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1 # Safety and immunogenicity of a live recombinant Newcastle disease virus-based
2 # COVID-19 vaccine (Patria) administered via the intramuscular or intranasal
3 # route: Interim results of a non-randomized open label phase I trial in Mexico

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56 **Abstract**

57 There is still a need for safe, efficient and low-cost coronavirus disease 2019 (COVID-19) vaccines that can
58 stop transmission of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Here we evaluated
59 a vaccine candidate based on a live recombinant Newcastle disease virus (NDV) that expresses a stable
60 version of the spike protein in infected cells as well as on the surface of the viral particle (AVX/COVID-12-
61 HEXAPRO, also known as NDV-HXP-S). This vaccine candidate can be grown in embryonated eggs at low
62 cost similar to influenza virus vaccines and it can also be administered intranasally, potentially to induce
63 mucosal immunity. We evaluated this vaccine candidate in prime-boost regimens via intramuscular,
64 intranasal, or intranasal followed by intramuscular routes in an open label non-randomized non-placebo-
65 controlled phase I clinical trial in Mexico in 91 volunteers. The primary objective of the trial was to assess
66 vaccine safety and the secondary objective was to determine the immunogenicity of the different vaccine
67 regimens. In the interim analysis reported here, the vaccine was found to be safe and the higher doses
68 tested were found to be immunogenic when given intramuscularly or intranasally followed by
69 intramuscular administration, providing the basis for further clinical development of the vaccine
70 candidate. The study is registered under ClinicalTrials.gov identifier NCT04871737. Funding was provided
71 by Avimex and CONACYT.

72 **Introduction**

73 Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in China in late 2019 and has
74 since then caused the coronavirus disease 2019 (COVID-19) pandemic (1, 2). Vaccines against SARS-CoV-
75 2 were rapidly developed and have been shown to be safe and efficacious (3). However, in many low- and
76 middle-income countries (LMICs) access to vaccines is still limited. In addition, mRNA based COVID-19
77 vaccines require frozen storage and transportation - severely restricting their usability in LMICs.
78 Furthermore, production of many of the available COVID-19 vaccines is costly, affecting the price per dose.
79 In addition, all currently approved COVID-19 vaccines are injected intramuscularly leading to strong
80 systemic but absent or weak mucosal immunity (4) which is thought to be critical for achieving sterilizing
81 immunity against SARS-CoV-2. Moreover, for more infectious variants like B.1.617.2 (Delta) the rate of
82 breakthrough infections has increased (5) and has now peaked with the emergence of B.1.1.529 (Omicron)
83 (6-13). These breakthrough infections are often asymptomatic or mild if symptomatic, and protection
84 from severe disease remains high (14, 15). However, the fact that they occur is likely a consequence of
85 the absence of persistent mucosal immunity, which can neutralize virus right at its entry point into the
86 body, on mucosal surfaces of the upper respiratory tract. Vaccines that potentially induce mucosal
87 immunity are better suited to induce sterilizing immunity and block transmission of a virus.

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88 To address the issues raised above, we have developed a live Newcastle disease virus (NDV)-based SARS-
89 CoV-2 vaccine. NDV is an avian paramyxovirus which is highly attenuated in mammals and has been tested
90 in humans as an oncolytic virus and in preclinical models as a live vaccine vector (16-24). We engineered
91 the LaSota vaccine strain of NDV to express the spike protein of SARS-CoV-2 (25-28). The version of the
92 spike protein used is based on an enhanced immunogen design, which includes six proline mutations and
93 a deletion of the polybasic cleavage site keeping the spike in a stable pre-fusion conformation (29). In
94 addition, the ectodomain of the spike protein was grafted onto the transmembrane domain and
95 cytoplasmic domain of the NDV fusion protein to ensure optimal incorporation into the Newcastle disease
96 virion. The vaccine vector therefore carries spike on its surface and expresses it in cells that it infects.

97 NDV is an avian virus and it can be grown in embryonated chicken eggs to very high titers. Embryonated
98 eggs are used for production of the majority of influenza virus vaccines used and therefore production
99 capacity for this NDV-vectored vaccine already exists in high-income and LMICs (30). This also allows the
100 vaccine to be produced at very low cost.

101 We have previously shown that an inactivated, as well a live version of this NDV-vectored vaccine, are
102 safe, well tolerated, highly immunogenic and protective in animal models including in a swine model using
103 different routes of administration, that contributed to the design of the phase I protocol reported herein
104 (25-28, 31-33). Inactivated versions of the vaccine are currently in clinical development in Vietnam
105 (NCT04830800), Brazil (NCT04993209) and Thailand (NCT04764422). Interim results from Thailand show
106 that the inactivated formulation – which is injected intramuscularly - is safe and highly immunogenic (34).
107 Here, we tested a live version of the vaccine, AVX/COVID-12-HEXAPRO (Patria, also known as NDV-HXP-
108 S), in an open label non-randomized non-placebo-controlled phase I trial in 91 healthy volunteers. Vaccine
109 was administered either via an intramuscular prime-boost regimen or, for optimal induction of mucosal
110 immunity, via an intranasal prime-boost regimen. In addition, intranasal immunization followed by an
111 intramuscular administration was also tested. Below, we report the interim safety and immunogenicity
112 results from this trial in Mexico (NCT04871737).

113 **Methods**

114 **Study design and participants**

115 The phase I study was designed to evaluate the safety and immunogenicity of NDV-HXP-S given via three
116 different vaccination strategies: intramuscular vaccination on day 0 and day 21, intranasal vaccination on
117 day 0 followed by intramuscular vaccination on day 21 and intranasal vaccination on day 0 and day 21. In
118 addition, three different dose levels were tested, $10^{7.0}$-$10^{7.49}$ 50% egg infectious doses ($EID_{50,}$ low dose
119 (LD)), $10^{7.5}$-$10^{7.99}$ EID_{50} (medium dose, MD) and $10^{8.0}$-$10^{8.49}$ EID_{50} (high dose, HD), resulting in 9 groups with
120 10 participants each (**Table 1**). Female and male participants between 18 and 55 years of age without
121 prior immunity to SARS-CoV-2 were enrolled. The protocol was designed by ProcliniQ Investigación Clínica,
122 S. A. de C. V. with input from the Instituto Mexicano del Seguro Social (IMSS) and Laboratorio Avi-Mex, S.
123 A. de C. V. (Avimex®), the later as sponsor with the statistical help of iLS Clinical Research, S. C. The study
124 was approved by the Federal Commission for the Protection against Sanitary Risks (COFEPRIS) in Mexico
125 under number 213300410A0063/2021, after approval by the Ethics, Biosafety and Research Committees
126 of the clinical research site Hospital Medica Sur (03-2021-CI/CEI/CB-156) in full compliance of the Mexican
127 regulation and under the principles of the Declaration of Helsinki and Good Clinical Practice. The samples
128 for the immunological assays were processed at the National Institute for Respiratory Diseases (INER) in
129 Mexico City.

