

1 Safety and immunogenicity of booster vaccination and fractional dosing 2 with Ad26.COV2.S or BNT162b2 in Ad26.COV2.S-vaccinated participants

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46 Abstract: 251 words, Full text: 11,773 words, Reference: 58.
47 Table: 1, Figures: 11, Supp tables: 2, Supp figure: 1.

48

49 **ABSTRACT**

50 **Background.** We report the safety and immunogenicity of fractional and full dose
51 Ad26.COVS.S and BNT162b2 in an open label phase 2 trial of participants previously
52 vaccinated with a single dose of Ad26.COVS.S, with 91.4% showing evidence of previous
53 SARS-CoV-2 infection.

54 **Methods.** A total of 286 adults (with or without HIV) were enrolled >4 months after an
55 Ad26.COVS.S prime and randomized 1:1:1:1 to receive either a full or half-dose booster of
56 Ad26.COVS.S or BNT162b2 vaccine. B cell responses (binding, neutralization and antibody
57 dependent cellular cytotoxicity-ADCC), and spike-specific T-cell responses were evaluated at
58 baseline, 2, 12 and 24 weeks post-boost. Antibody and T-cell immunity targeting the Ad26
59 vector was also evaluated.

60 **Results.** No vaccine-associated serious adverse events were recorded. The full- and half-
61 dose BNT162b2 boosted anti-SARS-CoV-2 binding antibody levels (3.9- and 4.5-fold,
62 respectively) and neutralizing antibody levels (4.4- and 10-fold). Binding and neutralizing
63 antibodies following half-dose Ad26.COVS.S were not significantly boosted. Full-dose
64 Ad26.COVS.S did not boost binding antibodies but slightly enhanced neutralizing antibodies
65 (2.1-fold). ADCC was marginally increased only after a full-dose BNT162b2. T-cell responses
66 followed a similar pattern to neutralizing antibodies. Six months post-boost, antibody and T-
67 cell responses had waned to baseline levels. While we detected strong anti-vector immunity,
68 there was no correlation between anti-vector immunity in Ad26.COVS.S recipients and spike-
69 specific neutralizing antibody or T-cell responses post-Ad26.COVS.S boosting.

70 **Conclusion.** In the context of hybrid immunity, boosting with heterologous full- or half-dose
71 BNT162b2 mRNA vaccine demonstrated superior immunogenicity 2 weeks post-vaccination
72 compared to homologous Ad26.COVS.S, though rapid waning occurred by 12 weeks post-
73 boost.

74 **Trial Registration:** South African National Clinical Trial Registry (SANCR): DOH-27-012022-
75 7841; **Funding:** South African Medical Research Council (SAMRC) and South African
76 Department of Health (SA DoH).

77 **Introduction**

78 The development of vaccines against SARS-CoV-2 was unparalleled, with numerous
79 platforms suited to rapid production dominating use in initial vaccination programs globally.
80 This included adenovirus-vectored vaccines, such as the Janssen Ad26.COV2.S, replication-
81 incompetent adenovirus 26 vectored SARS-CoV-2 spike protein vaccine. This vaccine is
82 registered as a single dose, with subsequent boosters recommended. This was the first
83 vaccine available nationally in South Africa, and health care workers, and later other essential
84 workers, were offered this vaccine as part of the Sisonke trial [1]. The mRNA-based Pfizer
85 BNT162b2 Comirnaty vaccine became available in South Africa subsequently as part of the
86 national vaccine rollout.

87 The emergence of SARS-CoV-2 variants of concern (VOCs) including the Beta, Delta and
88 Omicron variants reduced vaccine effectiveness against infection [2-6]. All vaccines based on
89 the sequence of the SARS-CoV-2 ancestral spike, including the Janssen Ad26.COV2.S and
90 the Pfizer Comirnaty BNT162b2 vaccines, elicited dramatically lower titers of neutralizing
91 antibodies against the Omicron subvariants [4, 7-20]. Vaccines incorporating Omicron
92 subvariant sequences [21] are not available in South Africa. However, the main driver of
93 increased neutralizing capacity against Omicron variants is hybrid immunity, which is the
94 combination of vaccine and infection elicited immunity. Population studies in SA showed
95 seroprevalence levels in excess of 95% by the end of the Omicron BA.1 Wave [22].

96 However, given the waning of humoral and cellular immunity over time, COVID-19 vaccine
97 boosting may be beneficial, especially in individuals at high risk for severe disease. The
98 choice of booster, timing and dose remain largely dependent on regulatory and national
99 considerations, including fiscal constraints and capacity of the health care system. Several
100 studies have demonstrated a more robust humoral and cellular immune response with a
101 heterologous boost compared to a homologous boost, in particular when boosting is with an
102 mRNA vaccine [12, 15, 23]. A further consideration is that due to pressure on vaccine
103 development, cost and equitable access, which are significantly impacted if multiple boosters

104 are needed, strategies such as fractional dosing should be considered. Fractional dosing has
105 previously been used with other vaccines such as yellow fever [24].

106 South Africa is burdened by more HIV infections than any other country in the world, with
107 approximately 8 million people living with HIV (PLWH) [25, 26]. PLWH, especially those with
108 low CD4 T-cell counts, have moderately worse COVID-19 outcomes [27-35]. This is
109 associated with lower and delayed neutralizing antibody titers in response to SARS-CoV-2
110 infection [19, 34, 36], more pronounced in PLWH with HIV viremia [19]. However, similar to
111 results reported for the AstraZeneca ChAdOx and the Pfizer BNT162b2 vaccines [37-41],
112 there was no observed difference between PLWH and HIV-negative individuals in neutralizing
113 antibody titers against SARS-CoV-2 spike after Ad26.COVS2 vaccination [19, 36].

114 Vaccines which use a viral vector to deliver the immunogen may be inhibited by pre-existing
115 immunity to the virus on which the vector is based [42, 43]. In addition to eliciting immunity to
116 the vaccine target, vaccination with an adenovirus vectored vaccine has been shown to elicit
117 neutralizing antibody and T-cell immunity to the vector itself [44-46]. This may reduce the
118 ability of repeated doses of the vectored vaccine to infect cells or reduce vaccine vector-
119 infected cell survival. The degree of elicited anti-vector immunity could therefore potentially
120 determine the effectiveness of vectored immunization in a population with previous immunity
121 to the virus on which the vector is based, or effectiveness if the vaccinees have been
122 previously immunized with the same vector. However additional factors, including transgene
123 persistence as well as local or systemic persistence may also impact the effectiveness of viral
124 vectored vaccines.

125 In this study, we evaluated the immunogenicity and safety of diverse boost strategies after
126 primary Ad26.COVS2 vaccination. We tested fractional and full dose heterologous and
127 homologous booster Ad26.COVS2 and BNT162b2 vaccinations. We also determined the
128 effects of HIV status, and degree of anti-adenovirus 26 vector immunity before and after
129 boosting. We observed that heterologous boosting of Ad26.COVS2 with the BNT162b2
130 mRNA vaccine was the most effective regimen to transiently enhance humoral and T-cell
131 immunity and HIV status did not have a substantial effect on the outcome. We show that

132 homologous boosting with Ad26.COV2.S occurred in the context of high anti-Ad26 vector
133 immunity. Although, Ad26.COV2.S gave a weak increase in antibody and cellular responses
134 against SARS-CoV-2 spike, we observed no correlation between vaccine response and anti-
135 Ad26 neutralizing antibody levels, as previously reported [47, 48].

136 **Methods and Materials**

137 **Study Design and Participants.** This study, **Booster After Sisonke Study** (BaSiS), is an
138 ongoing phase 2, randomized, open-label trial to evaluate the safety and immunogenicity of a
139 booster vaccination in participants who received a single Ad26.COVID.S vaccine through the
140 Sisonke phase 3B implementation study or via the South African National Department of
141 Health COVID-19 vaccine rollout. Participants were healthy adults, who met study eligibility
142 criteria and included participants with well-controlled comorbidities, except for HIV infection
143 where there were no immunological or virological exclusions. Full eligibility criteria are
144 provided in the protocol. We aimed to enrol at least one third of participants as PLWH and at
145 least 10% >55 years of age. Participants were included if they had no SARS-CoV-2 infection
146 at least 28 days prior to randomization. The study was conducted at four research sites in
147 South Africa, two in Johannesburg, one in Durban and one in Cape Town.

148 **Randomization.** Participants were randomized 1:1:1:1 to one of four booster vaccinations
149 including: Arm A: full-dose Ad26.COVID.S (5×10^{10} vp/mL, 0.25 mL); Arm B: half-dose
150 Ad26.COVID.S (2.6×10^{10} vp/mL, 0.13 mL); Arm C: full-dose BNT162b2 Comirnaty vaccine
151 (30mcg) and Arm D: half-dose BNT162b2 Comirnaty vaccine (15mcg). No masking was
152 required since the study was open-label.

153 **Procedures.** Study visits took place at baseline (randomization), 2 weeks, 12 weeks and 24
154 weeks. At each visit, medical history, COVID-19 infection history (symptoms or positive test)
155 and vaccination history (COVID-19 vaccine or other vaccination) was taken, followed by a
156 targeted clinical examination when necessary. At the baseline visit, participants received a
157 single booster vaccination, as per the randomization arm. Diary cards were issued and
158 participants were trained to collect data up to 7 days post booster. Telephonic contact at day 7
159 was conducted to enquire about severity or ongoing nature of reactogenicity. Blood samples
160 were drawn at each visit and included HIV testing at baseline (in all except those known to be
161 PLWH), CD4 count and HIV viral load (in PLWH), full blood count (BL, W2 and additional visits
162 in PLWH), D-dimers (BL and W2). A nasopharyngeal swab for SARS-CoV-2 PCR was

163 conducted at baseline. Interim visits were held for any safety concerns or if a participant had
164 COVID-19 symptoms or a positive SARS-CoV-2 PCR or antigen test outside of the study.

165 **Safety.** Reactogenicity was collected and graded according to criteria included in Food and
166 Drug Administration (FDA) “The Guidance for Industry: Toxicity Grading Scale for Healthy
167 Adult and Adolescent Volunteers Enrolled in Preventive Vaccine Clinical Trials” [49]. All grade
168 3 reactogenicity events, SAEs, SUSARs and adverse drug reactions were reported. A Data
169 Safety and Monitoring Committee was established to evaluate protocol-defined safety events
170 and immunogenicity data.

171

172 **Immunogenicity**

173 **Live virus neutralization assay**

174 Live virus neutralization assay was performed as previously described in our previous work [4,
175 6, 18]. Briefly, ACE2-expressing H1299-E3 (CRL-5803, ATCC) cells were seeded at 4.5×10^5
176 cells per well and incubated for 18-20 h. After washing, the sub-confluent cell monolayer was
177 inoculated with 500 μ L universal transport medium diluted 1:1 with filtered growth medium.
178 Cells were incubated for 1 h. Wells were then filled with 3 mL complete growth medium. After
179 4 days of infection (completion of passage 1), cells were trypsinized, centrifuged and
180 resuspended in 4 mL growth medium. Then, all infected cells were added to Vero E6 cells
181 (CRL-1586, ATCC). The coculture of ACE2-expressing H1299-E3 and Vero E6 cells was
182 incubated for 4 days. The viral supernatant from this culture (passage 2 stock) was used for
183 experiments.

184 H1299-E3 cells were plated at 30,000 cells/well 1 day pre-infection. Aliquots of cryo-preserved
185 plasma samples were heat-inactivated and clarified by centrifugation. Virus stocks were used
186 at approximately 50-100 focus-forming units per microwell and added to diluted plasma.

187 Antibody–virus mixtures were incubated for 1 h at 37°C. Cells were infected with 100 μ L of the
188 virus–antibody mixtures for 1 h, then 100 μ L of a RPMI 1640 (Sigma-Aldrich), 1.5%
189 carboxymethylcellulose (Sigma-Aldrich) overlay was added without removing the inoculum.

190 Cells were fixed 18 h post-infection. Foci were stained with a rabbit anti-spike monoclonal

191 antibody (0.5 µg/mL, BS-R2B12, GenScript) overnight at 4°C, washed and then incubated
192 with a horseradish peroxidase (HRP) conjugated goat anti-rabbit antibody (1 µg/mL, Abcam
193 ab205718) for 2 h. TrueBlue peroxidase substrate (SeraCare) was then added and incubated
194 for 20 min. Plates were imaged in an ImmunoSpot Ultra-V S6-02-6140 Analyzer ELISPOT
195 instrument with BioSpot Professional built-in image analysis (C.T.L). All statistics and fitting
196 were performed using custom code in MATLAB v.2019b. Neutralization data were fit to:
197 $T_x = 1/1 + (D/ID_{50})$. T_x is the number of foci normalized to the number of foci in the absence of
198 plasma on the same plate at dilution D and ID_{50} is the plasma dilution giving 50%
199 neutralization. $FRNT_{50} = 1/ID_{50}$. Values of $FRNT_{50} < 1$ are set to 1 (undiluted), the lowest
200 measurable value. As, the most concentrated plasma dilution was 1:25, $FRNT_{50} < 25$ were
201 extrapolated. To calculate confidence intervals, $FRNT_{50}$ or fold-change in $FRNT_{50}$ per
202 participant was log-transformed and arithmetic mean plus and minus two standard deviations
203 were calculated for the log transformed values. These were exponentiated to obtain the upper
204 and lower 95% confidence intervals on the geometric mean $FRNT_{50}$ or the fold-change in
205 $FRNT_{50}$ geometric means.

206 Sequences of outgrown ancestral SARS-CoV-2 and the Omicron BA.5 subvariant have been
207 deposited in GISAID with accession EPI_ISL_602626.1 (ancestral, D614G) and
208 EPI_ISL_12268493.2 (Omicron/BA.5).

209

210 **SARS-CoV-2 spike and nucleocapsid enzyme-linked immunosorbent assay (ELISA)**

211 For ELISA, Hexapro SARS-CoV-2 full spike protein with the D614G substitution were
212 expressed in Human Embryonic Kidney (HEK) 293F suspension cells by transfecting the cells
213 with the respective expression plasmid. After incubating for 6 days at 37°C, proteins were first
214 purified using a nickel resin followed by size exclusion chromatography. Relevant fractions
215 were collected and frozen at -80°C until use. Two µg/mL of D614G spike or nucleocapsid
216 protein was used to coat 96-well, high-binding plates (Corning) and incubated overnight at
217 4°C. The plates were incubated in a blocking buffer consisting of 1x PBS, 5% skimmed milk
218 powder, 0.05% Tween 20. Plasma samples were diluted to 1:100 starting dilution in a blocking

219 buffer and added to the plates. IgG secondary antibody (Merck) was diluted to 1:3000 in
220 blocking buffer and added to the plates followed by TMB substrate (Thermofisher Scientific).
221 Upon stopping the reaction with 1 M H₂SO₄, absorbance was measured at 450 nm. For spike
222 ELISA, mAbs CR3022 was used as a positive control and Palivizumab was used as a
223 negative control.

