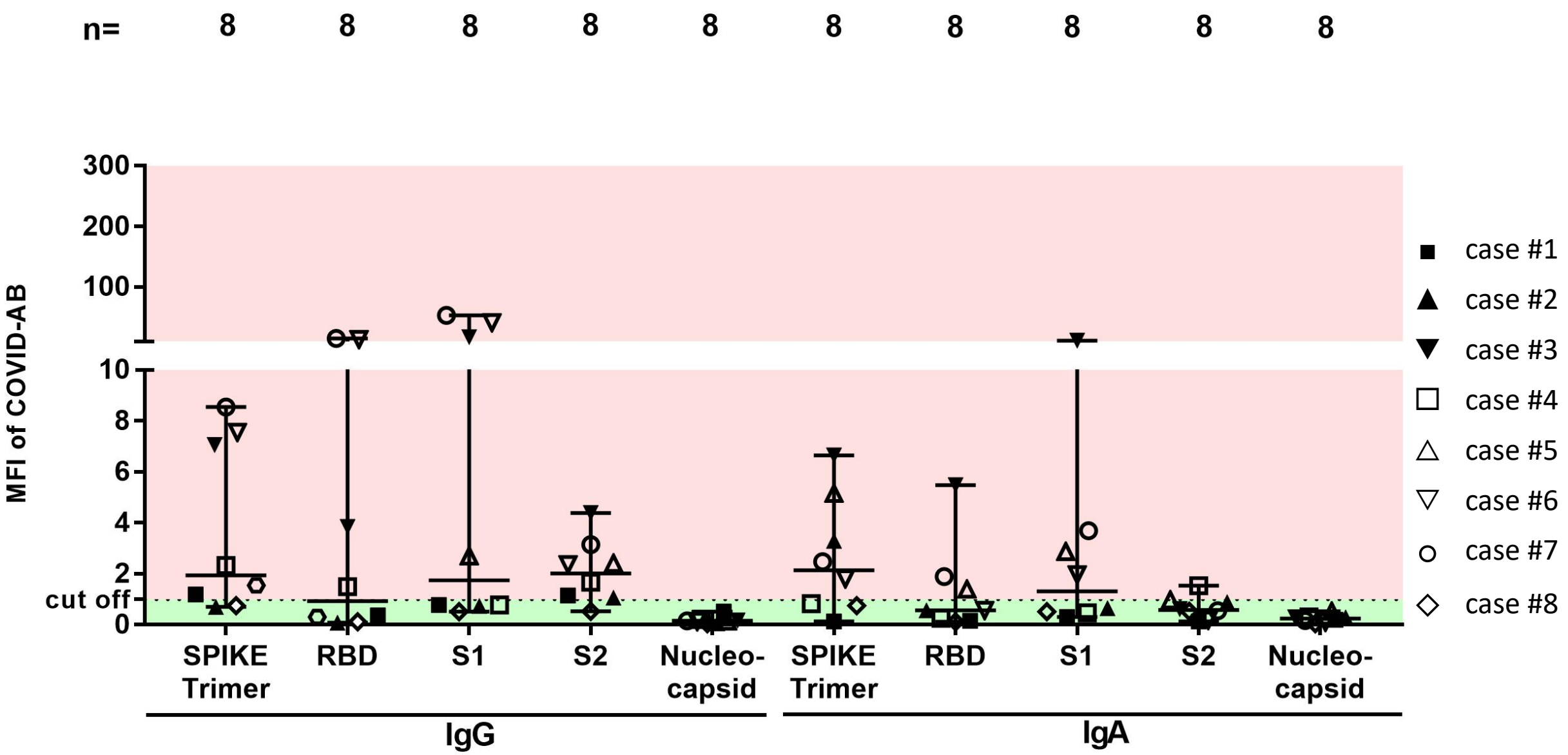
Supplemental Figure 4: Representative dot plot histograms of procoagulant platelets

Washed platelets (PLTs) were incubated with serum from VITT patients. I-VIII show representative dot plots in presence of [I] buffer HC, [II] buffer case #8, [III] 0.2 heparin case #8, [IV] 100 heparin case #8, [V] IV.3 case #8, [VI] IVIG case #8, [VII] PF4 case #8 and [VIII] VI.3 + PF4 case #8.

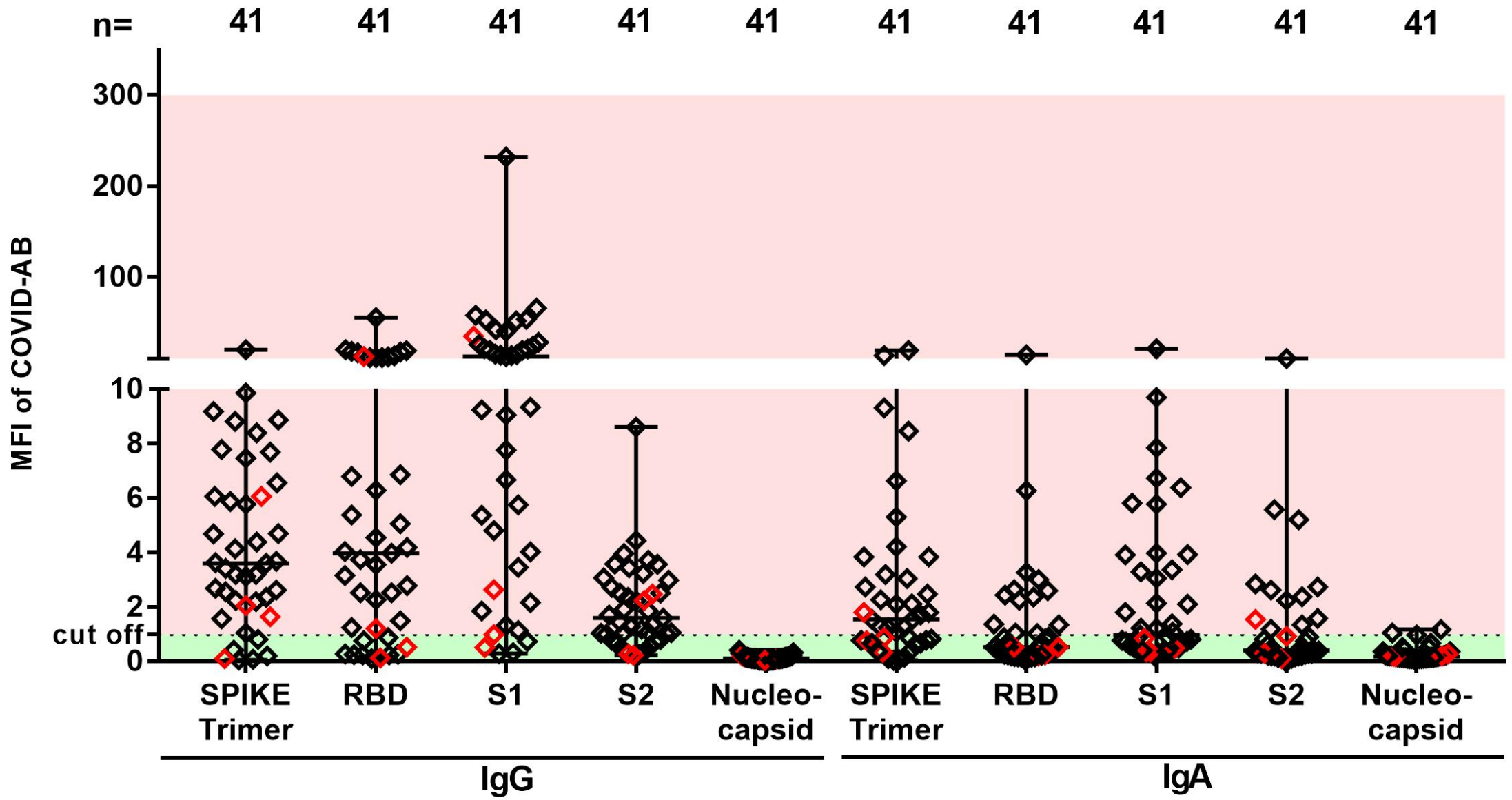
Supplemental Figure 5: Procoagulant platelets in vaccinated volunteers und COVID-19 patients