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130 The primary outcomes were to evaluate the safety of the three concentrations (viral titers) and three
131 administration routes across nine groups. Immunogenicity measurements including the induction of IgM
132 and IgG, neutralizing antibodies, cellular responses and induction of mucosal immunity (mucosal IgA,
133 neutralizing IgA) were secondary outcomes.

134 **Study groups**

135 This Phase I study was designed as a non-randomized open label study without placebo control group.
136 Ninety volunteers were assigned to one of nine treatment groups in the order of enrolment according to
137 **Table 1**. The first intervention of each treatment group was made sequentially to 18 sentinel subjects
138 according to **Table 2**. The first 18 subjects (S1 to S18) received incrementally a dose from the lowest to
139 the highest viral titer with no more than one subject per day, per dosage and route of administration. The
140 safety data of the sentinel subjects was then evaluated by an independent Safety Data Monitoring
141 Committee (SDMC) before authorizing the administration of the first vaccine dose to the rest of the
142 subjects, who were then sequentially enrolled according to **Table 3**. The SDMC also evaluated the safety
143 data of the full cohort after the first dose before the administration of the second dose to the nineteenth
144 subject enrolled (first outside the sentinel group) on day 21 after the first dose.

145 There was a deviation with one of the subjects who reported negative results in the PCR and IgG/IgM tests
146 for SARS-CoV-2 at screening, and who was therefore enrolled in the clinical trial and received the first
147 intramuscular vaccine dose (Day 0) in the low dose (LD) group. However, a subsequent test of anti-spike
148 antibodies, post-administration of the vaccine, showed a low, yet positive antibodies level (148.8 AU/mL,
149 Elecsys® Anti-SARS-CoV-2 S, Roche Diagnostics). The investigators reviewed the case and considered that
150 it was in the best interest of the subject to remain in the study, since the safety of the subject was not at
151 risk and vaccination for the age group to which the subject belongs was at the time of the study not
152 available under the Mexican national vaccination program. This decision would also be consistent with an
153 ethical obligation of properly monitoring the safety of the volunteer. The subject consented to continue
154 participation in the study with the sponsor's authorization. Safety data was included in the safety analysis,
155 but immunogenicity data from this subject was not considered for immunogenicity assessment.

156 For those subjects who received the first dose intranasally (IN), the second dose was administered by
157 alternating the administration route. The first subject was given the second dose via the IN route followed
158 by the second subject who was dosed by the IM route. This alternation continued until the IN/IN and
159 IN/IM groups were dosed at each dose level according to **Table 1**. All subjects who received the first dose
160 via IM also received the second dose via IM in order to complete the corresponding IM/IM groups.

161 As an additional circumstance around the protocol, it is important to stress that the study was conducted
162 almost concurrently with a COVID-19 wave in Mexico driven by the emergence of the B.1.617.2 (Delta)
163 variant in Mexico City (34). This circumstance affected the clinical trial as some of the participants were
164 infected either between the first and the second dose or after the administration of the second dose as
165 reported in **Table 4**.

166 According to the above, the total N for safety assessment was 91 participants and for immunogenicity
167 assessments, the N was variable per group since subjects who acquired an infection (see **Table 4**) were
168 excluded from analysis.

169 **Procedures**

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170 As mentioned above, AVX/COVID-12-Hexapro (Patria) is a Newcastle disease virus (NDV)-based SARS-CoV-
171 2 vaccine based on the LaSota vaccine strain of NDV (25-27). It was engineered to express a version of the
172 spike protein of SARS-CoV-2 which includes six proline mutations and a deletion of the polybasic cleavage
173 site, keeping the spike in a stable pre-fusion conformation (29). In addition, the ectodomain of the SARS-
174 CoV-2 spike was grafted onto the transmembrane and cytoplasmic domains of the NDV fusion protein to
175 ensure optimal incorporation into the Newcastle disease virion. The vaccine was obtained as reported
176 previously (25-27, 31) and manufactured under Good Manufacturing Practices at the COFEPRIS approved
177 facilities of Laboratorio Avi-Mex, S. A. de C. V. in Mexico City. The vaccine was formulated without
178 adjuvants in three different viral titers per dose (LD, MD, HD) as described above. For the intramuscular
179 (IM) administration, it was formulated in single dose vials with the corresponding viral titer contained in
180 0.5 mL for administration as a single injection into the deltoid muscle. In the case of the intranasal (IN)
181 administration, it was formulated in single dose vials as a 0.2 mL solution containing the corresponding
182 viral titer, for administration of 0.1 mL in each nostril. The vaccines formulated as described were stored
183 under refrigeration (4°C).

184 The 0.5 mL intramuscular dose was administered through a regular syringe and needle, and for the 0.2 mL
185 intranasal route a nasal sprayer device coupled to the syringe (MAD Nasal™ - Intranasal Mucosal
186 Atomization Device) was used instead.

187 The study was conducted at Hospital Medica Sur in Mexico City. A written informed consent was obtained
188 from each participant, as approved by COFEPRIS, to voluntarily participate in the study for 12 months
189 including 11 visits to the site plus at least six telephone follow-up calls scheduled according to the date of
190 the first visit.

191 A screening visit was conducted 3 days before vaccination where each participant underwent a full
192 medical history and examination. A medical history was obtained, including recording of all vaccines and
193 medications received within the last 30 days, and daily activities that posed a high risk for getting infected
194 with SARS-CoV-2. The physical examination included measurement of vital signs (blood pressure, heart
195 rate, respiratory rate, and temperature), oxygen saturation, weight and height. At the screening visit
196 participants were also subject to urine and blood testing, hematology, blood cell count, kidney and liver
197 function test, blood lipids, and testing for human immunodeficiency virus (HIV), hepatitis B and C virus
198 and syphilis, pregnancy tests for woman of childbearing potential, electrocardiogram, and thorax CT scan.
199 In addition, the participants were subject to COVID-19 testing (nucleic acid-GeneFinder™ COVID-19 Plus
200 RealAmpKit, and antibody-as above) to exclude prior or active infection, as such infection was part of the
201 exclusion criteria. Further details on eligibility are provided in the trial protocol (**Appendix 1**).