224

225 **Lentiviral pseudovirus production and neutralization assay**

226 Virus production and pseudovirus neutralization assays were done as previously described.
227 Briefly, 293T/ACE2.MF cells modified to overexpress human ACE2 (provided by M. Farzan,
228 Scripps Research) were cultured in DMEM (Gibco) containing 10% FBS and 3 µg/mL of
229 puromycin at 37°C. Cell monolayers were disrupted at confluency by treatment with 0.25%
230 trypsin in 1 mM EDTA (Gibco). The SARS-CoV-2, Wuhan-1 spike, cloned into pCDNA3.1 was
231 mutated using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies)
232 and NEBuilder HiFi DNA Assembly Master Mix (NEB) to include D614G (wild-type) or lineage
233 defining mutations for Beta (L18F, D80A, D215G, 241-243del, K417N, E484K, N501Y, D614G
234 and A701V), Delta (T19R, 156-157del, R158G, L452R, T478K, D614G, P681R and D950N),
235 Omicron BA.1 (A67V, Δ69-70, T95I, G142D/Δ143-145, Δ211/L212I, ins214EPE, G339D,
236 S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S,
237 Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K,
238 Q954H, N969K, L981F) and Omicron BA.4/5 (T19I, L24S, 25-27del, 69-70del, G142D, V213G,
239 G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N,
240 T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K,
241 D796Y, Q954H, N969K). Pseudoviruses were produced by co-transfection in 293T/17 cells
242 with a lentiviral backbone (HIV-1 pNL4.luc encoding the firefly luciferase gene) and either of
243 the SARS-CoV-2 spike plasmids with PEIMAX (Polysciences). Culture supernatants were
244 clarified of cells by a 0.45 µm filter and stored at -70°C. Plasma samples were heat-inactivated
245 and clarified by centrifugation. Pseudovirus and serially diluted plasma/sera were incubated
246 for 1 h at 37°C. Cells were added at 1×10⁴ cells per well after 72 h of incubation at 37°C.

247 Luminescence was measured using PerkinElmer Life Sciences Model Victor X luminometer.
248 Neutralization was measured as described by a reduction in luciferase gene expression after
249 single-round infection of 293T/ACE2.MF cells with spike-pseudotyped viruses. Titers were
250 calculated as the reciprocal plasma dilution (ID_{50}) causing 50% reduction of relative light units.

251

252 **Antibody-dependent cellular cytotoxicity (ADCC) assay**

253 The ability of plasma antibodies to cross-link Fc γ R1IIa (CD16) and spike expressing cells was
254 measured as a proxy for ADCC as previously described. HEK293T cells were transfected with
255 5 μ g of SARS-CoV-2 wild-type variant spike (D614G), Beta, Delta and Omicron BA.1 spike
256 plasmids using PEI-MAX 40,000 (Polysciences) and incubated for 2 days at 37°C. Expression
257 of spike was confirmed by binding of CR3022 and P2B-2F6 and their detection by anti-IgG
258 APC (Biolegend) measured by flow cytometry. Subsequently, 1×10^5 spike-transfected cells per
259 well were incubated with heat inactivated plasma (1:100 final dilution) or control mAbs (final
260 concentration of 100 μ g/mL) in RPMI 1640 media supplemented with 10% FBS and 1%
261 Pen/Strep (R10; Gibco) for 1 h at 37°C. Jurkat-Lucia™ NFAT-CD16 cells (Invivogen) (2×10^5
262 cells/well) were added and incubated for 24 h at 37°C. Twenty μ l of supernatant was then
263 transferred to a white 96-well plate with 50 μ l of reconstituted QUANTI-Luc secreted luciferase
264 and read immediately on a Victor 3 luminometer with 1s integration time. Cells were gated on
265 singlets, live cells (determined by Live/dead™ Viability dye; ThermoFisher Scientific), and
266 those cells that were positive for IgG and spike specific monoclonal antibodies binding to their
267 surface. Relative light units (RLU) of a no antibody control were subtracted as background.
268 Palivizumab was used as a negative control, while CR3022 was used as a positive control,
269 and P2B-2F6 to differentiate the Beta from the D614G variant. To induce the transgene, 1x
270 cell stimulation cocktail (ThermoFisher Scientific) and 2 μ g/ml ionomycin in R10 was added as
271 a positive control.

272

273 **Measurement of antigen-specific T cells by flow cytometry**

274 T-cell responses to SARS-CoV-2 spike or human adenovirus 26 (Ad26) hexon and penton
275 were measured as previously described [3]. Briefly, cryopreserved PBMC were thawed,
276 washed and rested in RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated FBS
277 (HyClone) for 4 h prior to stimulation. PBMC were seeded in a 96-well V-bottom plate at
278 $\sim 2 \times 10^6$ PBMC per well and stimulated with either a commercial ancestral SARS-CoV-2 spike
279 (S) pool (1 $\mu\text{g}/\text{mL}$, Miltenyi Biotec) or a Ad26 peptide pool containing 293 peptides (15mers
280 with 10-aa overlap) spanning the Ad26 hexon and penton proteins (1 $\mu\text{g}/\text{mL}$). All stimulations
281 were performed in the presence of Brefeldin A (10 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) and co-stimulatory
282 antibodies against CD28 (clone 28.2) and CD49d (clone L25) (1 $\mu\text{g}/\text{mL}$ each, BD
283 Biosciences). As a negative control, PBMC were incubated with co-stimulatory antibodies,
284 Brefeldin A and an equimolar amount of DMSO. After 16 h of stimulation, cells were washed,
285 stained with LIVE/DEAD™ Fixable Near-IR Stain (Invitrogen) and subsequently surface
286 stained with the following antibodies: CD14 APC-Cy7 (HCD14), CD19 APC-Cy7 (HIB19), CD4
287 BV785 (OKT4), CD8 FITC (RPA-T8), CD45RA BV570 (HI100) (Biolegend) and CD27 PE-Cy5
288 (1A4, Beckman Coulter). Cells were then fixed and permeabilized using a Cytotfix/Cytoperm
289 buffer (BD Biosciences) and stained with CD3 BV650 (OKT3), IFN- γ BV711 (4S.B3), TNF- α
290 PE-Cy7 (Mab11) and IL-2 PE/Dazzle™ 594 (MQ1-17H12) from Biolegend. Finally, cells were
291 washed and fixed in CellFIX (BD Biosciences). Samples were acquired on a BD Fortessa flow
292 cytometer and analyzed using FlowJo (v10.8.1, FlowJo LLC). Results are expressed as the
293 frequency of total memory CD4 $^{+}$ or CD8 $^{+}$ T cells expressing IFN- γ , TNF- α or IL-2. Due to high
294 TNF- α backgrounds, cells producing TNF- α alone were excluded from the analysis. All data
295 are presented after background subtraction.

296

297 **Outcomes**

298 The primary objectives of the study were to evaluate safety and reactogenicity and humoral
299 and cellular immunogenicity to full and half dose homologous and heterologous booster
300 vaccinations, at each study visit. Primary endpoint measures included measuring
301 nucleocapsid binding antibody titers, neutralization titers and T-cell response magnitudes.

302 Safety was measured by participant self-report using diary cards and graded according to
303 FDA standards [49]. The primary immunogenicity endpoint was defined as any study arm
304 eliciting <75% of the highest geometric mean titre (GMT) response in the study. Together with
305 the DSMB, the study team used this criteria to determine whether a subsequent booster
306 should be offered to participants, and which booster if so. In this study, based on results, all
307 participants except those who received the full dose BNT162b2 vaccination, were offered a
308 full dose BNT162b2 booster in addition to that received according to their original
309 randomisation.

310

311 **Statistical Analysis**

312 A two-tailed Wilcoxon signed-rank test or a Friedman test with Dunn's correction was used to
313 assess statistical differences between paired samples. A Mann-Whitney test or a Kruskal-
314 Wallis test with Dunn's corrections was used to compare multiple groups. Correlations were
315 tested by a two-tailed non-parametric Spearman's rank test. In all cases, P values of less than
316 or equal to 0.05 were considered significant.

317

318 **Data Sharing**

319 Protocol and data underlying the findings described in this manuscript may be obtained from
320 the lead authors upon request.

321

322 **Study Approval**

323 This study has been approved by the South African Health Products Regulatory Authority
324 (SAHPRA, number: 20210423) and all site-specific Human Research Ethics Committees
325 (Wits: 211001B, UKZN: BREC/00003487/2021, UCT: 680/202). All participants provided
326 written informed consent..

327 **Results**

328 **Participants and follow up.**

329 Between December 8, 2021, and July 27, 2022, 333 participants were screened and 289
330 randomized to 4 arms: Arm A: Full-dose Ad26.COV2.S (5×10^{10} vp/mL, 74 participants); Arm B:
331 half-dose Ad26.COV2.S (2.6×10^{10} vp/mL, 69 participants); Arm C: Full-dose BNT162b2
332 (30mcg, 73 participants); and Arm D: half-dose BNT162b2 (15mcg, 73 participants) (**Table 1**
333 and **Figure 1**). Participants were followed for 24 weeks, overall retention was 93.1% at 24
334 weeks (**Table 1**).

335 The majority of participants (53.6%, 155/289) were between 30 and 45 years old, with 36/189
336 (12.5%) 55 years and older, and 238/289 (82.4%) female. In the cohort, 116/289 (40.1%)
337 were PLWH, 16 (13.8%) were considered viremic (VL >200cps/ml) and had a median CD4
338 count of 452 cells/mm³ (IQR: 132-538), whereas participants who were virologically
339 suppressed (VL <200cps/ml) had a median CD4 count of 708 cells/mm³ (IQR: 558-922). At
340 randomisation, subsequently referred to as baseline, 61/289 (21.1%) of participants had
341 hypertension, 30/289 (10.4%) anaemia, and 174/289 (60.2%) were obese. Other
342 comorbidities were less common (asthma and diabetes mellitus 4.2% each, arthritis 2.4% and
343 tuberculosis 2.1% respectively).

344 Evidence of previous SARS-CoV-2 infection (positive nucleocapsid antibody detected by
345 ELISA) was present in 91.4% (213/223) of participants tested at baseline indicating a high
346 degree of hybrid immunity in the trial cohort. This was similar amongst the four vaccination
347 arms, namely 91.2% (52/57), 87.9% (51/58), 93.2% (55/59) and 93.2% (55/59) for half-dose
348 Ad26.COV2.S, full-dose Ad26.COV2.S, half-dose BNT162b2 and full-dose BNT162b2,
349 respectively. These high levels of nucleocapsid positivity were sustained 2, 12 and 24 weeks
350 after the booster dose, with greater than 80% nucleocapsid positivity at any given time point.
351 Participants received the booster vaccine at a median of 271 days (IQR: 255-296) after the
352 Ad26.COV2.S prime. Nine breakthrough infections (BTI) were confirmed by SARS-CoV-2
353 PCR during the trial and all were mild infections that resolved within 4-12 days, 7/9 in the

354 Ad26.COVS arms. Three BTI occurred between W2 and W12, and the remainder between
355 W12 and W24 (median: 17.1 weeks post boost).

356

357 **Safety and reactogenicity of the booster vaccination regimens**

358 Reactogenicity was measured through participant-completed diary cards, recording solicited
359 local and systemic adverse reactions, as well as unsolicited adverse reactions through day-7
360 post booster, or longer if adverse events persisted. Overall, safety profiles were comparable
361 between the four trial arms. Localised pain, headache, localised tenderness and weakness
362 were reported with highest frequency, mostly of grade 1 and 2 severity. Grade 3 or 4 events
363 included localised pain (2.0%), tenderness (1.7%), nausea (0.7%), diarrhoea (1.0%),
364 headache (1.7%), weakness (2.8%), myalgia (1.0%), chills (0.7%), cough (0.3%), loss of smell
365 (0.3%) and loss of taste (0.3%). Unsolicited AEs were uncommon, occurring in 8.7% of
366 participants (**Figure 2**).

367 No events of thrombosis with thrombocytopenia syndrome (TTS) were reported;
368 thrombocytopenia was reported in three participants, two had thrombocytopaenia at baseline.
369 There were 14 serious AEs (SAEs) on study, non-related to study product, and no AEs of
370 special interest (AESIs).

371

372 **Antibody responses after booster vaccination**

373 We evaluated spike binding antibody titers at baseline (BL) and week 2 (W2), week 12 (W12)
374 and week 24 (W24) post-boost in the four vaccination arms using a trimeric spike ELISA for
375 ancestral D614G virus. Relatively high spike binding antibody titers were detected in all
376 groups at BL (geometric mean titer (GMT), EC_{50} : >1000; **Figure 3A**). This is consistent with
377 our observation that the majority of participants showed evidence of prior infection (**Table 1**).
378 Spike antibody titers in the half- and full-dose Ad26.COVS arms were not significantly
379 boosted by W2 (1.15 and 1.29 fold change in GMT, respectively), and did not change
380 significantly up to W24. In contrast, half- and full-dose BNT162b2 arms demonstrated a
381 significant increase in binding antibody titers at W2 (3.9 and 4.54 fold change, respectively), to

382 a GMT >4500 (**Figure 3A**). Spike binding antibodies in the BNT162b2 arms reduced to half
383 that of W2 titers by W12, and had returned to BL levels by W24. These kinetics were reflected
384 in a significantly higher fold-change between W2 and BL in the BNT162b2 arms compared to
385 the Ad26.COVS2.S arms (**Figure 3B**). Although waning had occurred by W12, EC₅₀ titers in the
386 BNT162b2 arms were still significantly higher than the Ad26.COVS2.S arms (**Figure 3C**). While
387 some differences between the arms persisted at W24, relatively high antibody titers, similar to
388 those observed at BL and prior to a boost, were noted in all four arms, with GMT of 1016,
389 1140, 1563 and 1686 for half-dose Ad26.COVS2.S, full-dose Ad26.COVS2.S, half-dose
390 BNT162b2 and full-dose BNT162b2, respectively.