Procoagulant platelets (CD62P/Phosphatidylserine (PS) positive cells in different settings with sera from vaccinated volunteers (Panel A) and COVID-19 patients with anti PF4 antibodies (Panel B). Data are presented as mean ± standard deviation (SD) of the measured fold increase compared to control, not significant, and *$p<0.05$. Dot lines represent the cutoffs determined testing sera from healthy donors as mean of fold increase (FI).The number of sera tested is reported in each graphic.

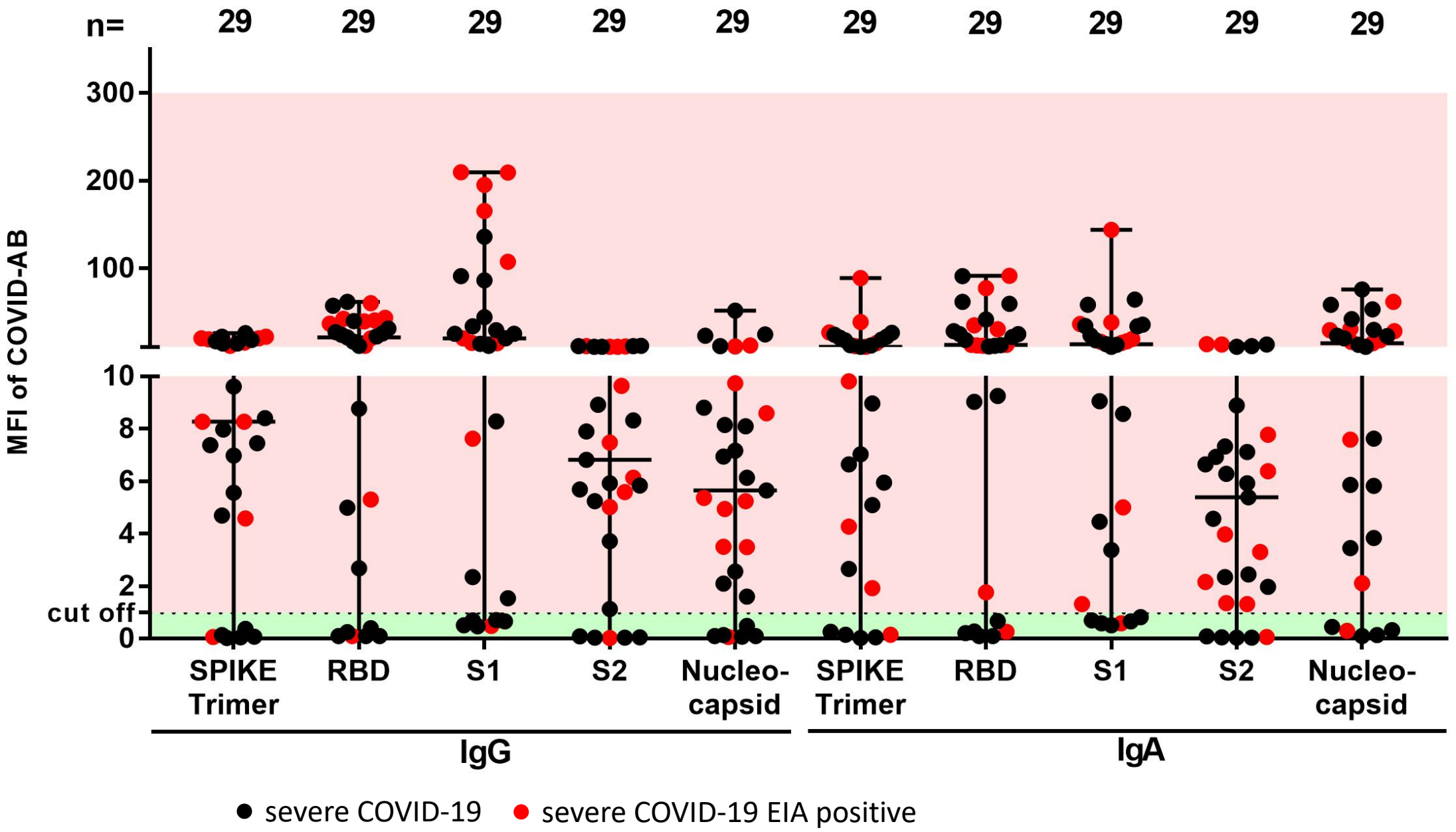
Supp Figure 1A