202 Eligible subjects were enrolled and were administered the first vaccine dose corresponding to their group
203 and given a patient diary at basal visit (D0). Vital signs were measured prior to the administration of each
204 dose and at 90 minutes thereafter. Subjects were observed on-site during that period. Further daily
205 telephone interviews were conducted from days 1 to 6 for collection of safety data and participants
206 returned to the site on day 7 (D7) after the first dose administration (D0) followed by scheduled visits on
207 days 14, 21, 28, 42, 90, 180 and 365. All on site visits included measurement of vital signs, weight, and
208 determination of body mass index (BMI). Data for visits on days 90, 180 and 365 are not yet available since
209 the trial is still ongoing.

210 Day 14, 21, 28, 42, 90, 180 and 365 visits include blood sampling for IgM – IgG – IgA antibodies, neutralizing
211 antibodies, and T cell responses. In addition, those subjects who received at least one IN dose provided

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212 also saliva and nasal swab samples on these same dates. According to the study protocol, basal samples
213 of saliva and nasal fluids were not collected as there was no previous infection and specific antibodies
214 were likely negative.

215 A PCR test was also performed prior to the application of the second dose of AVX/COVID-12-Hexapro . As
216 described above, participants positive for SARS-CoV-2 infection were considered for the safety
217 assessment but excluded from the immunogenicity analysis.

218 Adverse events were documented based on standardized terms (MedDRA) and classified as Adverse
219 Events (AE), Serious Adverse Events (SAE), both as defined by ICH/E6R2 Good Clinical Practices definitions.
220 Adverse Events of Special Interest (AESI) were defined in the Protocol as those that appeared within 7
221 days from vaccination and categorized as local, when related to the injection or the intranasal
222 administration (inflammation, redness, local increased temperature, itching, low-grade fever), or systemic
223 or related to COVID-19 disease (fever, chills, cough, difficult breathing, muscular or articular pain,
224 headache, anosmia, ageusia, odynophagia, nasal congestion or secretion, nausea or vomiting, diarrhea or
225 fatigue).

226 The number and percentage of AEs, SAEs and AESIs were recorded after every vaccine administration.
227 AESIs were considered associated with vaccination and evaluated 7 days after each vaccination, while AEs
228 were assessed after 21 days as of vaccination. AEs intensity was generally registered as low, mild or severe
229 according to the protocol. Clinically relevant abnormalities in laboratory tests or at physical examination
230 were recorded by groups and then correlated to the vaccine viral titer and administration route.

231 **Immunological assay**

232 **Sample collection**

233 Blood samples, nasal exudates, and saliva samples were obtained as described above, according to the
234 group, at the clinical research site and transported to INER at room temperature. Blood samples were
235 processed within two and half hours of vein puncture. Biological samples were obtained before and 14,
236 21, 28 and 42 days after the first vaccination.

237 Venous blood was obtained using standard procedures and was collected into separator tubes (SST BD
238 vacutainer tubes, Franklin Lakes, NJ, USA). Vacutainer tubes were centrifuged at 1200 rpm (centrifuge:
239 Rotanta 460R; Rotor: 5624, Hettich, Tuttlingen DE) for 10 minutes to separate serum. The serum was then
240 removed from the upper portion of the tube, aliquoted, and stored at -20°C until use.

241 Serum samples from convalescent individuals (N=51, collected at a median of 41 days post onset (standard
242 deviation 12 days, range 21-65 days)) with SARS-CoV-2 infection confirmed by real-time reverse
243 transcriptase PCR (RT-PCR) were collected after recovery on the day they resumed regular activities to be
244 used as positive controls, and serum samples from healthy individuals obtained between 2014-2018
245 (prepandemic) were used as negative controls (INER approved protocol number B20-21and B22-12).

246 All blood samples and blood products, nasal exudates and saliva were handled in a BSL-2 laboratory with
247 the use of appropriate personal protective equipment and safety precautions using processing protocols
248 approved by the INER Institutional Biosafety Committee.

249 Venous blood was collected in sodium heparin tubes (BD vacutainer tubes, Franklin Lakes, NJ, USA) and
250 diluted 1:1 within two and half hours for whole blood stimulation with 0.99 μg/mL of S1 subunit of the

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251 spike protein (RayBiotech, Peachtree Corners GA) in the presence of anti-CD28/CD49d (BD, San Jose CA)
252 for 18h 20min at 37ºC in 5% CO_2.

253 **SARS-CoV-2-spike protein specific antibodies enzyme-linked immunosorbent assay (ELISA)**

254 S1-specific IgG in serum samples were measured using two commercial kits from EuroImmun, following
255 manufacturer's instructions and using an analyzer (Euroimmun AG, Lübeck, Germany). Serum samples
256 were diluted 1:100, and 100 μL of samples, calibrator, negative and positive control were added to each
257 well and incubated at 37°C for 60 minutes. This step was followed by three washes using 300 μL of washing
258 buffer per well. Then, 100 μL of the anti-human IgG, labeled with peroxidase were added and incubated
259 at 37°C for 30 minutes for IgG detection. The plates were subsequently washed before the addition of 100
260 μL of substrate solution. After incubation for 30 minutes at room temperature, 100 μL of stop solution
261 was added and the optical density (OD) was read at 450 nm in the analyzer (EuroImmun) within 30
262 minutes after adding the stop solution. The results were reported as the ratio between the extinction of
263 the sample and the extinction of the calibrator, and a ratio of > 1.1 was considered positive (35). For
264 serum analysis, twofold serial dilutions of sera were processed as described above, and the end-point titer
265 was calculated as the most diluted serum concentration that gave a ratio >1.1. The limit of detection was
266 1:100, samples with activity below the limit of detection were assigned a titer of 1:50 for graphing
267 purposes. Samples ran across multiple plates were calibrated using a manufacturer-provided calibrator
268 solution.