391
392 Using a live virus neutralization assay, we evaluated neutralizing antibody titers to the
393 ancestral D614G virus and Omicron BA.5, which was the most recent dominant sub-lineage at
394 the time of W24 collection. At BL, neutralization activity against the D614G virus was similar
395 between the arms (GMT FRNT₅₀ values of 546, 345, 393 and 334 for half-dose Ad26.COVS2.S,
396 full-dose Ad26.COVS2.S, half-dose BNT162b2 and full-dose BNT162b2, respectively; **Figure**
397 **4A**). When measured at W2, half-dose Ad26.COVS2.S did not boost BL neutralizing titers,
398 while full-dose Ad26.COVS2.S led to a 2.14-fold increase (**Figure 4B**). Both doses of
399 BNT162b2 demonstrated a superior ability to boost neutralizing antibodies at W2 (8.5 to 10.2-
400 fold compared to BL), with all but one participant increasing neutralizing titer (**Figure 4B**). As
401 expected from its known immune evasion properties, FRNT₅₀ values for BA.5 neutralization
402 were considerably lower than for ancestral virus (GMT of 84, 75, 84 and 63 for the four arms,
403 respectively; **Figure 4C**). The kinetics mirrored those for D614G neutralization, with both
404 doses of BNT162b2 demonstrating enhanced boosting of neutralizing antibodies compared to
405 Ad26.COVS2.S (**Figure 4D**). By week 24, neutralizing titers had waned significantly for the
406 D614G virus, to a GMT of 732 and 840 for the BNT162b2 half- and full-doses, which was
407 significantly higher than full-dose Ad26.COVS2.S, with a GMT of 292 (**Figure 4E**).
408 Neutralization of BA.5 was poor at W24 (GMT of 73-192), with no differences between the

409 groups. Five participants with documented BTI between W2 and W24 (**Figure 4A,C, red**
410 **lines**) showed boosted neutralization activity, as expected.

411
412 In parallel, we assessed neutralizing antibody titers against an expanded panel of virus
413 variants, namely D614G, Beta, Delta, Omicron BA.1 and BA.4/5 (which share identical spikes)
414 using a lentiviral pseudovirus assay, limited to HIV-negative participants in the cohort. The
415 data were consistent with our observations from the live virus assay, where significant
416 boosting of neutralizing antibodies occurred at W2 against all variants for BNT162b2, but not
417 for either the half- or full- dose Ad26.COVS.S (**Figure 5**). Titers against all variants declined
418 from the W2 peak by W12, with a 2.4 to 5.7-fold drop, and levels remained constant to W24,
419 with BNT162b2 arms trending to higher titers, regardless of the variant at W24.

420
421 Finally, we investigated antibody-dependent cellular cytotoxicity (ADCC) responses against
422 the D614G, Beta, Delta and BA.1 spikes (**Figure 6A and 6B**) at BL and 2 weeks post-boost.
423 As observed for the binding and neutralizing antibody responses, there was no increase in
424 titers at W2 for either the half- or full-dose Ad26.COVS.S arms (median fold change 0.97 and
425 0.91, respectively), consistent with the fact that binding antibodies and ADCC potential are
426 generally correlated. However, the half-dose BNT162b2 failed to trigger increased ADCC
427 (median change 1.01), in contrast to the binding and neutralization results. For the full-dose
428 BNT162b2 we observed marginally but nevertheless significantly higher ADCC titers against
429 D614G, Beta and BA.1 spike at W2 compared to BL, but not to the same extent as binding or
430 neutralizing responses.

431
432 **T-cell responses before and after booster vaccination**

433 We also measured SARS-CoV-2 spike-specific T-cell responses before and after homologous
434 and heterologous vaccination with full or half-dose vaccines (n=214). Prior to boosting, spike-
435 specific CD4+ responses were detected in most participants (>94.4%), with a frequency
436 comparable between the four arms (**Figure 7A**). Two weeks after booster immunization, the

437 frequency of spike-specific CD4+ T cells was significantly increased in participants who
438 received BNT162b2 compared to BL (median: 0.23% vs 0.12% for half dose BNT162b2 and
439 0.25% vs 0.11% for full dose BNT162b2, respectively), while in participants boosted with
440 Ad26.COVS.2, the median frequency of SARS-CoV-2 spike-specific CD4+ T cells, while
441 statistically higher than BL, demonstrated only a marginal increase (0.12% vs 0.10% for half
442 dose and 0.15% vs 0.11% for full dose) (**Figure 7B**). The median fold change in CD4+
443 response between BL and W2 was significantly greater after a BNT162b2 booster (1.8 for half
444 dose and 1.98 for full dose) compared to an Ad26.COVS.2 booster (1.1 for both half and full
445 dose; **Figure 7C**). Overall, ~80% of participants boosted with BNT162b2 (regardless of the
446 dose) had an increased spike-specific CD4+ T-cell response, while only 35.3% of participants
447 who received half dose Ad26.COVS.2 and 43.4% in those receiving a full dose Ad26.COVS.2
448 booster expanded their CD4+ T-cell responses (**Figure 7D**). Spike-specific CD8+ T-cell
449 responses were less frequent than CD4+ responses, detected in only ~55% of the participants
450 at BL, and for those with a CD8+ response, the frequency of spike-specific CD8+ T cells was
451 comparable between the four groups at BL (**Figure 7E**). Two weeks after boosting, a
452 significant increase in the median CD8+ T-cell response was observed after a full-dose of
453 Ad26.COVS.2 ($p = 0.002$) and both half- or full-dose of BNT162b2 ($p = 0.003$ and $p < 0.001$,
454 respectively; **Figure 7F**). Assessing only participants with a spike CD8+ response at both time
455 points, the median fold change at W2 post-boost was significantly higher after a BNT162b2
456 booster (1.91 for half-dose and 2.24 for full-dose) compared to an Ad26.COVS.2 booster (1.22
457 for half-dose and 1.24 for full-dose; **Figure 7G**). However, it is important to note that individual
458 responses were highly variable within all groups, with spike-specific CD8+ responses
459 contracting, remaining negative or expanding. Overall, approximately half of participants
460 boosted with BNT162b2 displayed an increase in spike-specific CD8+ T-cell responses,
461 whereas an expansion of the CD8+ response was observed in only 1/3 of participants who
462 received a half-dose Ad26.COVS.2 booster and ~40% in those receiving a full-dose
463 Ad26.COVS.2 booster (**Figure 7H**).

464

465 The durability of T-cell responses was then assessed by measuring spike-specific T cells 24
466 weeks after the booster vaccination in a subset of participants (n=190). Pairwise comparison
467 of spike-specific CD4+ T-cell frequencies at W24 relative to W2 demonstrated a significant
468 contraction in the CD4+ response for all groups (p <0.001 for both BNT162b2 boosters and
469 half-dose Ad26.COV2.S and p = 0.0047 for full-dose Ad26.COV2.S), subsiding to BL levels
470 (**Figure 8A**). Consequently, 24 weeks post-boosting, the magnitude of spike-specific CD4+ T
471 cells was comparable in all trial arms (**Figure 8B**). In contrast, the median frequency of spike-
472 specific CD8+ T cells appeared to increase between W2 and W24 in the Ad26.COV2.S group,
473 although individual responses were highly variable, with some participants showing
474 substantial waning or stable levels while others demonstrated gain of a CD8+ T-cell response
475 that could be related to an undocumented BTI (**Figure 8C**). Overall, as for the CD4+ T-cell
476 response, the frequency of spike-specific CD8+ T cells was similar across the four vaccine
477 regimens when examined 24 weeks after booster vaccination (**Figure 8D**).

478

479 **PLWH develop comparable antibody and T-cell responses after boosting**

480 To determine whether HIV infection impacts vaccine booster responsiveness, participants
481 were stratified by their HIV status. Prior to boosting, significantly lower titers of both binding
482 and live virus neutralizing antibodies were found in PLWH who were viremic (viral load >200
483 HIV-1 mRNA copies/mL, n = 10) compared to virally suppressed individuals, who had similar
484 antibody profiles to HIV-negative individuals (**Figure 9A-B**). Examination of baseline T-cell
485 responses also revealed deficiencies, where significantly fewer viremic PLWH displayed
486 spike-specific T-cell responses compared to aviremic and HIV-negative participants at BL
487 (50% vs 100% and 97.6% for CD4+ and 10% vs 58.2% and 57.6% for CD8+; **Figure 9C-D**).
488 Two weeks after booster vaccination, there was no difference in the degree of boosting (as
489 measured by median fold-change between W2 and BL) for binding or neutralizing antibody
490 titers between PLWH and HIV-negative participants for all vaccination groups (**Figure 9E-F**).
491 For T-cell responses, however, the median fold change in CD4+ response in the full dose
492 BNT162b2 group was significantly lower in PLWH compared to the HIV-negative participants

493 (1.52 vs 2.21, respectively, $p = 0.037$), with a trend towards lower fold-change in CD8+ spike
494 T-cell responses in PLWH ($p = 0.065$; **Figure 9G-H**). Of note, a high proportion of viremic
495 participants did not mount any CD4+ (4/10) or CD8+ T-cell responses (4/10) after any of the
496 vaccine boosts, indicative of the impact of immunosuppression (**Supplementary Fig S1**).

497
498 At W24, comparable titers of binding and neutralizing antibodies were detected between
499 PLWH and HIV-negative participants in all vaccination groups, and T-cell frequencies did not
500 show any deficiencies in PLWH (**Figure 9I-L**). In fact, significantly higher frequencies of spike-
501 specific CD4+ T cells were observed in PLWH in some vaccination groups (**Figure 9K**),
502 possibly reflecting persistence of the higher baseline CD4+ T-cell responses observed in
503 PLWH. Overall, these data suggest that some differences in the degree of boosting in PLWH,
504 but by 24 weeks after revaccination, the frequencies of binding and neutralizing antibodies as
505 well as spike-specific CD4+ and CD8+ T cells were comparable between HIV-negative
506 participants and PLWH. This suggests that SARS-CoV-2 specific responses persist to a
507 similar extent in those with well-controlled HIV infection compared to HIV-negative individuals.
508 A proportion of viremic individuals, however, may be at risk of impaired T-cell responses and
509 less durable antibody responses over the long term.

510

511 **Anti-vector immunity is detectable but may not account for the limited ability of**
512 **Ad26.COVS to boost spike-specific responses**

513 We hypothesized that the muted capacity of Ad26.COVS to boost spike responses,
514 compared to BNT162b2, may be due to anti-vector immunity, given that prior studies
515 demonstrated that pre-existing Ad5 vector-specific nAbs and T cells have the capacity to limit
516 responses to the transgene immunogen [44-46]. To measure anti-Ad26 neutralizing antibody
517 activity, we established an Ad26 neutralization assay in which dilutions of plasma from
518 vaccinees were mixed with the Ad26.COVS vaccine, and vector infection was measured by
519 spike expression in the Ad26 vector infected cells (**Figure 10A-B**). To quantify Ad26-specific
520 neutralizing activity before and after boosting, we titrated participant plasma to obtain the

521 plasma concentration needed to inhibit 50% of cellular infections with the Ad26 vector
522 represented as ID₅₀, the reciprocal of this plasma dilution (**Figure 10C**). We tested 24
523 participants who had received full dose Ad26.COVS2.S, and compared them to 24 participants
524 who were boosted with BNT162b2, where no expansion of the vector-specific response would
525 be expected. At BL, the majority of participants in both groups had Ad26-specific neutralizing
526 antibody responses above the level of quantification (**Figure 10D**). Two weeks later, there was
527 a significant increase in vector-specific ID₅₀ in the Ad26.COVS2.S boosted group ($p = 0.0006$),
528 but no boosting in the group receiving BNT162b2 (median fold-change in nAb ID₅₀ between
529 W2 and BL: 1.83 for Ad26.COVS2.S and 0.87 for BNT162b2; **Figure 10E**). To determine the
530 level of neutralizing immunity against Ad26.COVS2.S before primary Ad26.COVS2.S vaccination,
531 we investigated an independent cohort where participants were vaccinated with Ad26.COVS2.S
532 for the first time, with a subset accessing a second Ad26.COVS2.S vaccine (**Supplementary**
533 **Table 1**). The levels of anti-Ad26 neutralizing immunity prior to Ad26.COVS2.S prime
534 vaccination were below the level of quantification in all but one participant. Titers were
535 significantly higher after an Ad26.COVS2.S prime ($p < 0.0001$; **Figure 10F**) but did not further
536 increase after a boost, and were of similar magnitude to those in the BaSiS trial (**Figure 10F**).
537 Finally, we investigated the association between Ad26-specific nAb titer at BL and the fold-
538 change in spike neutralizing titer from W2 to BL, but found no correlation (**Figure 10G**).

539
540 To measure Ad26-specific T-cell responses, we stimulated PBMC with a pool of peptides
541 spanning the viral hexon and penton proteins of Ad26 and characterized T-cell cytokine
542 responses by flow cytometry. Ad26-specific T cells were readily detectable at BL and 2 weeks
543 post-boosting (**Figure 11A**). We tested 44 participants who had received full-dose
544 Ad26.COVS2.S, and compared them to 20 participants who were boosted with BNT162b2, as a
545 control group. At baseline, all participants in both groups had a CD4+ T-cell response to Ad26
546 (**Figure 11B**). Two weeks after Ad26.COVS2.S booster vaccination, there was no overall
547 increase in the Ad26-specific CD4+ response, which resembled the group who received the
548 BNT162b2 booster. When comparing the fold-difference in the response from BL to W2, there

549 was a minor increase in the Ad26.COVS.S-boosted group (1.08 for Ad26.COVS.S and 0.91 for
550 BNT162b2), reflecting a proportion of participants who had a marginal increase in the
551 frequency of Ad26-specific CD4+ T cells (**Figure 11C**). We also investigated an independent
552 unvaccinated healthcare worker cohort prior to Ad26.COVS.S vaccination (**Supplementary**
553 **Table 2**); cross-sectional analysis indicated a significant, 3-fold higher magnitude of Ad26-
554 specific CD4+ T-cell responses 4 weeks after vaccination ($p = 0.017$; **Figure 11D**), similar to
555 what we observed for Ad26 nAb responses. Interestingly, 85% of participants had an existing
556 adenovirus CD4+ response even before the priming dose, which is the likely result of
557 conserved T-cell cross-reactivity due to infection with other adenoviral types.