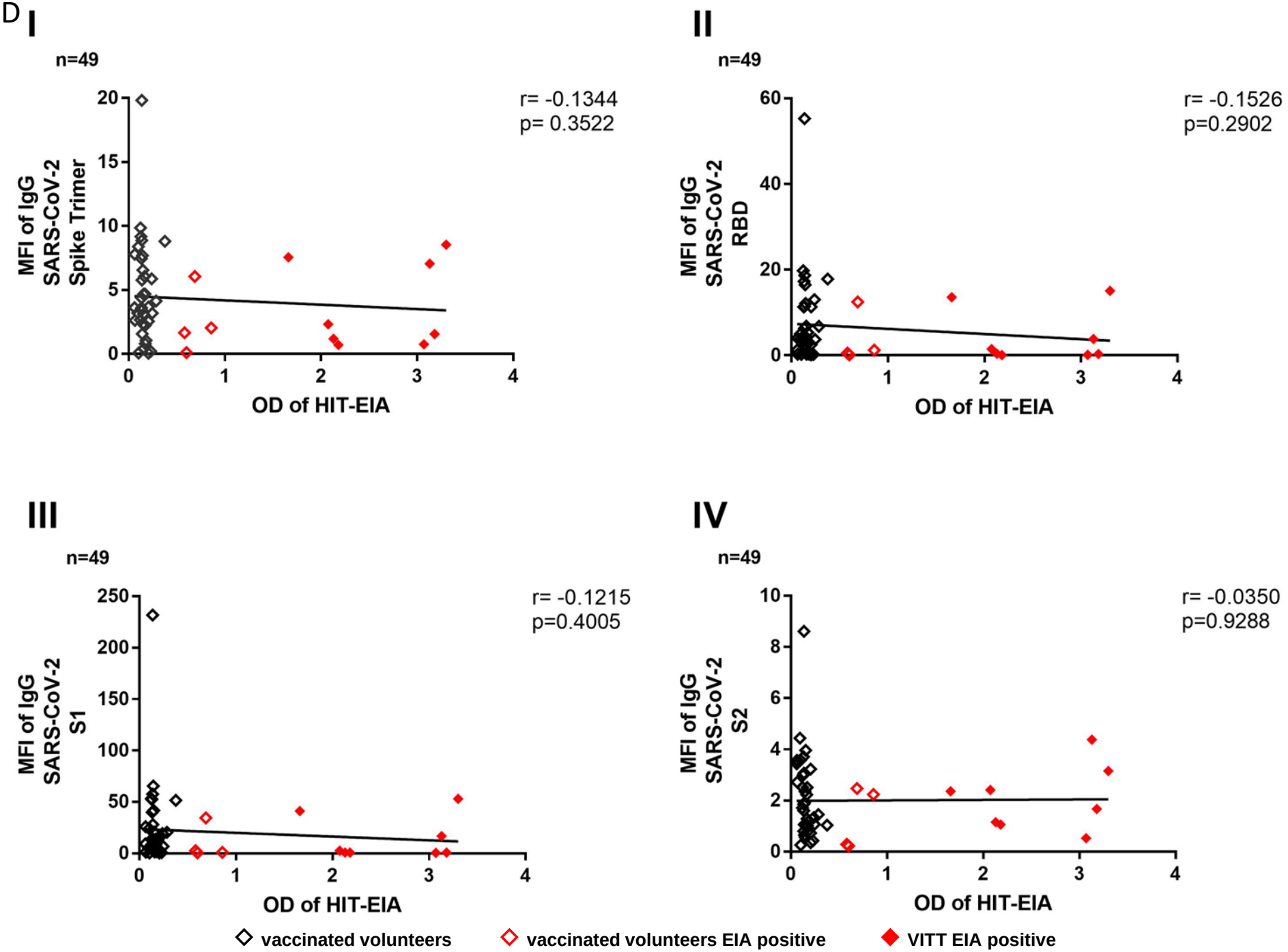
Supp Figure 1B



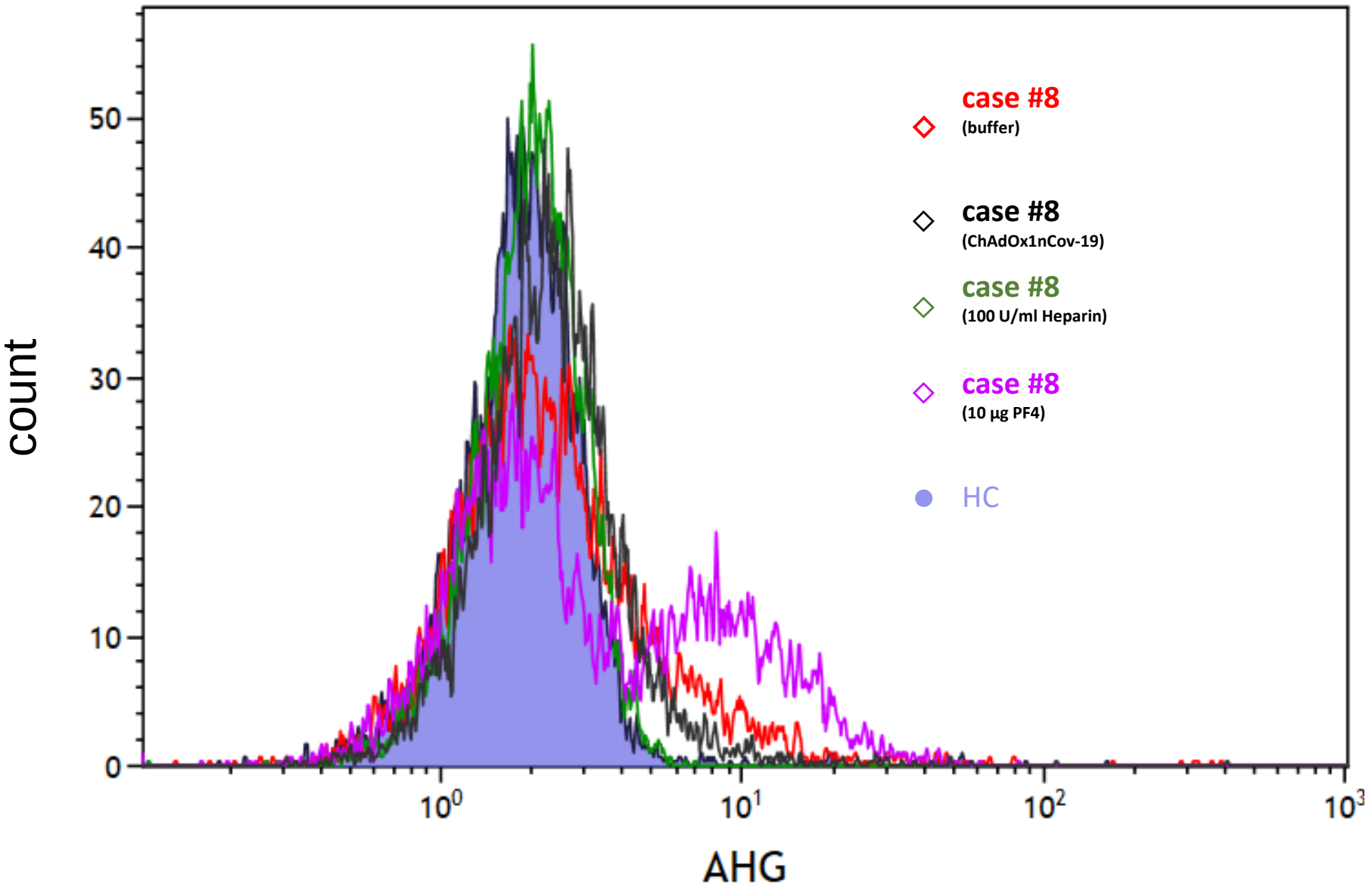
Supp Figure 1C



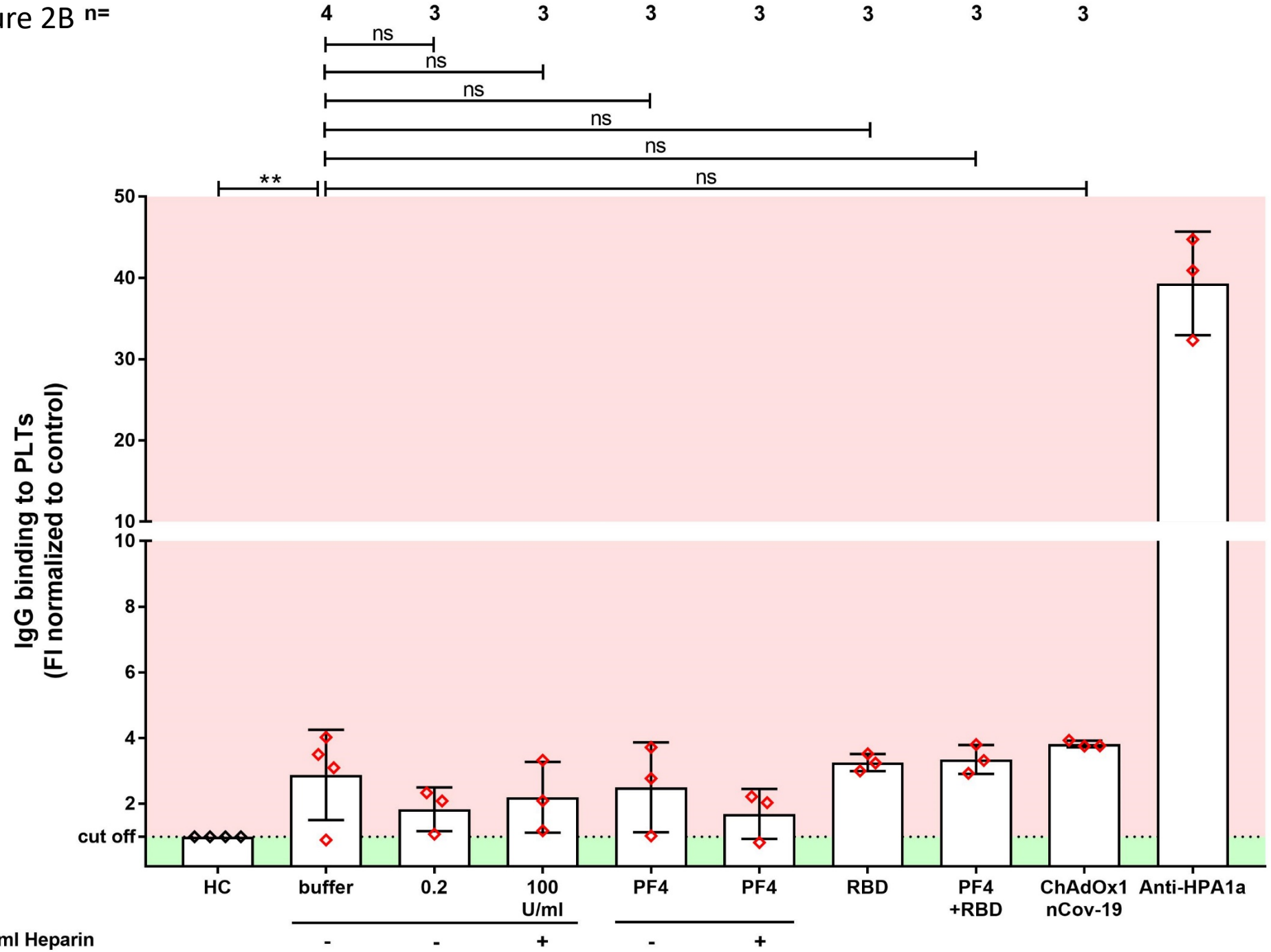
Supp Figure 1D