269 **Receptor binding domain (RBD) – angiotensin converting enzyme 2 (ACE2) interaction inhibition assay**
270 **(RAIIA)**

271 To determine the presence of antibodies that block interaction between the spike receptor binding
272 domain (RBD) and the angiotensin converting enzyme 2 (ACE2) receptor, we used a commercial assay
273 from GenScript, which is a protein-based surrogate neutralization assay (36). Samples were analyzed
274 following the manufacturer's instructions (GenScript version RUO 3.0 update 01/02/2021). Briefly,
275 samples and controls were diluted 1:10 in kit sample buffer and mixed 1:1 with horseradish peroxidase
276 (HRP)-conjugated recombinant SARS-CoV-2 RBD fragment (HRP-RBD) and incubated at 37°C for 30
277 minutes to allow binding of circulating antibodies to HRP-RBD. The mixture was then added to the capture
278 plate which is pre-coated with the human ACE2 protein. Unbound HRP-RBD as well as any HRP-RBD bound
279 to non-neutralizing antibody was captured on the plate, while circulating neutralization antibodies-HRP-
280 RBD complexes remained in the supernatant and get removed during washing. Then 3,3',5,5'-
281 tetramethylbenzidine (TMB) solution was added. By adding stop solution, the reaction was quenched and
282 the plates were read at 450 nm using Analyzer 1 (EuroImmun). Absorbance of a sample is inversely
283 correlated with blocking RBD-ACE2 interactions. The results are expressed as the percentage (%) of
284 inhibition and 30% inhibition was used as cutoff as previously established (36).

285 **Intracellular cytokine staining assay**

286 Whole blood diluted 1:1 was stimulated with RPMI 1640 medium (Lonza, Walkersville, MD) 0.99 μg/mL
287 of S1 subunit of spike protein in the presence of anti-CD28/CD49d (BD, San Jose CA) for 18 h20 min at
288 37°C in 5% CO_2. GolgiStop (BD, San José, CA) was added, and the samples were cultured additionally for
289 4 h. Medium was used as a negative control and 10 μg/mL of PHA (Sigma-Aldrich) as a positive control.
290 Samples were washed with phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+} (Lonza), and stained
291 with Live/Dead near-IR Dead Cell Stain Kit for 633 or 635nm excitation (Invitrogen, Eugene, OR), for 15

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292 min at room temperature in the dark. Then red blood cells (RBCs) were lysed with RBC lysis buffer (BD)
293 for 10 min followed by a washing step with staining buffer PBS without Ca^2+ and Mg^2+ supplemented with
294 1% fetal bovine serum (FBS) and 0.1% NaN_3). Cell surface staining was performed using a cocktail of anti-
295 human CD3, CD4 and CD8 antibodies in staining buffer for 15 min at room temperature in the dark. After
296 an additional washing step with staining buffer, the cells were fixed and further permeabilized using BD
297 Cytofix/Cytoperm following the manufacturer's instructions. Intracellular staining was performed in
298 Cytoperm using a anti-human IFN-γ antibodies for 30 min at room temperature in the dark. Cells were
299 washed with BD Perm/Wash buffer and further resuspended in PBS. Cells were kept at 4°C in the dark
300 until acquisition and analysis. Unstained and fluorescence minus one (FMO) controls were included.
301 Details of the antibodies used in the flow cytometry assay are listed in Supplementary Table 1, and the
302 rest of the reagents in Supplementary Table 2. At least 200,000 events of the lymphocyte region in a
303 forward scatter (FSC) vs side scatter (SSC) scatter plot were acquired in a fluorescence-activated cell sorter
304 (FACS) Aria II (BD). Analysis was performed using FACS Diva 8.0. The gates applied for the identification of
305 SARS-CoV-2 antigen-specific cytokine-producing CD3+, CD4+ or CD8+ cells were defined using the FMO
306 controls and used for limit of detection (LOD) (37).

307 **Outcome**

308 **Primary outcomes**

309 Primary outcomes of the study were established as follows:

310 - To evaluate the safety of three concentrations ($10^{7.0-7.49}$, $10^{7.5-7.99}$, $10^{8.0-8.49}$ $EID_{50\%}$/dose) of the
311 recombinant vaccine against SARS-CoV-2 based on a Newcastle Disease virus (rNDV)
312 administered twice intramuscularly, twice intranasally or intranasally followed by
313 intramuscularly in healthy volunteers

314 **Secondary outcomes**

315 - To evaluate the immunogenicity of three concentrations ($10^{7.0-7.49}$, $10^{7.5-7.99}$, $10^{8.0-8.49}$ $EID_{50\%}$/dose)
316 of the recombinant vaccine against SARS-CoV-2 based on a Newcastle Disease virus (rNDV)
317 administered twice intramuscularly, twice intranasally or intranasally followed by
318 intramuscularly in healthy volunteers
319 - To evaluate the nasal mucosal humoral immunity of three concentrations ($10^{7.0-7.49}$, $10^{7.5-7.99}$,
320 $10^{8.0-8.49}$ $EID_{50\%}$/dose) of the recombinant vaccine against SARS-CoV-2 based on a Newcastle
321 Disease virus (rNDV)

322 This manuscript describes an interim analysis which focuses on initial safety data and binding and
323 ACE2/RBD interaction inhibiting antibodies and T-cell based immunity. Other readouts will be described
324 in future publications.

325 **Statistical analysis**

326 Interim analyses were scheduled for days 21, 28, 42, and after 6 and 12 months (end of study). This
327 report includes data obtained up to day 42. For continuous variables, one-way ANOVA and Student t test
328 were used, and non-parametric tests were used for discrete (count) variables. Safety endpoints were
329 expressed as frequencies (%) with 95% binomial exact confidence intervals (Cis), while immunological
330 assessment are expressed as median and IQRs. In this report all analyses are descriptive only, as samples

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331 are still pending further analyses and the results reported here are preliminary in nature. IgG titers are
332 reported per group as geometric mean titers (GMT) with a 95% CI at days 0 (basal), 14, 21, 28 and 42. For
333 logarithmically transformed antibody titers ANOVA and Wilcoxon's rank sum test were used for data not
334 distributed normally, and significances between groups were paired and differences assessed with a 95%
335 CI. Bilateral CIs for GMT were calculated by back-transformed 95% CI based on Student t tests for titers
336 with $\log_{10}$ transformation. The proportion of subjects with a titer above a predetermined parameter for
337 IgG, IgM and IgA with 95% CI at days 14, 21, 28, and 42. Days 90, 180, as well as 6 and 12 months will also
338 be analyzed after completion of the study. Seroconversion rate and 95% CI with respect to the basal titer
339 was also determined as the proportion of subjects with detected titers of specific antibodies for the spike
340 protein of SARS-CoV-2 as determined by ELISA and an ANOVA with 95% CI was used for assessing the
341 capacity of circulating antibodies to inhibit the interaction between RBD and ACE2. T cell mediated
342 responses were assessed as a proportion of positive responders through a $\chi 2$ test or Fisher's exact test for
343 categorical data. Finally, the titers of antibodies per administration form (IM/IM, IN/IN and IN/IM) were
344 compared using two-way ANOVA. The full statistical analyses details are provided in the trial protocol
345 (**Appendix 1**), and will be performed in full upon completion of all procedures.