558 Similarly, we characterized CD8+ T-cell responses specific for Ad26. These were detectable
559 in 75% of participants at baseline, and unlike CD4+ responses, were boosted significantly ($p =$
560 0.002) by a second dose of Ad26.COVS.S, demonstrating an increase in response of 1.2
561 compared to 0.95 for the BNT162b2-boosted group (**Figure 11E and F**). CD8+ responses
562 were more rare in the cohort not previously vaccinated with Ad26.COVS.S, detectable in only
563 35% of unvaccinated participants. These were significantly higher ($p = 0.005$) and detectable
564 in 81.8% of individuals 4 weeks after initial Ad26.COVS.S vaccination, at a magnitude and
565 range similar to the baseline responses in our trial (**Figure 11G**). Importantly, and consistent
566 with Ad26-specific nAb responses, there was no association between the magnitude of Ad26-
567 specific CD4+ or CD8+ T cells and spike-specific T-cell responses, either at BL or W2 (**Figure**
568 **11H**). Overall, anti-vector humoral and cellular immunity was abundantly detectable in our trial
569 participants, but neither appeared to have a clear effect on dampening spike-specific
570 responses.

571 **Discussion**

572 In this trial we evaluated the safety and immunogenicity of fractional and full doses of the
573 Janssen adeno-vectored Ad26.COVID.S vaccine and the Pfizer BNT162b2 mRNA vaccine in
574 an open label phase 2 trial of adults who had previously received a single dose of
575 Ad26.COVID.S. The overwhelming majority of participants had detectable anti-nucleocapsid
576 responses prior to receiving their boosts, indicating prior SARS-CoV-2 infection and boosting
577 on the background of extensive hybrid immunity. We investigated safety and immunogenicity
578 in both HIV-negative participants and PLWH, with the latter group generally well-controlled
579 with VL suppression, and only 13.8% of PLWH being viremic (VL >200 cps/ml). Safety profiles
580 in all four booster regimens were similar, with no regimen resulting in appreciable increased
581 reactogenicity beyond localized responses at the injection site and mild, transient symptoms
582 such as headache and weakness. Documented BTI, while few, mostly occurred in the
583 Ad26.COVID.S boosted participants.

584 Heterologous boosting with the BNT162b2 mRNA vaccine was superior to homologous
585 vaccination at early time points (2-12 weeks) by multiple humoral and cellular immune
586 measures, including binding and neutralizing antibodies, and CD4+ T-cell responses to SARS-
587 CoV-2 spike. This result is consistent with other studies showing better neutralizing antibody
588 and T-cell responses after heterologous relative to homologous boosting of Ad26.COVID.S
589 with an mRNA vaccine [23, 50]. Similar results were observed with mRNA vaccine
590 heterologous boosting of the ChAdOx1 chimpanzee adeno-vectored vaccine [51, 52].

591 In contrast to the BNT162b2 vaccine, a homologous Ad26.COVID.S boost elicited limited, if
592 any boosting of immune responses. Binding antibodies, neutralization, and T-cell responses
593 increased 2-fold or less, and halving the dose resulted in no detectable booster activity. In
594 contrast, boosting with BNT162b2 gave a 4- to 10-fold increase of antibody responses, with
595 the exception of ADCC (discussed below), and 80% of trial participants had increased spike
596 specific CD4+ T-cell responses. Unlike with Ad26.COVID.S, halving the dose of BNT162b2 did
597 not strongly decrease the response to the booster in terms of either elicited binding
598 antibodies, neutralizing antibodies, or CD4+ T-cell responses. Despite the strong boosting

599 effect of BNT162b2, waning of the binding and neutralizing antibody responses was
600 pronounced by 12 weeks post-boost and by week 24, titers had declined close to the relatively
601 high baseline titers. For ADCC, which generally correlates well with binding and neutralization,
602 the absence of boosting by Ad26.COV2.S is expected, given the lack of boosting overall.
603 However the marginal ADCC boosting observed only in the full-dose BNT162b2 arms (despite
604 increased binding and neutralization) may be a consequence of class switching towards the
605 IgG4 subclasses, which does not effectively mediate ADCC, as recently reported for repeated
606 mRNA vaccination [53, 54].

607 The effects of HIV status on the neutralizing antibody and T-cell responses were moderate,
608 and largely limited to reduced responses observed at baseline in PLWH with HIV viremia
609 (despite the small size of this group). Both binding neutralizing antibody responses to
610 ancestral SARS-CoV-2 were significantly lower in HIV viremic participants at baseline, while
611 there was no difference between aviremic PLWH and HIV-negative participants. CD4+ T-cell
612 responses showed a more marked difference with only 50% of viremic PLWH having
613 detectable spike-specific CD4+ T cells, compared with 100% of aviremic PLWH and 97% of
614 HIV-negative participants. For CD8+ T cells, the fraction of detectable spike-specific cells was
615 58% for both aviremic PLWH and HIV negative participants. In contrast, only 1 out of 10 HIV
616 viremic participants had a detectable spike-specific CD8+ T-cell response. Interestingly, the
617 median fraction of spike specific CD4+ T cells was significantly higher in aviremic PLWH than
618 the HIV-uninfected participants, perhaps indicative of a different SARS-CoV-2 course of
619 infection in PLWH [27] or interactions of SARS-CoV-2 immunity with HIV-mediated partial
620 immune activation in HIV suppressed PLWH [55].

621 Post-boost, binding antibodies and neutralization were generally similar between PLWH and
622 HIV negative participants. Even viremic PLWH showed increased binding and neutralizing
623 antibody responses after BNT162b2 vaccination, and these were within the range seen in the
624 other participants. These results are consistent with previous studies that PLWH have good
625 antibody responses to vaccination [19, 38, 39, 56] with the exception of those with CD4+ T cell
626 concentrations than 200 cells/mm³ [34, 36, 37, 57, 58]. T-cell responses were more strongly

627 affected by HIV status: they were significantly lower for spike-specific CD4+ T cells in PLWH
628 boosted with a full-dose of BNT162b2. We did not detect an increase in spike-specific CD8+
629 T-cell responses with full-dose BNT162b2, although the results did not reach statistical
630 significance. The interpretation of the CD4+ T cell results is complicated by the higher median
631 absolute fraction of spike-specific CD4+ T cells in PLWH post- BNT162b2 boost.
632 Although the Ad26.COVS.2 is known to trigger lower responses than BNT162b2, the almost
633 complete absence of boosting by this vaccine in our cohort was striking. We thus assessed
634 whether this was caused by anti-vector immunity. Vaccination with adenovirus 5 vectored
635 vaccines elicits both neutralizing antibody and T-cell immunity to the vector itself, decreasing
636 the efficiency of cellular infection with the vaccine vector and attenuating the vaccine response
637 [44-46]. Using the samples from this trial and supplementing with other cohorts where a pre-
638 Ad26.COVS.2 prime vaccination sample was available, we observed negligible Ad26.COVS.2
639 neutralizing activity in vaccine-naïve donors. However, a single dose of Ad26.COVS.2 strongly
640 increases both neutralizing titers against Ad26.COVS.2 and the fraction of Ad26-specific
641 CD4+ and CD8+ T cells. Nevertheless, as with other studies of anti-Ad26.COVS.2
642 neutralization, we could not find a clear association between the levels of anti-vector immunity,
643 either neutralizing or T cell, and the degree of immune response post-Ad26.COVS.2 boost, as
644 previously reported [47, 48].
645 To conclude, we show that in the context of high levels of hybrid immunity, heterologous
646 boosting with the BNT162b2 mRNA vaccine following a Ad26.COVS.2 prime demonstrated
647 superior immunogenicity and was safe and effective in both PLWH and HIV-negative
648 participants, although there was a rapid waning of binding and neutralizing antibody
649 responses. The Ad26.COVS.2 homologous boost was also safe but showed highly attenuated
650 immunogenicity relative to BNT162b2, both in antibody and T-cell immunity. While the
651 Ad26.COVS.2 vaccine elicited strong anti-vector immunity, we did not find clear evidence that
652 this was the cause of attenuation.

653 **Author contributions**

654 Study design: LF, ASi, PLM, WAB, GG, L-GB, HR, SS, EL in collaboration with the sponsor
655 (SAMRC). Data curation: JNB, TM-G, SS, NG, NN, EL. Experiment/investigation: RSK, RN,
656 MAO, HM, RB, ANg, AW, SG, AAN, SFJM, ASB, NB, PM, SC, YG, KK, TM-G, ZM, EV, SvG,
657 PK, QM, FA, SRB, TPM, TH, NPM, NBM, HK, MM, FP, JLR, SS, NG, NN, EL, RP, ANa.
658 Methodology: WAB, CR, ASi, PLM, JNB, SIR, FP, SS. Data Analysis: WAB, CR, MPB, GL,
659 ASi, PLM, JNB, SIR, LF, JLR, SS. Resource: AG, ASe. Project administration: WAB, ASi,
660 PLM, JNB, CC, LF, FP, JLR, SS, NG, NN, TPN, MD, KMG. Writing (original draft): LF, PLM,
661 ASi, WAB, CR, JNB, SS. Writing (review and editing): All authors reviewed and approved the
662 final draft.

663

664 **Acknowledgements and funding**

665 We thank all the trial participants who contributed to this study. This study was funded by the
666 South African Medical research Council (SAMRC), the Bill and Melinda Gates Foundation,
667 through the Global Immunology and Immune Sequencing for Epidemic Response (GIISER)
668 program (INV-030570) and the Wellcome Trust (226137/Z/22/Z). P.L.M is supported by the
669 SAMRC (96833) and is an Department of Science and Innovation-National Research
670 Foundation South African Research Chair (98341). A.Si. is supported by the Bill and Melinda
671 Gates foundation (INV-046743) and the SAMRC (D2112300-01). W.A.B. is supported by the
672 EDCTP2 program of the European Union's Horizon 2020 programme (TMA2016SF-1535-
673 CaTCH-22) and the EU-Africa Concerted Action on SARS-CoV-2 Virus Variant and
674 Immunological Surveillance (COVICIS), funded through the EU's Horizon Europe Research
675 and Innovation Programme (101046041). C.R. is supported by the EDCTP2 program
676 (TMA2017SF-1951-TB-SPEC). This project has also been funded in part by the National
677 Institute of Allergy and Infectious Diseases, NIH, Department of Health and Human Services,
678 under Contract No. 75N93021C00016 to A.G. and 75N93019C00065 to A.Se. The Wits RHI
679 site received grant funding from Janssen to conduct the following clinical trials: Ensemble
680 study (3UM1 AI068614-14SI), the Sisonke 1 study (96833), the Sherpa Study (96867) as well

681 as Pfizer for the Pfizer C4591015 study (C4591015), Horizon 1 (VAC31518COV2004) and
682 Horizon 2 (VAC31518COV3006). For the purposes of open access, the authors have applied
683 a CC-BY public copyright license to any author-accepted version.

684

685 **Declaration of interest:**

686 A.Se. is a consultant for AstraZeneca Pharmaceuticals, Calyptus Pharmaceuticals, Inc,
687 Darwin Health, EmerVax, EUROIMMUN, F. Hoffman-La Roche Ltd, Fortress Biotech, Gilead
688 Sciences, Granite bio., Gritstone Oncology, Guggenheim Securities, Moderna, Pfizer,
689 RiverVest Venture Partners, and Turnstone Biologics. A.G. is a consultant for Pfizer. LJl has
690 filed for patent protection for various aspects of T cell epitope and vaccine design work. All
691 other authors declare no competing interests.

692 **Figure legends**

693

694 **Figure 1: Study design and CONSORT diagram. (A)** Study design. **(B)** CONSORT flow
695 diagram. BL: baseline, nAbs (Live): Live virus neutralization assay; nAbs (Pseudo):
696 pseudovirus neutralization assay. Binding Abs: Spike-specific IgG ELISA. ADCC: Antibody-
697 dependent cellular cytotoxicity assay. T-cell response: Spike-specific T-cell intracellular
698 cytokine staining assay.

699

700 **Figure 2: Recorded adverse events in each study arm.** Distribution of participants
701 experiencing adverse events (pain, headache, tenderness, weakness, nausea, diarrhea,
702 cough, myalgia, swelling, chills, loss of taste, redness, loss of smell or fever) recorded 1 week
703 post booster vaccination in each study arm.

704

705 **Figure 3: Spike-specific IgG responses over time in the immunogenicity sub-study**
706 **population. (A)** Longitudinal spike-specific IgG titer (EC_{50}) at baseline (BL), W2, W12 and
707 W24 after vaccine booster. The color-coded dots and bold lines represent the geometric mean
708 titer (GMT) at each time point. Recorded BTI between W2 and W24 are depicted with a red
709 line. Fold-change in the GMT is indicated at the bottom of each graph. Statistical comparisons
710 were performed using a Friedman test with Dunn's correction. **(B)** Fold change in spike-
711 specific IgG titer between W2 and BL in each study arm. Bars represent median fold change.
712 Statistical comparisons were performed using a Kruskal-Wallis test with Dunn's correction. **(C)**
713 Comparison of spike-specific IgG titer between study arms at W12 (left panel) and W24 (right
714 panel). Bars represent GMT. Statistical comparisons (in B and C) were performed using a
715 Kruskal-Wallis test with Dunn's correction.

716

717 **Figure 4: Live-virus neutralization activity against ancestral D614G and BA.5 SARS-**
718 **CoV-2 variant after booster vaccination.** Neutralizing titer ($FRNT_{50}$) against ancestral
719 D614G **(A&B)** and Omicron BA.5 **(C&D)** at BL and post-vaccine booster. **A&C** show titer at

720 BL, W2 and W24 post-boost for D614G **(A)** and BA.5 **(C)**. Fold-change of the GMT is
721 indicated at the bottom of each graph. The color-coded dots and bold lines represent the GMT
722 at each time point. Recorded BTI between W2 and W24 are depicted with red lines. Statistical
723 comparisons were performed using a Friedman test with Dunn's correction. **B&D** show fold-
724 change in neutralizing titer against D614G **(B)** and BA.5 **(D)** between BL and W2 in each
725 study arm. Bars represent median fold-change. A Kruskal-Wallis test with Dunn's correction
726 was used to compare different arm groups. **(E)** Comparison of the neutralizing titer (FRNT₅₀)
727 against D614G (left panel) and BA.5 (right panel) between study arms at W24. Bars represent
728 GMT. Statistical comparisons were performed using a Kruskal-Wallis test with Dunn's
729 correction.