Supp Figure 2A

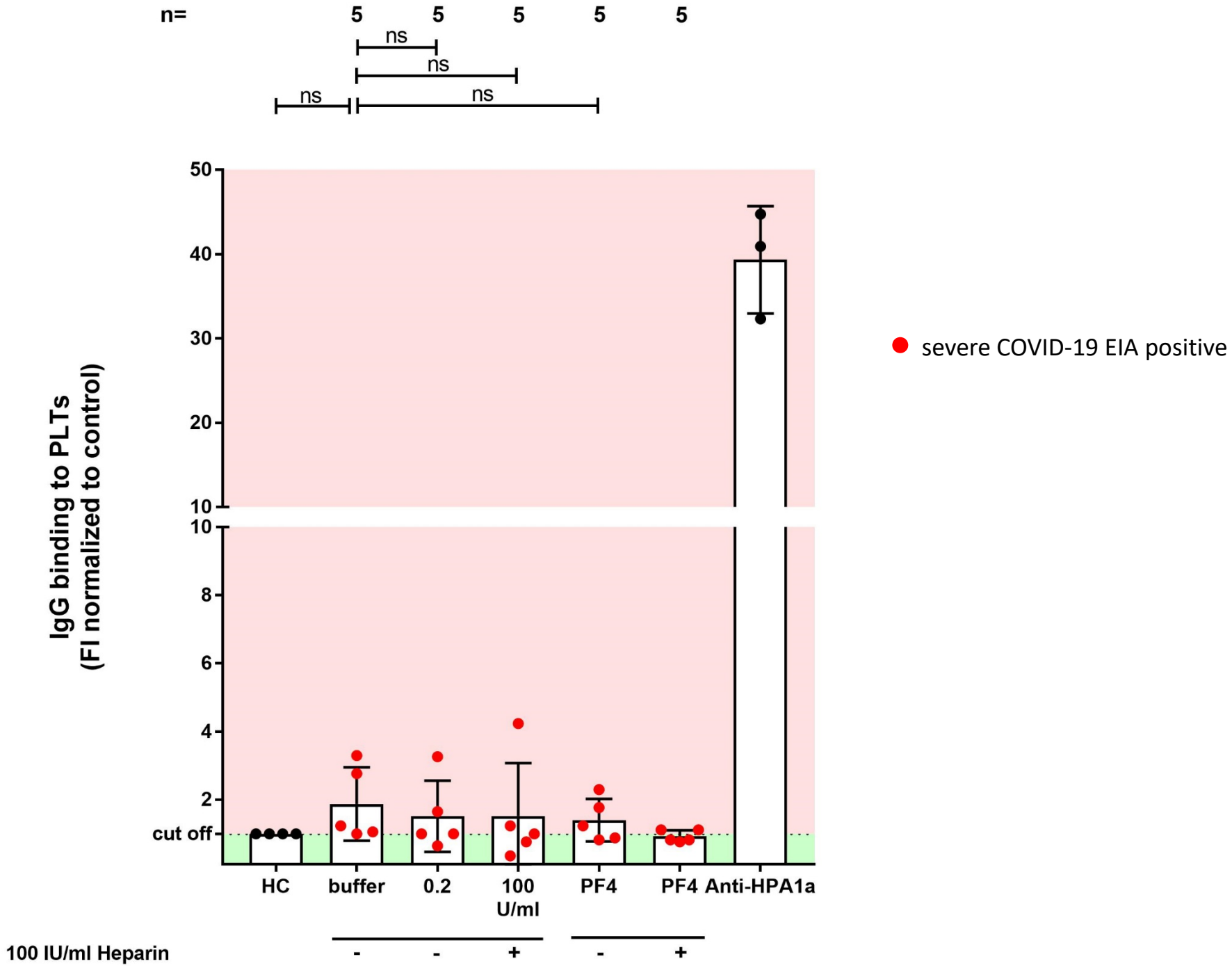


Supp Figure 2B

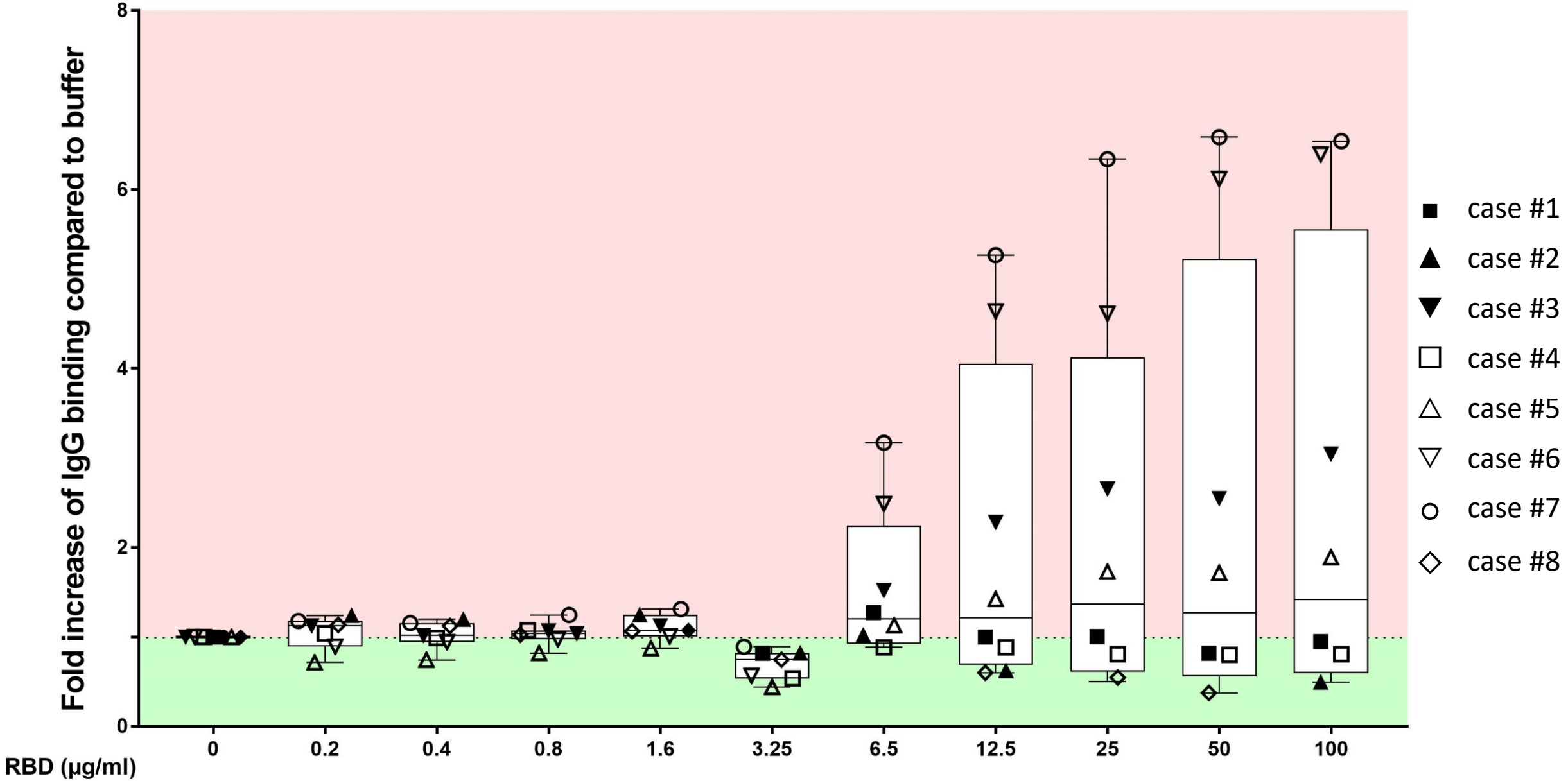


◇ vaccinated volunteers EIA positive

Supp Figure 2C



Supp Figure 2D