346 **Role of funding source**

347 The funding for the clinical study was provided by the National Council for Science and Technology
348 (CONACYT, México), except for all the production and vaccine product supply which was funded solely by
349 Avimex. CONACYT did not participate in the trial design but did evaluate it and approved the project
350 through their National Committee on Research, Development and Innovation on Public Health. Funding
351 was managed by Avimex and used to pay for all laboratory tests, clinical site and clinical professionals.
352 CONACYT also facilitated the identification, purchase and importation of certain supplies and the
353 communication with other entities of the Federal Mexican Government to facilitate the study.

354 **Results**

355 From May 24th 2021 to August 20th 2021, 153 volunteers were assessed. Two voluntarily withdrew from
356 the study, while 49 were excluded as they did not meet eligibility criteria. 91 volunteers were enrolled
357 into the nine different groups and either dosed twice IM (IM-IM groups), dosed sequentially IN followed
358 by IM (IN-IM groups) or received two IN vaccinations (IN-IN). groups) in a three week interval (**Figures 1**
359 **and 2, Tables 1-3**). Three different dose levels, low dose (LD), medium dose (MD) and high dose (HD) were
360 evaluated. Distribution of participants by gender between the safety population groups did not show
361 statistically significant differences according to the dose/route of administration. All the participants
362 identified themselves as Mestizo. Regarding distribution of patients according to age, there were no
363 significant differences either between groups that received low, medium, or high doses by any
364 administration routes . Average ages, age range of participants, gender distribution, weight, height, and
365 body mass index in each study group are indicated in **Figure 1C**. Up to day 45 post first vaccination none
366 of the enrolled individuals were excluded from the study for safety evaluation but one subject had to be
367 excluded from the immunogenicity evaluation due to a positive baseline titer and several subjects had to
368 be excluded due to SARS-CoV-2 infections (**Table 4**).
369 In general, all formulations were well tolerated with little reactogenicity detected (**Figure 3**). Up to day 45
370 post first vaccination of the latest enrollment of a subject, there had been 625 adverse events in total, of
371 which 319 occurred within the period considered as of special interest (within 7 days after either of the
372 two administrations). Of these 319 events within the special interest period, 66 were considered local and
373 253 systemic. In general, the distribution of AEs among the different groups of the study did not present

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374 statistically significant differences in terms of the number of individuals who presented at least one event,
375 nor differences in the incidence of events according to their severity, except for those of the IN route
376 which of course did not show the injection-related AEs. Additionally, none of the routes of administration
377 or dose evaluated were associated with serious adverse events.

378 Out of 625 adverse events, 552 (88.32%) were of mild intensity, 68 (10.88%) moderate and only 5 (0.8%)
379 severe intensity events were recorded. Distribution of adverse events between the different groups by
380 route of administration or by dose received was not statistically significant. No deaths or
381 significant/serious adverse events were reported, and no alterations of vital signs or clinically significant
382 events were reported.

383 To determine immunogenicity of the different vaccine doses and vaccination routes we first performed
384 ELISAs against the S1 domain of the spike protein (**Figure 4**). S1 was chosen as target because this
385 subdomain of the spike includes the N-terminal domain and the RBD, which host most of the neutralizing
386 epitopes. In addition, a reliable commercial ELISA focusing on that target was available locally. For the IM-
387 IM vaccination regimen, little induction of anti-S1 antibody was observed after the first dose. However,
388 the second dose boosted titers in a dose dependent manner with high reactivity in the HD group and
389 lower reactivity in the MD and LD groups. As expected, the response after IN vaccination was lower and
390 substantial reactivity was only detected in the HD group post-boost with 56% of the individuals in the
391 group having detectable titers. Finally, in the IN-IM regimen, the reactivity was similar to the IM-IM
392 regimen with an 89% response rate after the boost. Antibody titers induced by the HD IM-IM and IN-IM
393 regimens were in general comparable or higher than the titer of convalescent individuals.

394 While binding antibodies are important indicators of immunogenicity and have been correlated with
395 protection (38, 39), we also wanted to determine functional antibody titers. A neutralization assay was
396 unavailable for this interim analysis but we performed a surrogate assay, which measures inhibition of the
397 interaction between the RBD and ACE2 (36). The titers detected in this assay do reflect results from the
398 binding assay (**Figure 5**). For the HD IM-IM group the first vaccination increased the inhibitory titer just
399 slightly. However, strong inhibitory activity was observed at post boost time points. This was also observed
400 in the MD and LD groups, although more variability was detected there. For the IN-IN groups little
401 inhibitory activity was detected and only in the HD group subjects. The IN-IM groups showed an
402 intermediate phenotype with all individuals in the HD group having post-boost inhibitory antibodies. The
403 response rate in the MD group was lower and only 20% of individuals in the LD group had activity above
404 the limit of detection. Inhibition in the HD IM-IM regimen was in general comparable to inhibition of
405 convalescent individuals. Of note, this assay does not allow to determine differences between groups with
406 very strong responses. To summarize the binding and inhibition data, an analysis of the frequency of
407 individuals with detectable binding or inhibiting antibody titers was performed (**Supplementary Figure 1**).
408 High frequencies of subjects with binding and inhibiting antibodies were present in the all-dose groups
409 receiving an IM-IM regimen, while moderate to high frequencies were detected in the HD IN-IM group.

410 Cellular immune responses have been shown to be important for protection from SARS-CoV-2 infection,
411 especially when neutralizing antibody titers are low (40). Here we assessed specific cellular immune
412 responses by determining the percentage of CD3+, CD4+ and CD8+ cells that produced interferon γ (IFN
413 γ) upon stimulation with the spike protein. A significant induction of IFN producing CD3+ cells was
414 detected in all three HD vaccination regimens but not in the MD and LD groups when comparing day 42
415 with day 0 (**Figure 6**). While a trend was seen for IFN producing CD4+ cells in the HD IM-IM and IN-IN

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416 groups the increase was only statistically significant for the IN-IM HD group. No significant increases were
417 found for CD4+ in the MD and LD groups. For CD8+ IFN producing cells a trend was also observed for the
418 IM-IM HD group and the induction was significant for the HD IN-IN and IN-IM groups but not for any of
419 the MD and LD groups. As a control of the specificity of the assay, a comparison of medium vs spike
420 stimulated cells was performed (**Supplementary Figure 2**). Most of the participants had undetectable
421 levels of activated CD3+ T cells upon stimulation with medium.