730
731 **Figure 5: Pseudovirus neutralization activity against ancestral D614G, Beta, Delta, BA.1**
732 **and BA.4/5 SARS-CoV-2 variants after booster vaccination.** Longitudinal neutralizing titer
733 (ID₅₀) against ancestral D614G, Beta, Delta, Omicron BA.1 and BA.4/5 at BL, W2, W12 and
734 W24 after vaccine booster. Bars represent medians. Bars represent GMT. Statistical
735 comparisons were performed using a Kruskal-Wallis test with Dunn's corrections. Only HIV-
736 negative participants were included in these analyses.

737
738 **Figure 6: Antibody-dependent cellular cytotoxicity (ADCC) against ancestral (D614G),**
739 **Beta, Delta and Omicron BA.1 SARS-CoV-2 variants. (A)** ADCC (CD16 signalling) at BL
740 and W2 in each study arm. Bars represent medians. The grey shaded area indicates an
741 undetectable ADCC response. Statistical comparisons were performed using a Wilcoxon
742 matched-pairs signed rank test. **(B)** Fold change in ADCC activity between W2 and BL in each
743 study arm. Bars represent median fold change. Statistical comparisons were performed using
744 a Kruskal-Wallis test with Dunn's corrections.

745
746 **Figure 7: SARS-CoV-2 spike-specific T-cell responses before and 2 weeks after vaccine**
747 **boosting. (A)** Comparison of the frequency of spike-specific CD4+ T cells pre-boost in the

748 four study arms. The grey shaded area indicates undetectable response. **(B)** Frequency of
749 spike-specific CD4+ T cells before (BL) and after vaccine boost (W2). **(C)** Fold change in the
750 frequency of spike-specific CD4+ T cells between W2 and BL. **(D)** Overall profile of the
751 evolution of the spike-specific CD4+ T-cell response between BL and W2. **(E)** Comparison of
752 the frequency of spike-specific CD8+ T cells pre-boost in the four trial arms. **(F)** Frequency of
753 spike-specific CD8+ T cells before (BL) and after vaccine boost (W2). **(G)** Fold change in the
754 frequency of spike-specific CD8+ T cells between W2 and BL. **(H)** Overall profile of the
755 evolution of the spike-specific CD8+ T-cell response between BL and W2. Bars represent
756 medians. A two-tailed Wilcoxon signed-rank test was used to assess statistical differences
757 between paired samples and a Kruskal-Wallis with Dunn's corrections was used to compare
758 different groups.

759

760 **Figure 8: Kinetics of SARS-CoV-2 spike-specific T-cell response after vaccination. (A)**

761 Longitudinal frequencies of spike-specific CD4+ T-cell responses induced by the four different
762 booster vaccine regimens. **(B)** Comparison of the frequency of spike-specific CD4+ T cells
763 between the four arms at W24 post-boost. **(C)** Longitudinal spike-specific CD8+ T-cell
764 responses induced by the four booster vaccine regimens. **(D)** Comparison of the frequency of
765 spike-specific CD8+ T cells between the four arms at W24 post-boost. The proportion of spike
766 CD8+ responders is indicated at the top of the graph. The grey shaded area indicates
767 undetectable response. The color-coded dots and bold lines in (A) and (C) represent the
768 median at each time point. Recorded BTI between W2 and W24 are depicted with a red line. A
769 Friedman test with Dunn's correction was used to assess statistical differences between
770 paired samples and a Kruskal-Wallis with Dunn's corrections was used to compare different
771 groups.

772

773 **Figure 9: Humoral and cellular responses in study participants stratified by HIV status.**

774 **(A to D)** Spike-specific binding antibodies (A), live neutralization activity (B), spike-specific
775 CD4+ response (C) and spike-specific CD8+ response (D) against ancestral (D614G) SARS-

776 CoV-2 in HIV-negative (HIV-), PLWH with a viral load <200 HIV mRNA copies/ml (HIV+ Avir)
777 and PLWH with a viral load >200 HIV mRNA copies/ml (HIV+ Vir) before vaccine booster (BL).
778 Bars represent GMT for A, B, I, J and medians for all other graphs. Statistical comparisons
779 were performed using a Kruskal-Wallis test with Dunn's corrections. The proportion of T-cell
780 responders is indicated on top of the graph. **(E to H)** Fold change in spike-specific binding
781 antibodies (E), live neutralization activity (F), spike-specific CD4+ response (G) and spike-
782 specific CD8+ response (H) between W2 and BL in each study arm, stratified by HIV status. **(I**
783 **to L)** Spike-specific binding antibodies (I), live neutralization activity (J), spike-specific CD4+
784 response (K) and spike-specific CD8+ response (L) against ancestral (D614G) SARS-CoV-2
785 at W24 after vaccine booster. Viremic PLWH are identified with a cross. Bars represent
786 medians. Statistical comparisons between PLWH and HIV-negative groups were performed
787 using a Mann-Whitney test.

788
789 **Figure 10: Ad26-specific neutralizing activity. (A)** Schematic representation of the Ad26-
790 specific neutralization assay. **(B)** Representative example of spike expression in
791 Ad26.COVS-infected H1299 cells measured by flow cytometry. **(C)** Representative example
792 of the inhibition of spike expression on Ad26.COVS-infected H1299 cells when
793 Ad26.COVS was pre-incubated with plasma (serial dilution) from a participant vaccinated
794 with one full dose of Ad26.COVS. **(D)** Ad26 neutralization activity (IC₅₀) pre- and W2 post full
795 dose-Ad26.COVS or a full-dose BNT162b2 booster. Statistical difference were assessed
796 using a Wilcoxon matched paired signed rank test. **(E)** Fold change in Ad26 neutralization
797 activity between W2 and BL in Ad26.COVS or BNT162b2 boosted participants. Bars
798 represent GMT for D and F and medians for E. Statistical differences were assessed using a
799 Wilcoxon matched paired signed rank test. **(F)** Comparison of Ad26 neutralization activity
800 (IC₅₀) in individuals who were vaccine naïve (n=14), received one full dose of Ad26.COVS
801 (n=14) or received two full doses of Ad26.COVS (n=6) from an independent cohort.
802 Statistical differences were assessed using a Kruskal-Wallis test with Dunn's correction. **(G)**
803 Relationship between the fold change in neutralizing titer against D614G SARS-CoV-2

804 between W2 and BL and Ad26 neutralization activity at BL. Correlation was tested by a two-
805 tailed non-parametric Spearman's rank test.

806

807 **Figure 11: Ad26-specific T-cell responses. (A)** Representative example of IFN-g production
808 in response to Ad26-specific peptide pool (hexon and penton) in one participant before
809 (baseline) and 2 weeks after a full dose-Ad26.COVS booster. **(B & E)** Frequency of Ad26-
810 specific CD4+ T cell (B) and CD8+ T cells (E) pre- and post a full dose-Ad26.COVS or a full-
811 dose BNT162b2 booster. Statistical difference were assessed using a Wilcoxon matched
812 paired signed rank test. **(C & F)** Fold change in the frequency of Ad26-specific CD4+ T cells
813 (C) and CD8+ T cells (F) between W2 and BL in full-dose Ad26.COVS or full-dose
814 BNT162b2 boosted participants. **(D & G)** Comparison of the frequency of Ad26-specific CD4+
815 T cells (D) and CD8+ T cells (G) in individuals who are vaccine naïve (n=20) or received one
816 full dose of Ad26.COVS (n=11) from an independent cohort. The proportion of Ad26 T- cell
817 responders is indicated at the top of each graph. Statistical differences were assessed using a
818 Kruskal-Wallis test with Dunn's correction and a Chi-test to compare proportions. Bars
819 represent medians. **(H)** Relationship between the frequency of spike-specific T-cell response
820 at BL or W2 and the frequency of Ad26-specific T-cell responses at BL. Correlations were
821 tested by a two-tailed non-parametric Spearman's rank test.

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Table 1

	Arm A Full-dose Ad26.COV2.S	Arm B Half-dose Ad26.COV2.S	Arm C Full-dose BNT162b2	Arm D Half-dose BNT162b2	Total
Vaccinated (N)	74	69	73	73	289
Age*	42 (35-48)	40 (35-45)	43 (35-50)	42 (35-49)	42 (35-49)
18-29 years	5 (6.8%)	5 (7.2%)	7 (9.6%)	6 (8.2%)	23 (8%)
30-44 years	41 (55.4%)	46 (66.7%)	34 (46.6%)	34 (46.6%)	155 (53.6%)
45-54 years	20 (27%)	10 (14.5%)	23 (31.5%)	22 (30.1%)	75 (26%)
≥ 55 years	8 (10.8%)	8 (11.6%)	9 (12.3%)	11 (15.1%)	36 (12.5%)
Sex					
Male	10 (13.5%)	8 (11.6%)	18 (24.7%)	15 (20.5%)	51 (17.6%)
Female	64 (86.5%)	61 (88.4%)	55 (75.3%)	58 (79.5%)	238 (82.4%)
Ethnicity					
Black African	69 (93.2%)	67 (97.1%)	69 (94.5%)	70 (95.9%)	275 (95.2%)
Other	5 (6.8%)	2 (2.9%)	4 (5.5%)	3 (4.1%)	14 (4.8%)
Days between prime and Booster*	265 (245-292)	271 (259-302)	273 (258-291)	272 (253-300)	271 (255-296)
BMI					
Underweight	0 (0%)	2 (2.9%)	0 (0%)	1 (1.4%)	3 (1%)
Normal	12 (16.2%)	8 (11.6%)	14 (19.2%)	6 (8.2%)	40 (13.8%)
Overweight	20 (27%)	41 (59.4%)	39 (53.4%)	47 (64.4%)	71 (24.6%)
Obese	41 (55.4%)	39 (56.5%)	47 (64.4%)	47 (64.4%)	174 (60.2%)
Not available	1 (1.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
HIV infection					
HIV Positive	27 (36.5%)	28 (40.6%)	31 (42.5%)	30 (41.1%)	116 (40.1%)
Viremic [§]	1 (3.7%)	5 (17.9%)	4 (12.9%)	6 (20%)	16 (13.8%)
CD4 count (cells/mm ³)*	223 (na)	452 (103-471)	1136	341 (132-538)	452 (132-538)
Viral load (copies/mL)*	818 na	5838 (3054-18252)	29911 (14863-47207)	19960 (5324-37713)	15376 (3054-27420)
Aviremic [§]	26 (96.3%)	23 (82.1%)	27 (87.1%)	24 (80%)	100 (86.2%)
CD4 count (cells/mm ³)*	698 (580-923)	677 (530-855)	812 (623-931)	666 (538-737)	708 (558-922)
Prior SARS-CoV-2 infection^{&}	51/58 (87.8%)	52/57 (91.2%)	55/59 (93.2%)	55/59 (93.2%)	213/223 (91.4%)
Co-morbidities					
Hypertension	19 (25.7%)	8 (11.6%)	17 (23.3%)	17 (23.3%)	61 (21.1%)
Anaemia	7 (9.5%)	8 (11.6%)	6 (8.2%)	9 (12.3%)	30 (10.4%)
Asthma	6 (8.1%)	3 (4.3%)	2 (2.7%)	1 (1.4%)	12 (4.2%)
Diabetes mellitus	3 (4.1%)	1 (1.4%)	4 (5.5%)	4 (5.5%)	12 (4.2%)
Arthritis	1 (1.4%)	0 (0%)	3 (4.1%)	3 (4.1%)	7 (2.4%)
Tuberculosis	0 (0%)	4 (5.8%)	2 (2.7%)	0 (0%)	6 (2.1%)
Retention					
W2 visits completed	74 (100%)	66 (95.7%)	72 (98.6%)	69 (94.5%)	281 (97.2%)
W12 visits completed	74 (100%)	67 (97.1%)	67 (91.8%)	70 (95.9%)	278 (96.2%)
W24 visits completed	69 (93.2%)	63 (91.3%)	69 (94.5%)	68 (93.2%)	269 (93.1%)

Table 1: Clinical Characteristics of study participants

Unless specified, all data are presented as n (%N); *median and interquartile range (IQR); [§]% is of all HIV-infected participants; Viremic individuals were defined as having a HIV-1 viral load (VL) >200 HIV mRNA copies/mm³; 3 participants in the viremic arm C had a missing CD4 count; [&]Prior SARS-CoV-2 infection was defined by the presence of Nucleocapsid-specific IgG; na: non-applicable.