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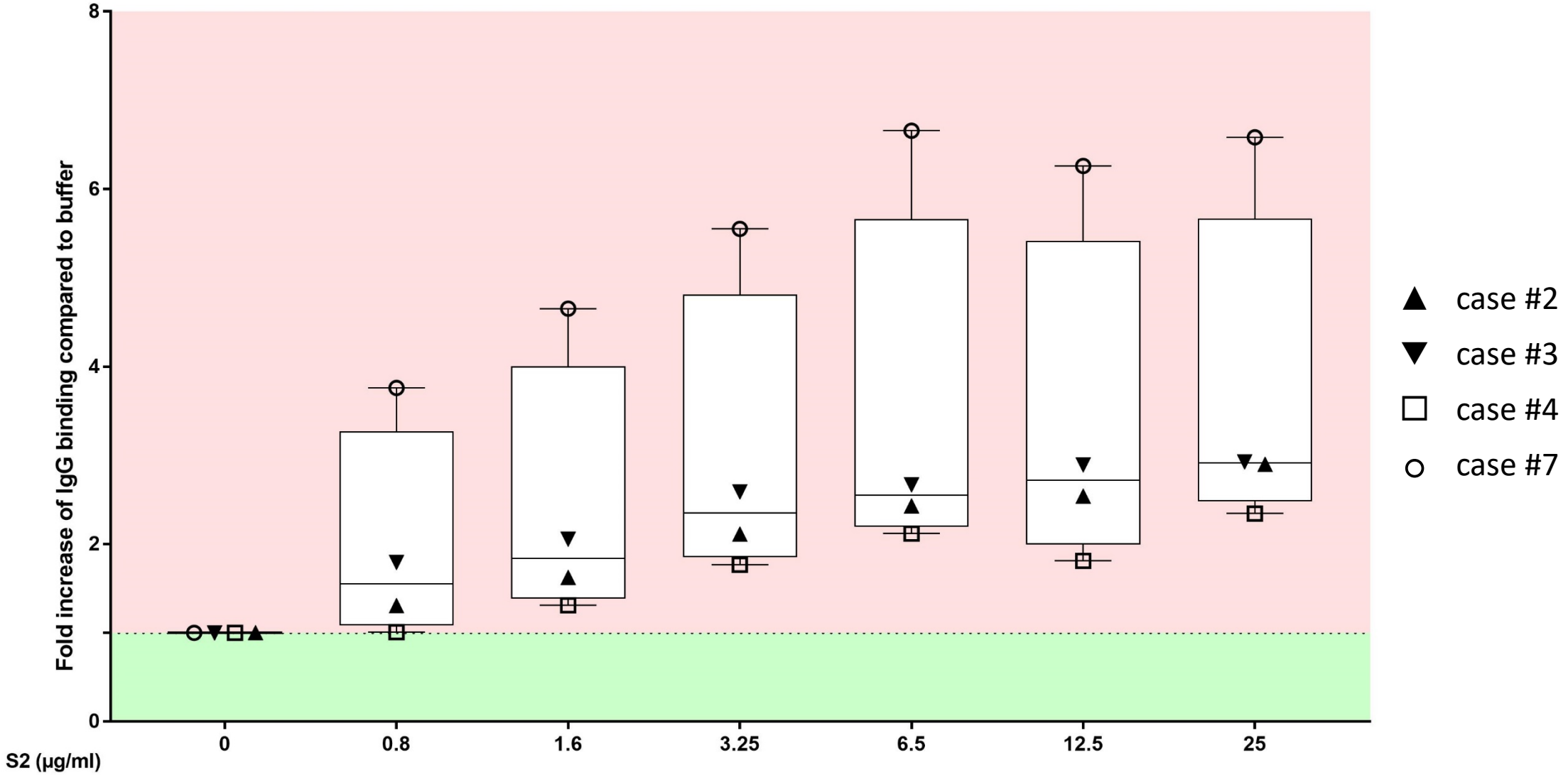
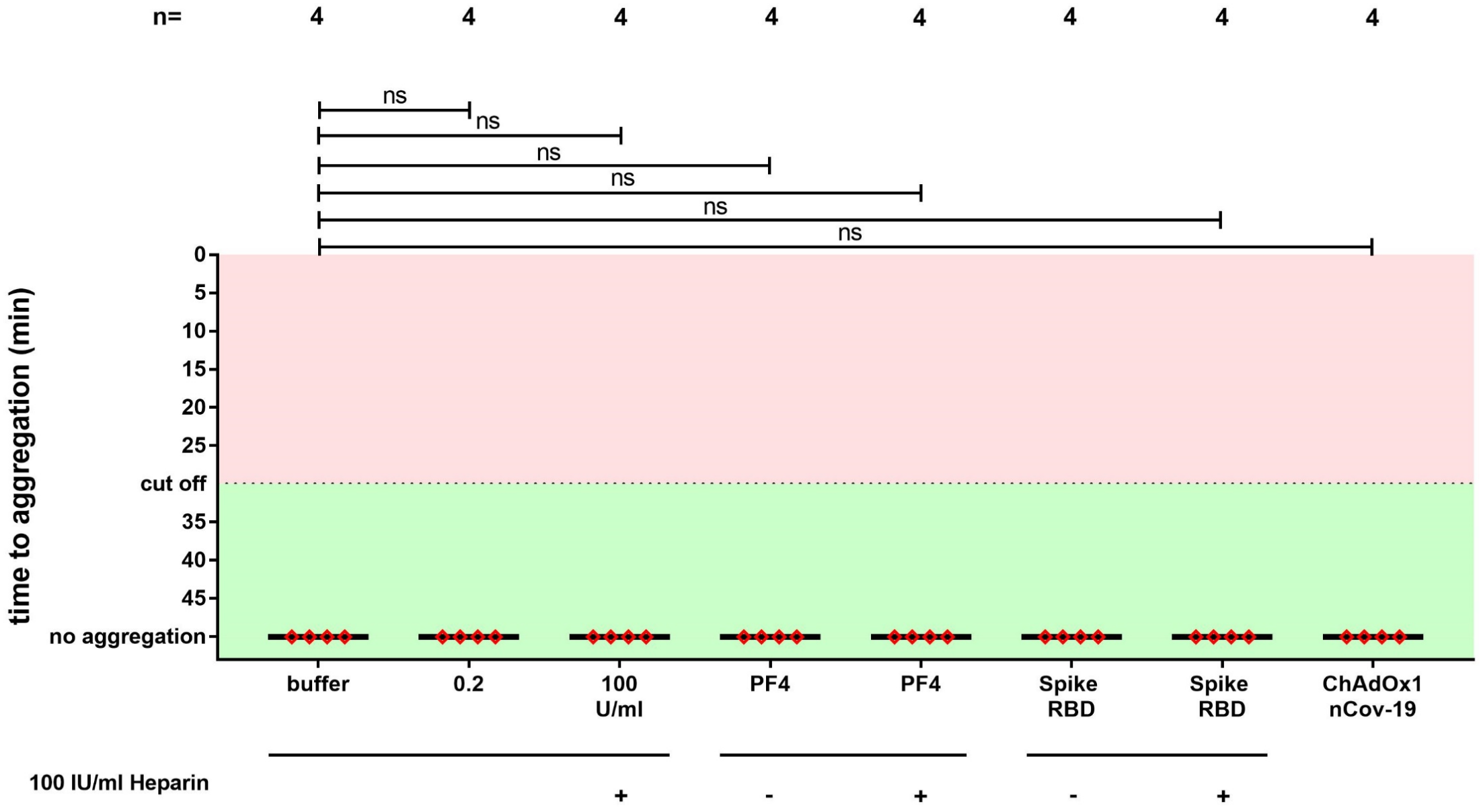
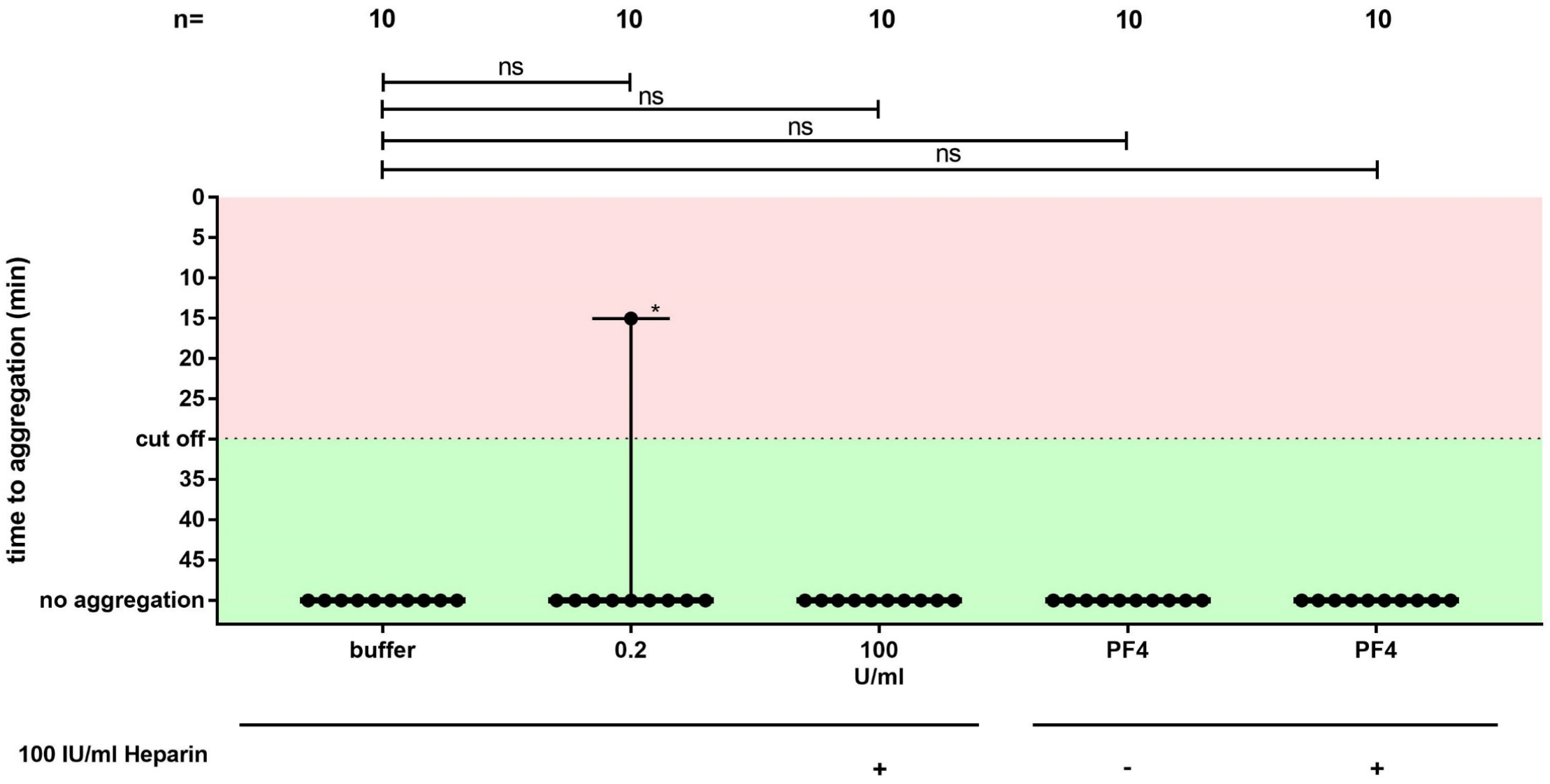