422 As described above, breakthrough infections did happen during the clinical trial. At day 42, there were 10
423 cases detected among groups, with no apparent trend dependent on dose or administration route (**Table**
424 **4**). The 10 cases were symptomatic, symptoms were mild, and none required hospitalization. 50% of the
425 cases occurred before the second dose and the other 50% of the cases occurred after the second dose.

426 Assessment of safety and immunogenicity will continue for 12 months with sampling for immunogenicity
427 planed at the 90-, 180-, and 365-day time points.

428 **Discussion**

429 NDV-HXP-S can be produced at low cost and large scale using traditional egg-based influenza virus
430 production processes. Influenza virus production capacity is available globally in high-income countries
431 but also in LMICs (30). In addition, veterinary vaccine producers often also have egg-based production
432 capacity which can be adapted for good manufacturing practice (GMP) production of human vaccines.
433 The development of AVX/COVID-12-HEXAPRO could therefore alleviate the unmet global need for
434 additional COVID-19 vaccine doses. Importantly, the superior spike antigen design of the NDV-vectored
435 SARS-CoV-2 vaccine (29) is an additional advantage. Also, the favorable reactogenicity profile (as
436 described here and in (41)), which is akin to that of influenza virus vaccines, makes the NDV-based vaccine
437 likely more tolerant than mRNA or adenovirus-vectored vaccines (42-45). Furthermore, as demonstrated
438 here, live NDV-vectored SARS-CoV-2 vaccine can be administered via the IN route. While assessment of
439 mucosal immunity is not part of this interim report, intranasal vaccination is known to induce mucosal
440 immunity that can potentially lead to sterilizing immunity and complete block of transmission.

441 Here we demonstrated that administration of live of AVX/COVID-12-HEXAPRO is safe and well tolerated
442 at all dose levels. However, only the HD vaccine regimen induced robust antibody and cellular immune
443 responses when given via the IM-IM or IN-IM routes, comparable to those in convalescent individuals.
444 Cellular immune responses were induced by the IN-IN route but systemic antibody responses were not as
445 robust. These results mirror those obtained with live AVX/COVID-12-HEXAPRO in the pig and rat models
446 (31, 33). Given the robust immunogenicity and the high tolerability of the HD IM-IM and IN-IM vaccination
447 regimens, it is justified to further develop these two modalities in Phase II trials. Importantly, given the
448 high seroprevalence for SARS-CoV-2 in many regions globally and given the need for booster doses in a
449 part of the population (elderly, immunosuppressed, health care workers etc.), the HD vaccination
450 regimens should certainly also be evaluated in individuals with pre-existing immunity, likely as single IM
451 or IN administrations. Currently, a single-dose booster trial is ongoing in Mexico City based on the data
452 reported here with one IM and IN HD, and a phase II/III Study based on the HD IM-IM scheme is about to
453 start in Mexico.

454 Our study has several limitations. Quantitative neutralization assays with authentic SARS-CoV-2 could not
455 be performed at the study site at the time of analysis due to biosafety restrictions. In addition, in this
456 interim analysis, neutralizing activity against variants of concern could not be assessed. Furthermore, we

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457 were not able to directly compare immune responses induced by inactivated NDV-vectored SARS-CoV-2
458 vaccines (41) with those observed following administration of other authorized/licensed COVID-19
459 vaccines. We expect to perform these additional assays and direct comparisons at later time points as
460 soon as reagents and materials become available. So far, the assessment of mucosal antibodies has also
461 not been possible. Finally, this was a non-randomized open label study without a placebo control group,
462 which is more prone to biases as compared to randomized and double-blinded study designs.

463 In conclusion, we show that the live AVX/COVID-12-HEXAPRO vaccine has a safety profile that is
464 remarkably independent of the dose and administration route with low frequency and intensity.
465 Furthermore, the HD IM-IM and IN-IM vaccination regimens showed strong evidence of immunogenicity
466 warranting further development of this vaccine candidate. Finally, it is important to note that the NDV
467 vector technology is amenable to rapid changes in antigens expressed allowing for strain changes to match
468 emerging viral variants. A B.1.1.529 (Omicron)-specific version of NDV-HXP-S is currently in development.

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496 management of import/export of Newcastle disease virus vectors at the Icahn School of Medicine at
497 Mount Sinai.

498 **Conflict of interest statement**

499 PP reports financial support from the U.S. NIAID (Centers of Excellence for Influenza Research and
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508 years. The NDV construction administered in this study was developed by faculty members at the Icahn
509 School of Medicine at Mount including WS, PP, AGS, and FK. Mount Sinai has filed patent applications
510 related to SARS-CoV-2 serological assays and the NDV-based SARS-CoV-2 vaccines; the institution and its
511 faculty inventors could benefit financially. The live vaccine used in the study was developed by members
512 of Avimex and Avimex filed patent applications with Mount Sinai and CONACYT. M.T-R., D.S.-M., E.S.P.,
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514 applications. The clinical study was entirely performed in Mexico. The rest of the participants are
515 employees of their corresponding institutions.

516

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705

706 **Tables**

707 **Table 1.** Distribution of subjects in groups per dose and administration route/regimen
708 (IM=intramuscular; IN=intranasal)
709

Dose	Administration route 1st Dose/2nd Dose		
	IM/IM	IN/IN	IN/IM
$10^{7.0}$ $EID_{50\%}$/dose (LD)	10	10	10
$10^{7.5}$ $EID_{50\%}$/dose (MD)	10	10	10
$10^{8.0}$ $EID_{50\%}$/dose (HD)	10	10	10

710
711 **Table 2.** Incremental dose administered per route/regimen for the first 18 subjects as a sentinel group
712 for safety monitoring (IM=intramuscular; IN=intranasal; S=subject)
713

1st Dose	Low Dose (LD) ($10^{7.0}$ $EID_{50\%}$/dose)			Medium Dose (MD) ($10^{7.5}$ $EID_{50\%}$/dose)			High Dose (HD) ($10^{8.0}$ $EID_{50\%}$/dose)		
	Day1	Day 2	Day3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
IM	S1	S3	S5	S7	S9	S11	S13	S15	S17
IN	S2	S4	S6	S8	S10	S12	S14	S16	S18
Evaluation by an Independent Safety Committee 7 days after last High Titer vaccination									