Figure 1

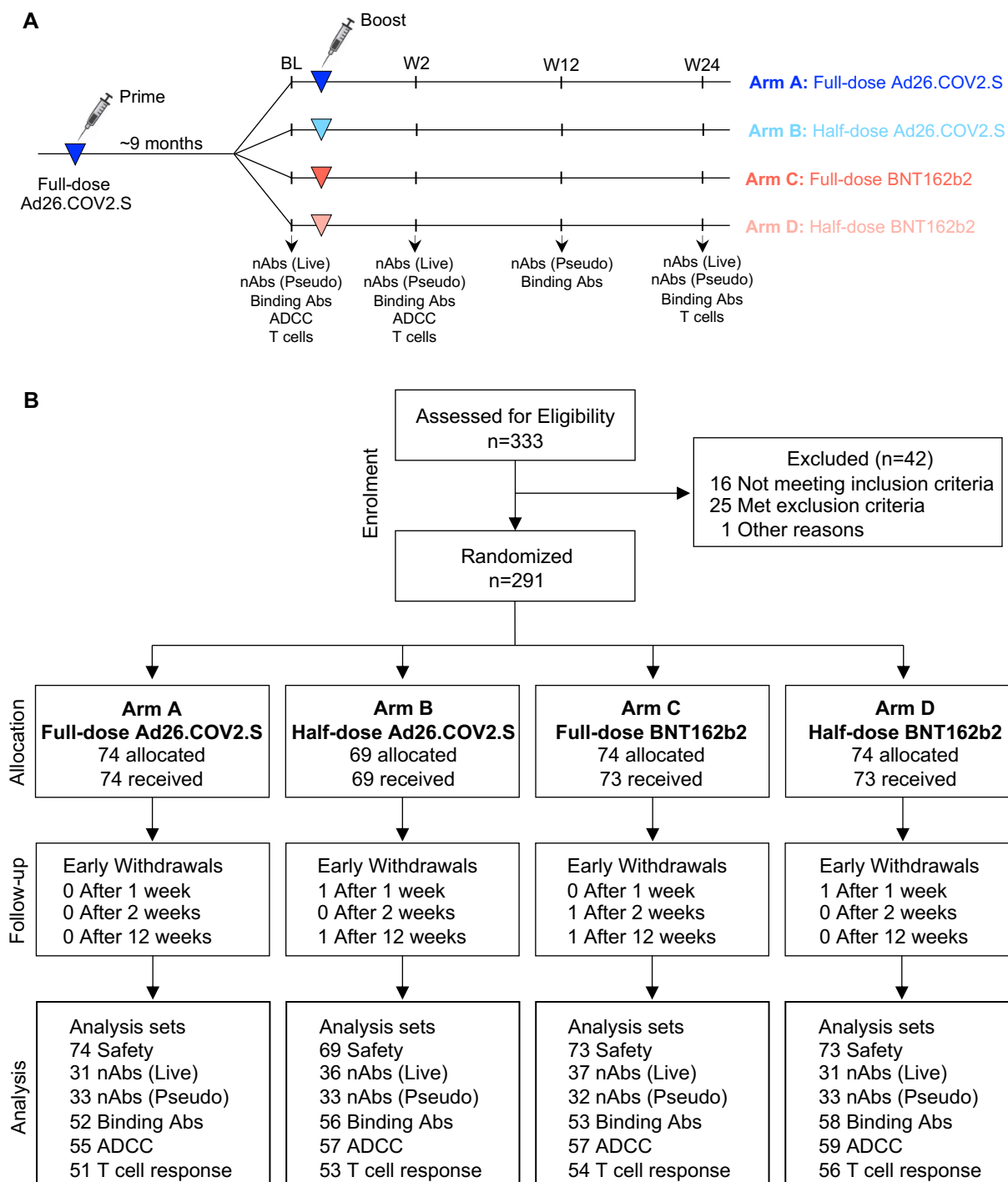
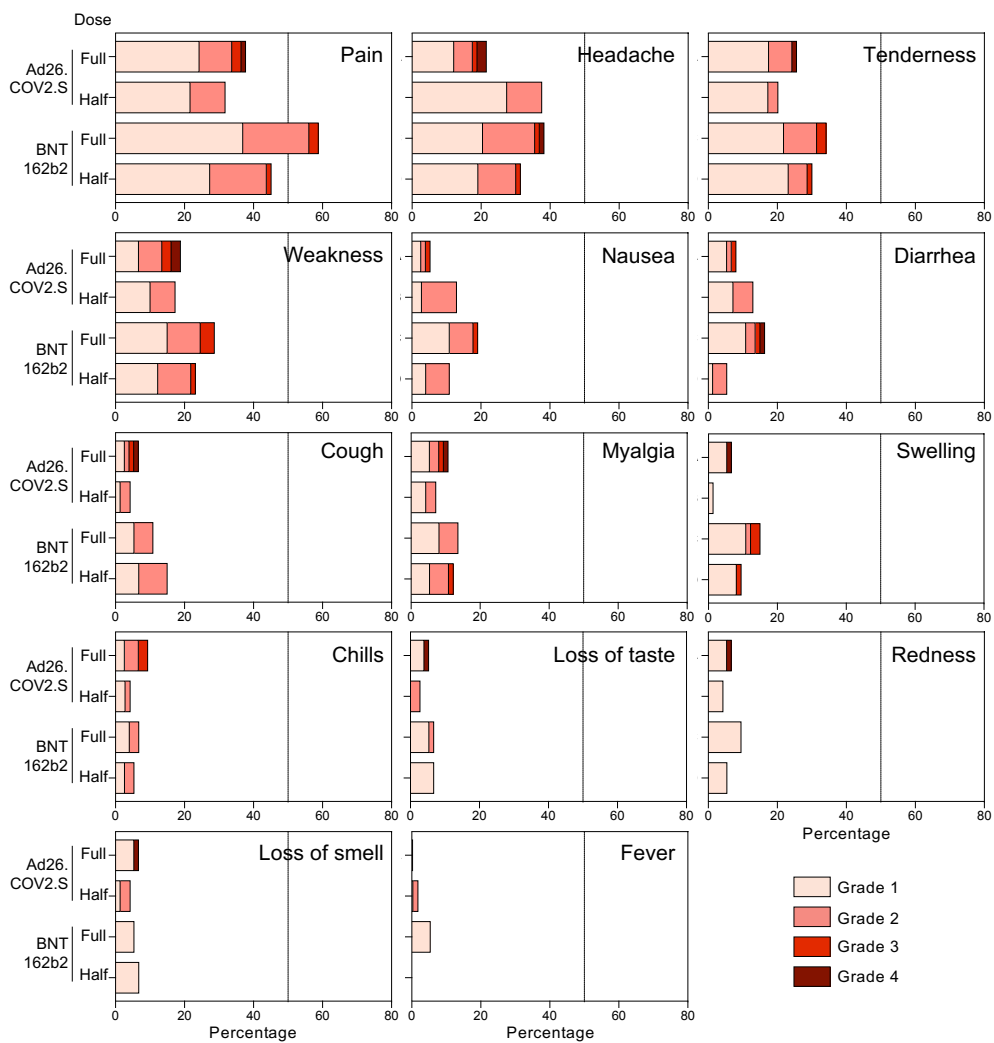
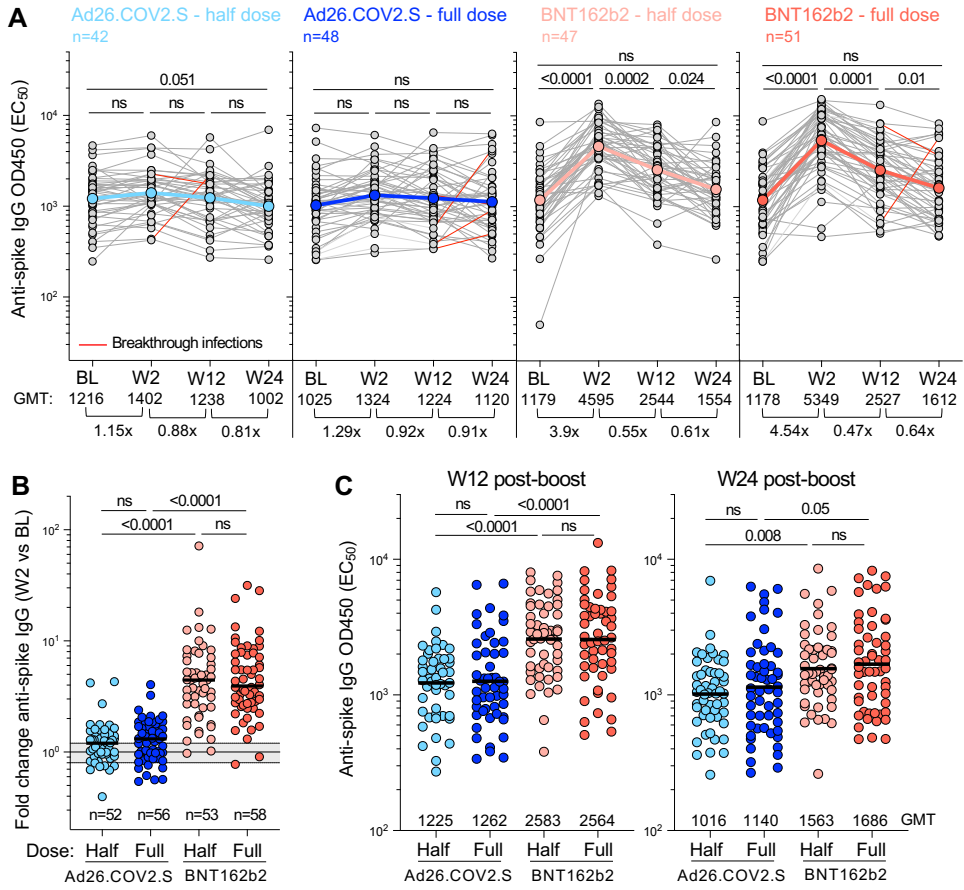