Supp Figure 3A



◇ vaccinated volunteers EIA positive

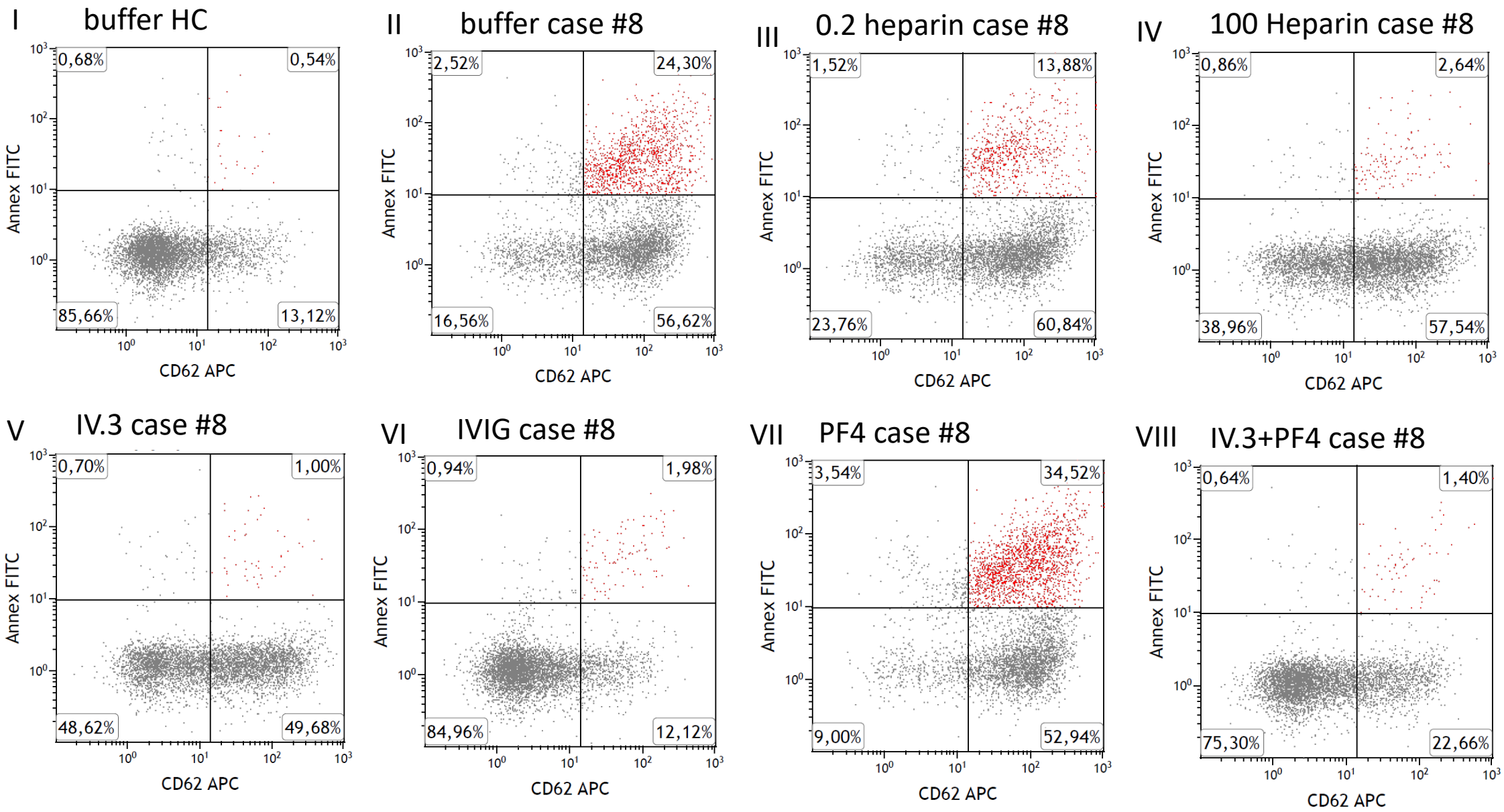
Supp Figure 3B



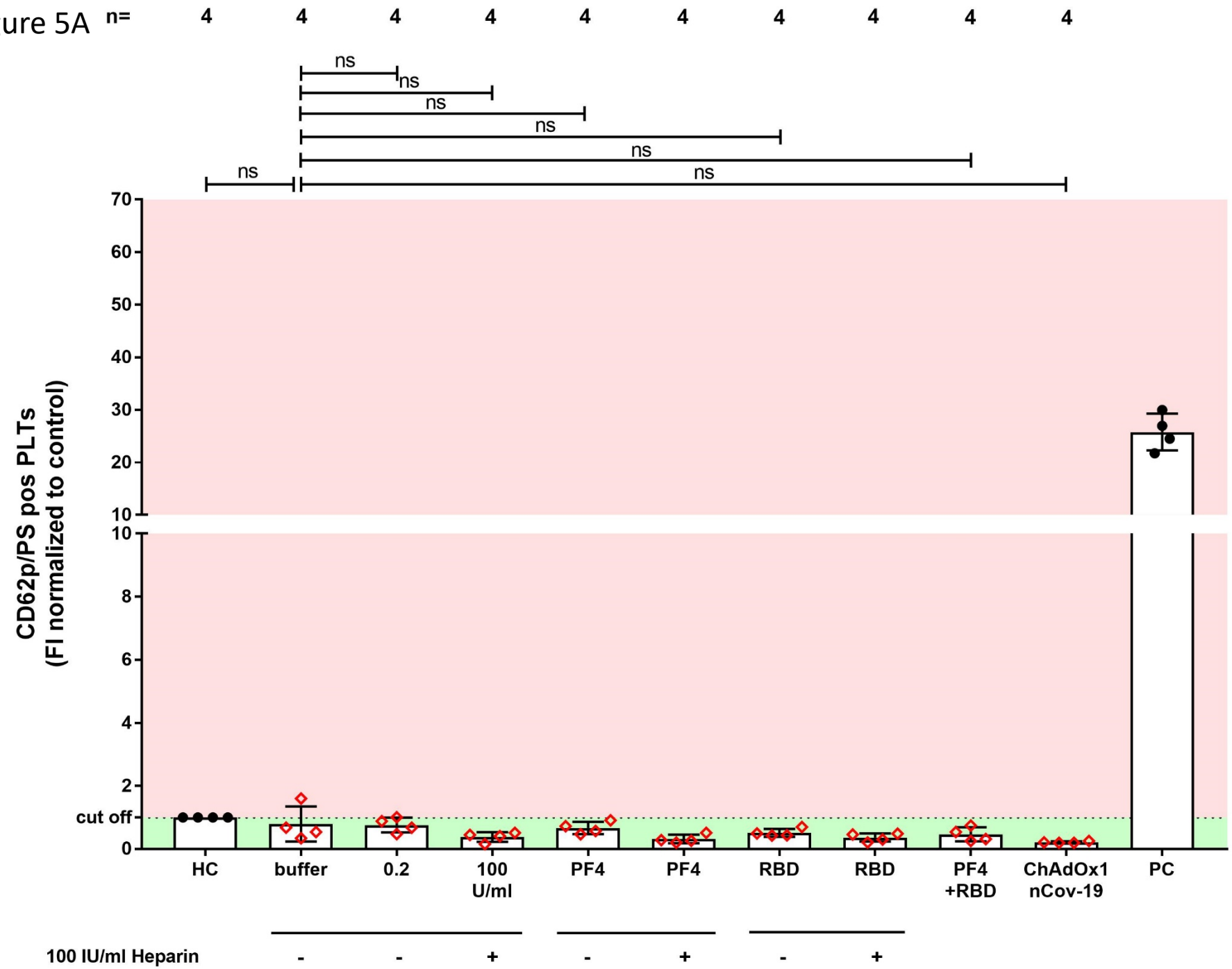
* 2 out of 4 donors

● severe COVID-19 EIA positive

Supp Figure 4



Supp Figure 5A



◇ vaccinated volunteers EIA positive

Supp Figure 5B