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714
715 **Table 3.** Assignment of first dose to subjects enrolled after sentinel group (IM=intramuscular;
716 IN=intranasal; S=subject)
717

1st Dose	Low Dose (LD) ($10^{7.0}$ EID$_{50\%}$/dose)	Medium Dose (MD) ($10^{7.5}$ EID$_{50\%}$/dose)	High Dose (HD) ($10^{8.0}$ EID$_{50\%}$/dose)
IM	S19, S21, S23, S28, S30, S32, S37	S43, S45, S47, S52, S54, S56, S61	S67, S69, S71, S76, S78, S80, S85
IN	S20, S22, S24, S25, S26, S27, S29, S31, S33, S34, S35, S36, S38, S39, S40, S41, S42	S44, S46, S48, S49, S50, S51, S53, S55, S57, S58, S59, S60, S62, S63, S64, S65, S66	S68, S70, S72, S73, S74, S75, S77, S79, S81, S82, S83, S84, S86, S87, S88, S89, S90
Evaluation by an Independent Safety Committee 7 days after last HD vaccination			

718
719 **Table 4.** Subjects infected by SARS-CoV-2 per group (IM=intramuscular; IN=intranasal)

Dose	Route/regimen Infected after 1st Dose			Route/regimen Infected after 2nd Dose			Total per dose level
	IM/IM	IN/IN	IN/IM	IM/IM	IN/IN	IN/IM	
$10^{7.0}$ EID$_{50\%}$/dose (LD)	1	0	0	0	0	0	1
$10^{7.5}$ EID$_{50\%}$/dose (MD)	0	2	0	0	3	1	6
$10^{8.0}$ EID$_{50\%}$/dose (HD)	1	1	0	0	0	1	4
Subtotal per route and administered doses	2	3	0	0	3	2	

720

721

722 **Supplementary Table 1. Flow Cytometry antibodies.**

Antibody	Fluorochrome	Clone	Dilution	Brand	Catalog Number
Anti-human IFN-γ	FITC	4S.B3	1:200	BD	554551
Anti-human CD3	Alexa Fluor© 700	SK7	1:33	BioLegend	344822
Anti-human CD4	PerCP/Cy5.5	RPA-T4	1:100	BioLegend	300530
Anti-human CD8	PE/Cy7	53-6.7	1:100	BioLegend	980910

723

724 **Supplementary Table 2. Flow Cytometry reagents.**

Reagent	Brand	Catalog Number
Live/Dead Fixable near 633 or 635nm (work dilution 1:1000)	Invitrogen	L34976A
Anti-CD28/CD49d	BD	347690
Phytohemagglutinin (PHA)	Sigma	
Recombinant Spike Protein, Subunit 1	Raybiotech	230-011101-500
RBC Lysis Buffer	Biolegend	420302

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Golgi Stop	BD	554715
Cytofix/Cytoperm	BD	51-2090KZ
Perm/Wash	BD	51-2091KZ
PBS	Lonza	17-516Q
RPMI 1640	Lonza	12-16Q

725

726 **Figure legends**

727 **Figure 1. Study design and groups distribution.** (A) Schematic representation of the study timeline,
728 indicating routes of administration, vaccination time points, and sample collection for immunogenicity
729 analyses. The three different vaccination regiments tested; intramuscular (IM) followed by intramuscular
730 (IM), intranasal (IN) followed by intranasal (IN), and intranasal (IN) followed by intranasal (IN)
731 administration are show on the left. Time points of sample collection (0, 14, 21, 28, 42, 90, 180 and 365
732 days after the first vaccine dose administration) and time points of vaccine administration (indicated by
733 the red syringe) are shown on the right. (B) Diagram depicting specimen types collected to assess
734 immunogenicity. (C) Subgroup characteristics and demographic information of participants of the trial
735 (n=91).

736 **Figure 2. Enrollment and sub-randomization.** Diagram representing number of participants initially
737 screened (n=142), failed enrollment criteria (n=48), early withdrawals (n=3), and eligible participants
738 (n=90) that were included in the trial and assigned to any of the three vaccination regimens (n=30, per
739 group) and dose (low n=10, medium n=10, high=10). A participant that initially was considered eligible
740 and received an IM-IM regimen, but subsequently failed study criteria is indicated on the left.

741 **Figure 3. Local and systemic solicited adverse reactions.** Adverse events (AE) were registered according
742 to the standardized MedDRA dictionary terms and classified as mild, moderate, or severe according to
743 ICH/E6R2 Good Clinical Practices definitions. (A) Adverse reactions reported by the trial participants within
744 7 days after the first and second vaccine doses are shown. (B) Systemic reactions reported by the trial
745 participants throughout the observational period are shown. In both A and B, mild, moderate, and severe
746 adverse reactions are shown in the individuals receiving either of the three vaccination regimens and data
747 is stratified by the vaccine dose received (LD=low dose, MD=medium dose, and HD=high dose).

748 **Figure 4. Spike-reactive antibody levels in sera from vaccinated volunteers.** Antibodies against the S1
749 subunit of the spike protein (which contains the receptor binding domain (RBD)) were measured in
750 vaccinees' sera by ELISA at baseline, and 14, 21, 28, and 42 days after the first vaccine dose administration.
751 Individuals receiving the IM-IM regimen (left column), IN-IN regimen (middle column), or IN-IM (right
752 column), with a high dose (top row), medium dose (middle row), or low dose (bottom row) of the vaccine
753 are shown. Human convalescent serum (HCS) samples were added as additional controls. The limit of
754 detection (LoD) is indicated by the horizontal dotted line. Negative values are indicated as half of the LoD.
755 Statistical significance is indicated as follows: *P < 0.05, **P < 0.01, ***P < 0.001.

756 **Figure 5. RBD-ACE2 interaction inhibiting antibodies in sera from vaccinated volunteers.** Antibodies
757 binding to the receptor binding domain (RBD) that inhibited its interaction with the angiotensin-
758 converting enzyme 2 (ACE2) were assessed in vaccinees' sera using an RBD-ACE2 interaction inhibition
759 assay at baseline and 14, 21, 28, and 42 days after the first vaccine dose administration. Individuals
760 receiving the IM-IM regimen (left column), IN-IN regimen (middle column), or IN-IM (right column), with

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761 a high dose (top row), medium dose (middle row), or low dose (bottom row) of the vaccine are shown.
762 Human convalescent serum (HCS) samples were added as additional controls. The cutoff established for
763 positivity (30%) in this assay is indicated by the horizontal dotted line. Statistical significance is indicated
764 as follows: *P < 0.05, **P < 0.01, ***P < 0.001, ***P < 0.0001.