Figure 2





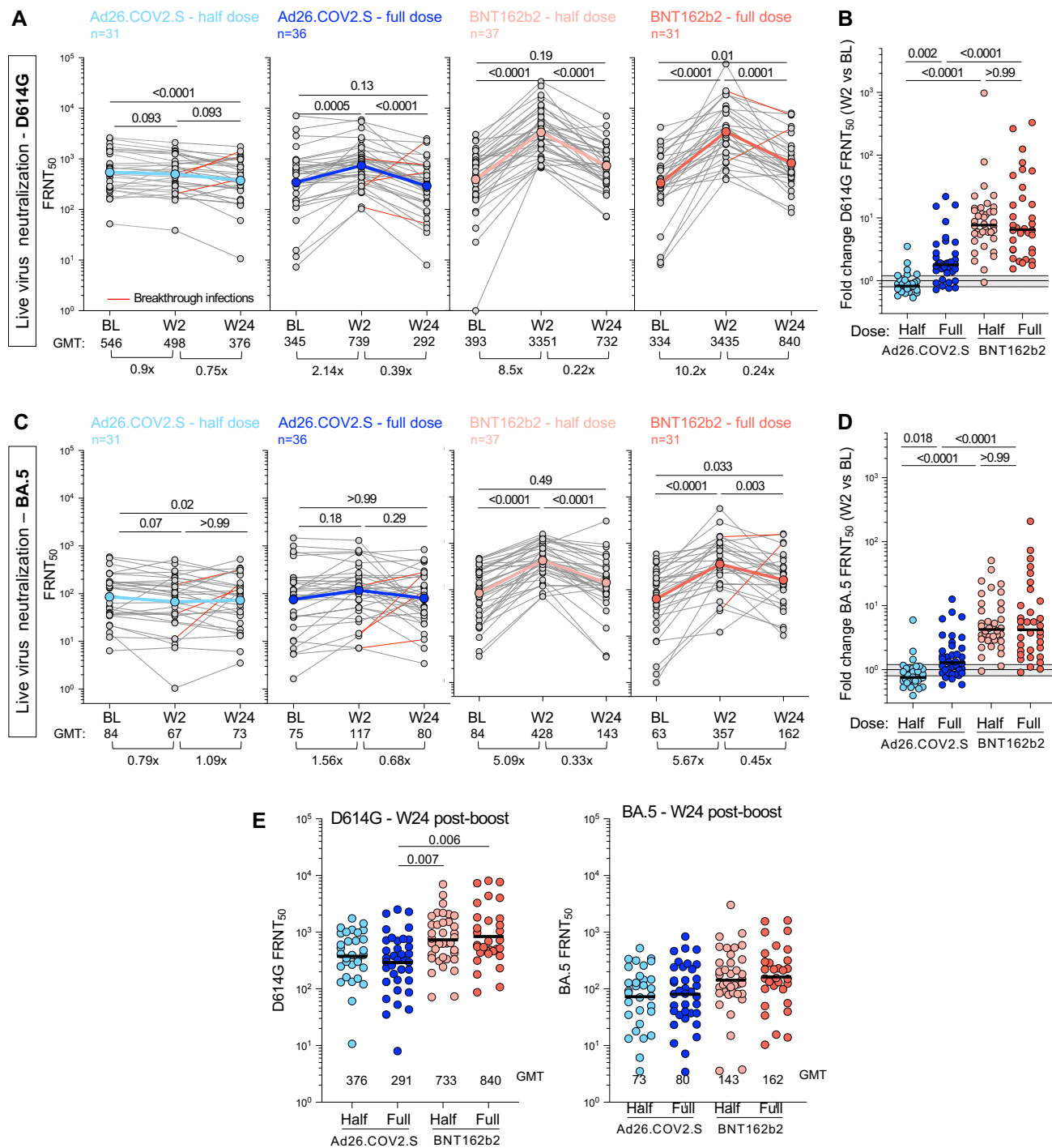
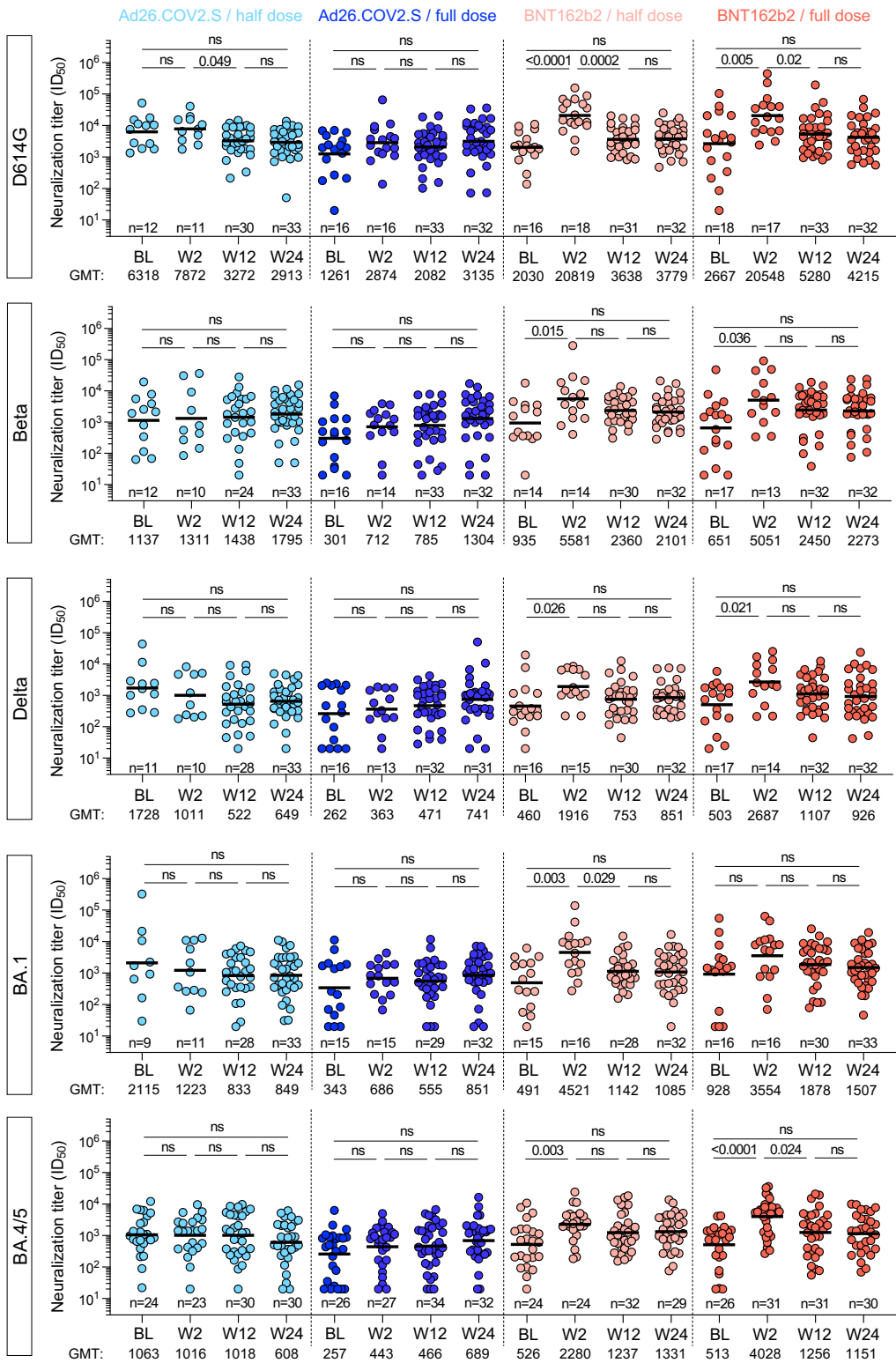
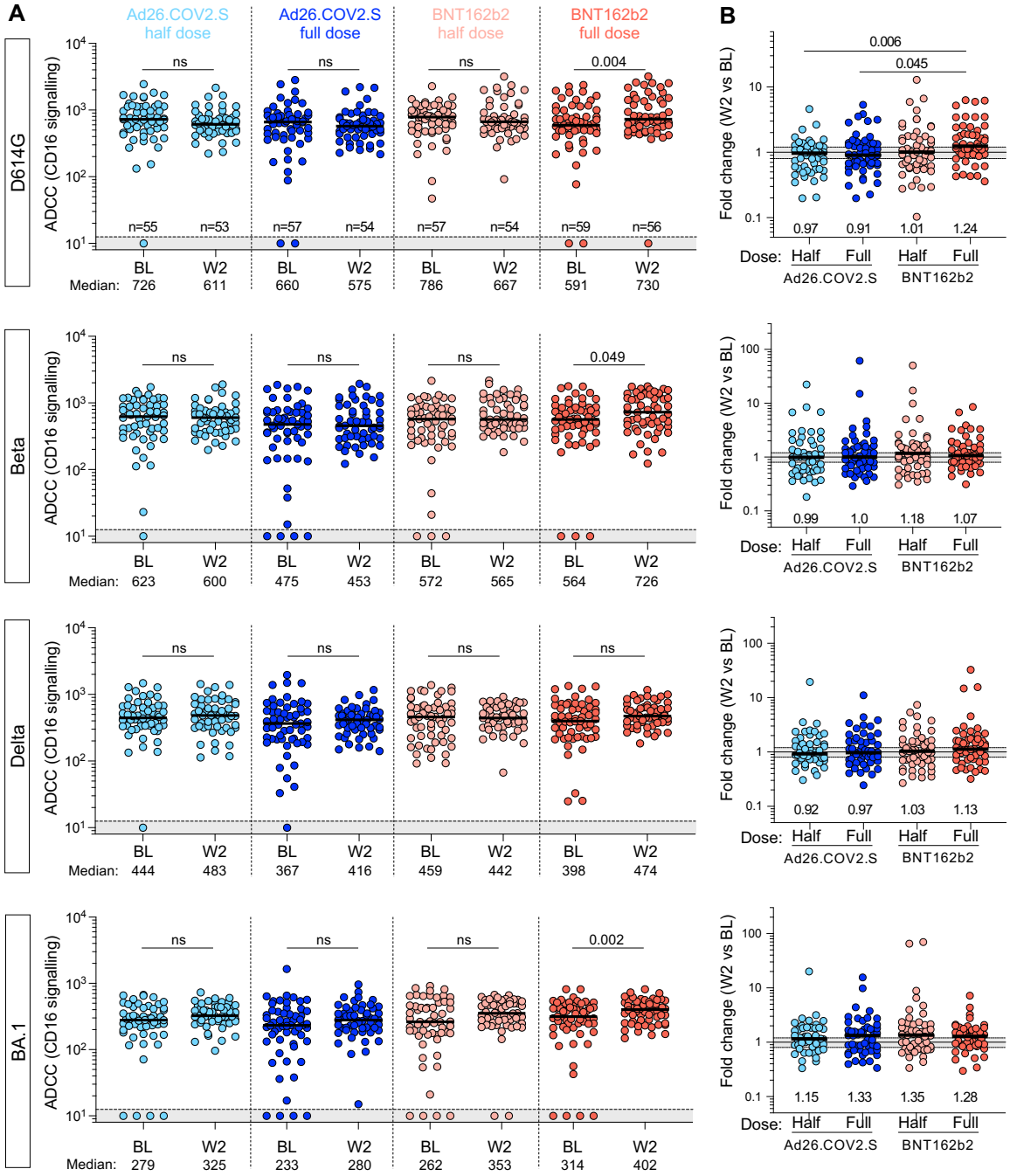
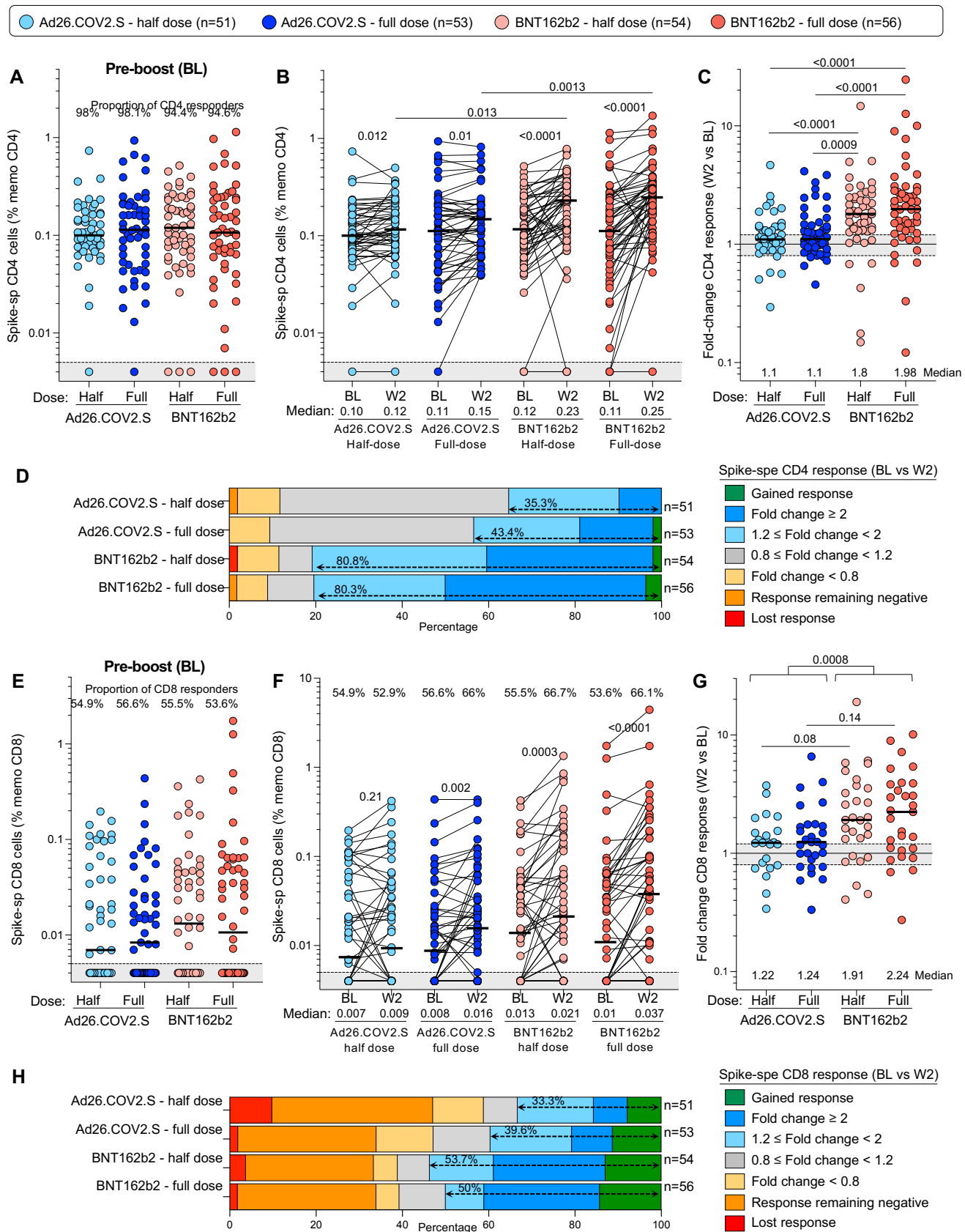
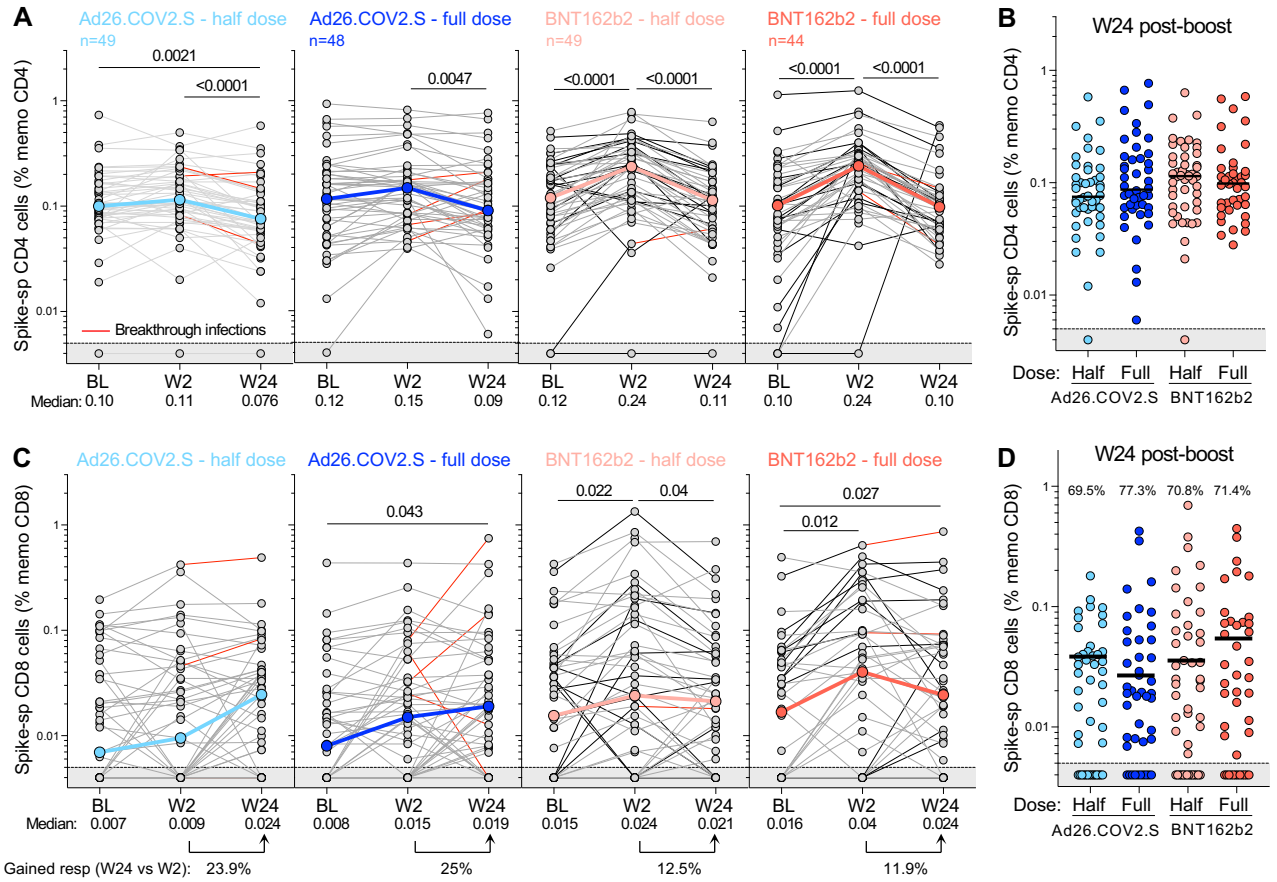


Figure 5

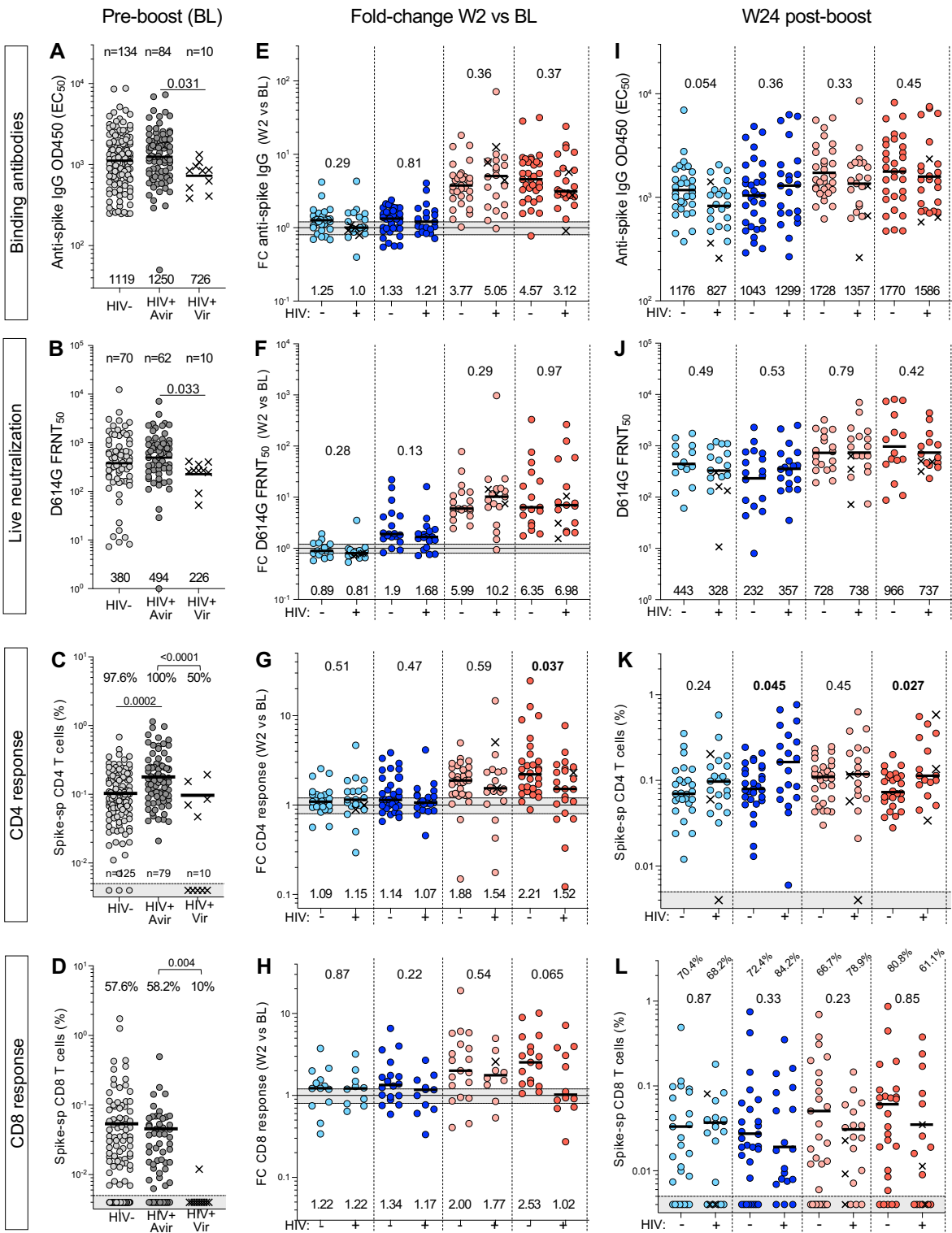


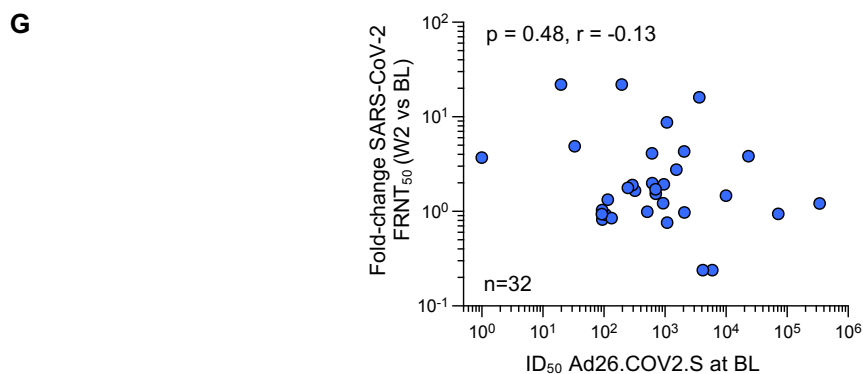
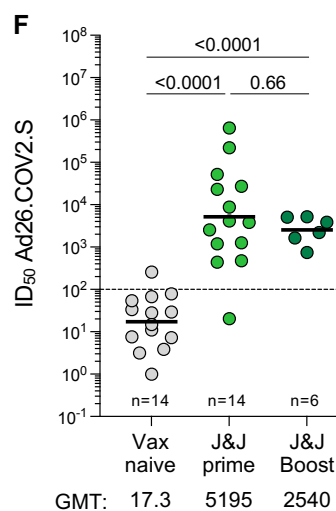
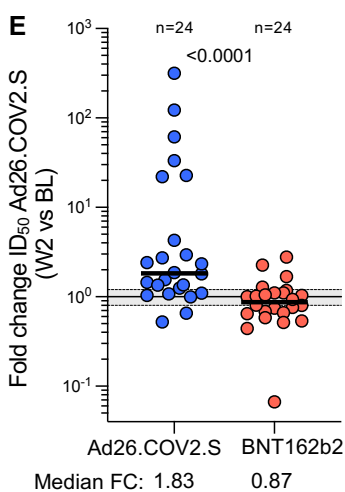
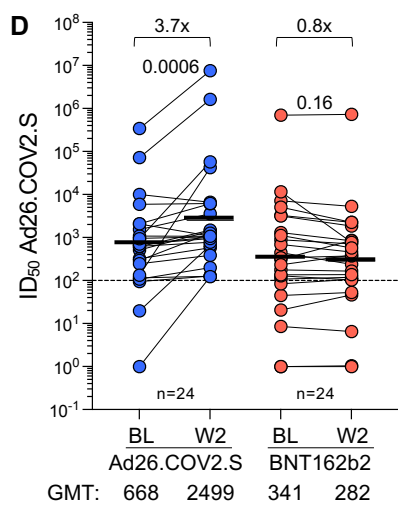
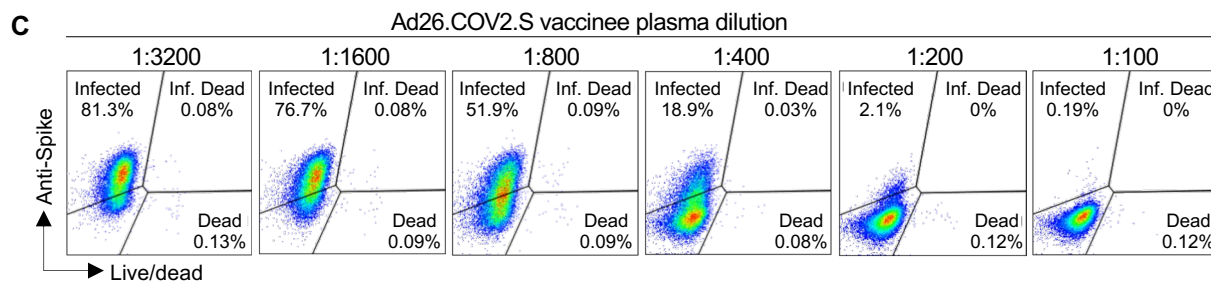
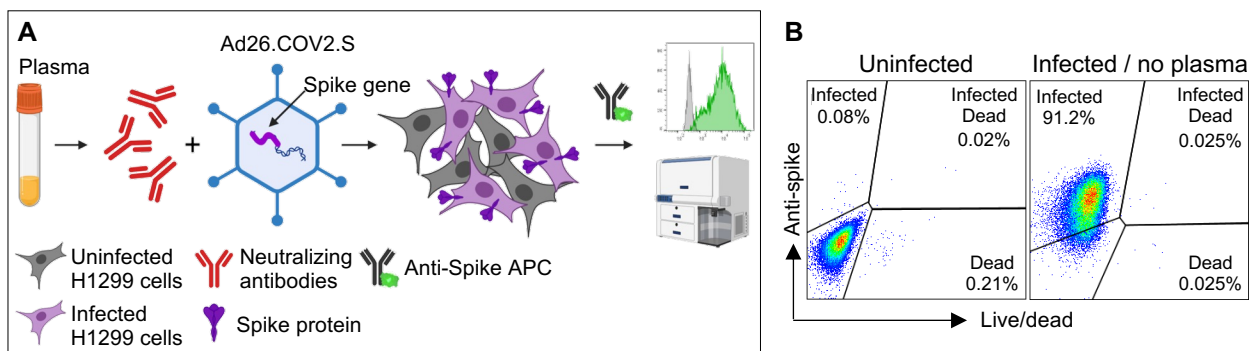


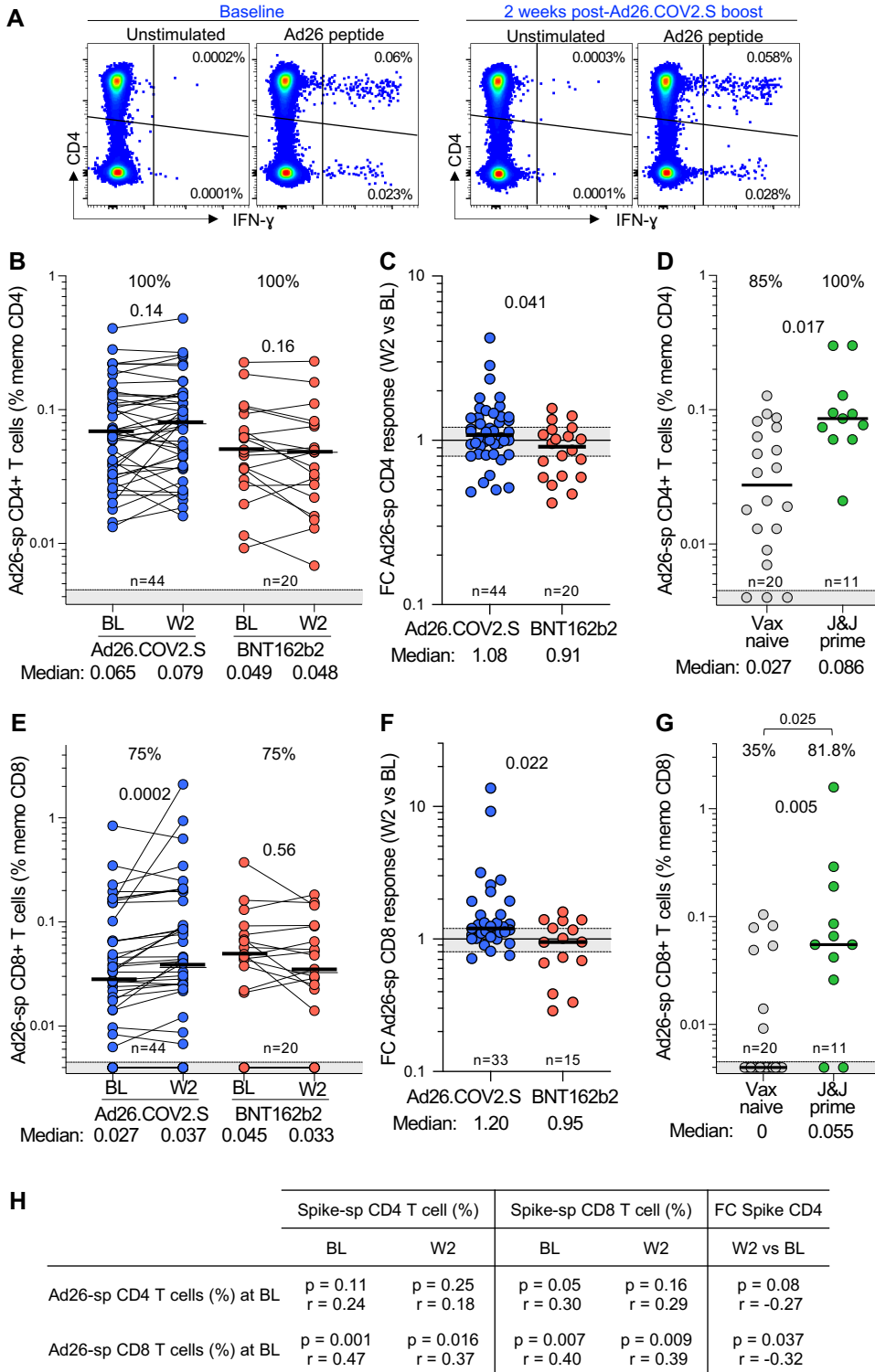


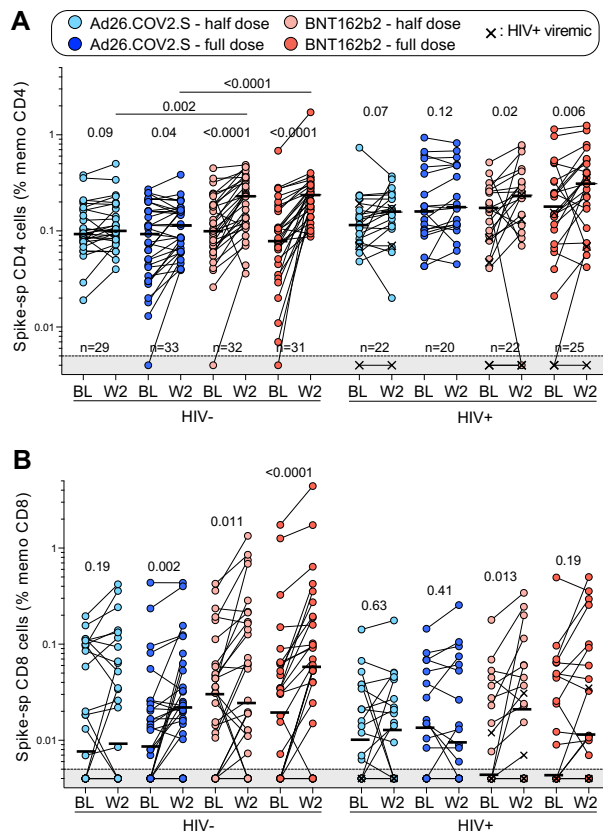


● Ad26.COVID.S - half dose ● Ad26.COVID.S - full dose ● BNT162b2 - half dose ● BNT162b2 - full dose x: HIV+ viremic









Supplemental Figure 1: SARS-CoV-2 spike-specific T cell responses in participants stratified by HIV status in each study arm. Frequency of spike-specific CD4⁺ T cells (**A**) and spike-specific CD8⁺ T cells (**B**) before (BL) and 2 weeks after vaccine boost (W2) in HIV-negative participants and in PLWH. Bars represent medians. Viremic PLWH are identified with a cross. Bars represent medians. A two-tailed Wilcoxon signed-rank test was used to assess statistical differences between paired samples and a Kruskal-Wallis with Dunn's corrections was used to compare different groups.

Supp Table 1

	Vax-naive	J&J primed	J&J boosted
N	14	14	6
Age*	46 (37-57)	46 (37-57)	55 (49-57)
Gender (% female)	100%	100%	100%
Time since last vaccination (days)*	na	44 (31-53)	110 (96-117)

Supplementary Table 1: Clinical characteristics of samples used for Ad26-specific antibody response assessment. (Related to Figure 10F). *: Median and Interquartile range (IQR), na: not applicable.

Supp Table 2

	Vax-naive	J&J primed
N	20	11
Age*	43 (33-53)	49 (32-53)
Gender (% female)	90%	81.8%
Time since vaccination (days)*	na	25 (20-45)

Supplementary Table 2: Clinical characteristics of samples used for Ad26-specific T cell response assessment. (Related to Figures 11D and 11G). All participants were HIV-uninfected. *: Median and Interquartile range (IQR). na: not applicable.