765 **Figure 6. Activation profile of spike-specific CD3+, CD4+ and CD8+ T cells after vaccination.** PBMCs were
766 collected from vaccinees at baseline and 42 days after the first vaccine dose administration. Individuals
767 receiving the IM-IM regimen (left column), IN-IN regimen (middle column), or IN-IM (right column)
768 stratified by vaccine dose received (low, medium, or high) are shown. Activated CD3+ (top row), CD4+
769 (middle row), and CD8+ (bottom row) T cells were determined by flow cytometry after 18 h incubation
770 with recombinant spike protein. Frequencies of T cells producing interferon gamma (IFN-γ) are presented.
771 Statistical significance is indicated as follows: *P < 0.05, **P < 0.01.

772

773 **Supplementary figure 1. Frequency of individuals with detectable spike-reactive and RBD-inhibiting**
774 **antibody titers.** Antibodies against the S1 subunit of the spike protein (which contains the receptor
775 binding domain (RBD)) and antibodies binding to the RBD that inhibited its interaction with the
776 Angiotensin-Converting Enzyme 2 (ACE2) were assessed in vaccinees' sera at baseline and 14, 21, 28, and
777 42 days after the first vaccine dose administration. Individuals receiving the IM-IM regimen (left column),
778 IN-IN regimen (middle column), or IN-IM (right column), with a high dose (top), medium dose (middle), or
779 low dose (bottom) of the vaccine are shown. S1-IgG = antibodies binding to the S1 spike subunit; NAb =
780 antibodies inhibiting RBD-ACE2 interactions.

781 **Supplementary figure 2. Medium vs antigen stimulation of CD3+ T cells from vaccinated volunteers.**
782 PBMCs were collected from vaccinees at baseline, and 14, 21, 28 and 42 days after the first vaccine dose
783 administration. Individuals receiving the IM-IM regimen (left column), IN-IN regimen (middle column), or
784 IN-IM (right column) stratified by the vaccine dose received (high, medium, or low) are shown. Activated
785 CD3+ T cells were determined by flow cytometry after 18 h incubation with the recombinant spike protein
786 or with medium only. Frequencies of T cells producing interferon gamma (IFN-γ) are presented. Statistical
787 significance is indicated as follows: *P < 0.05, **P < 0.01.

788

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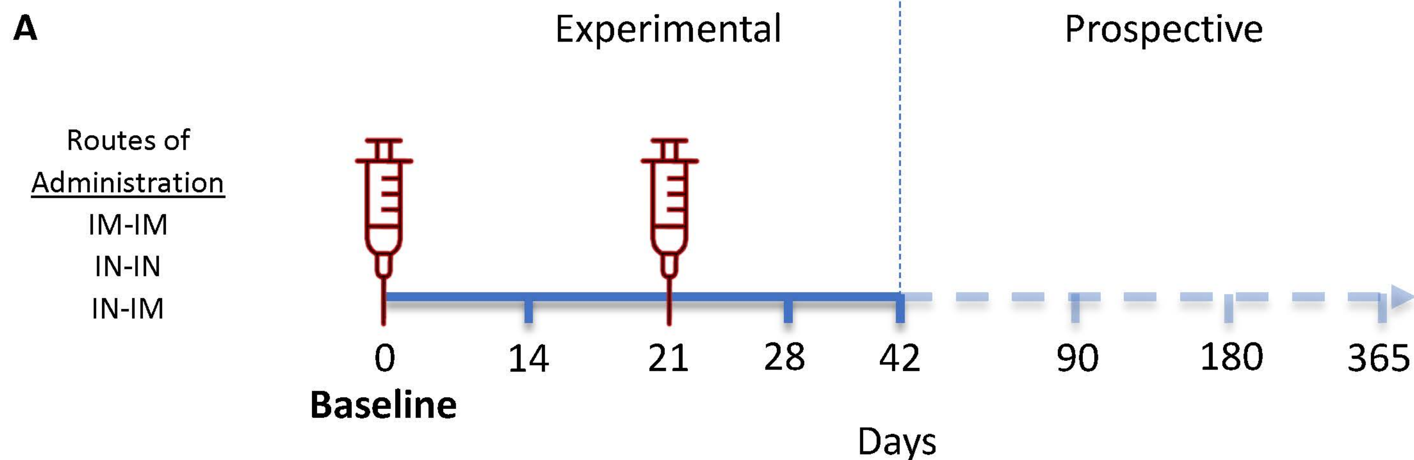
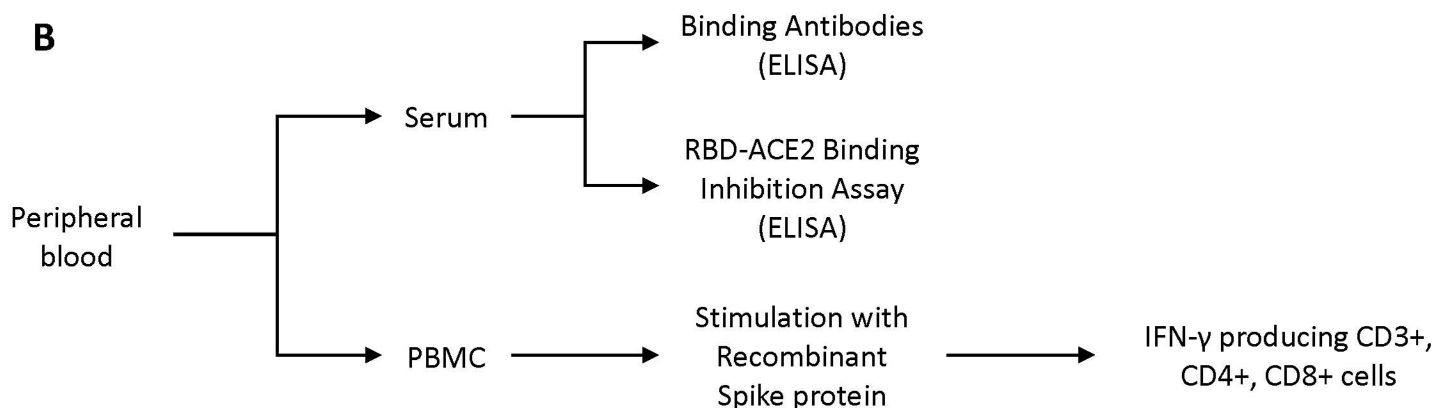
Figure 1





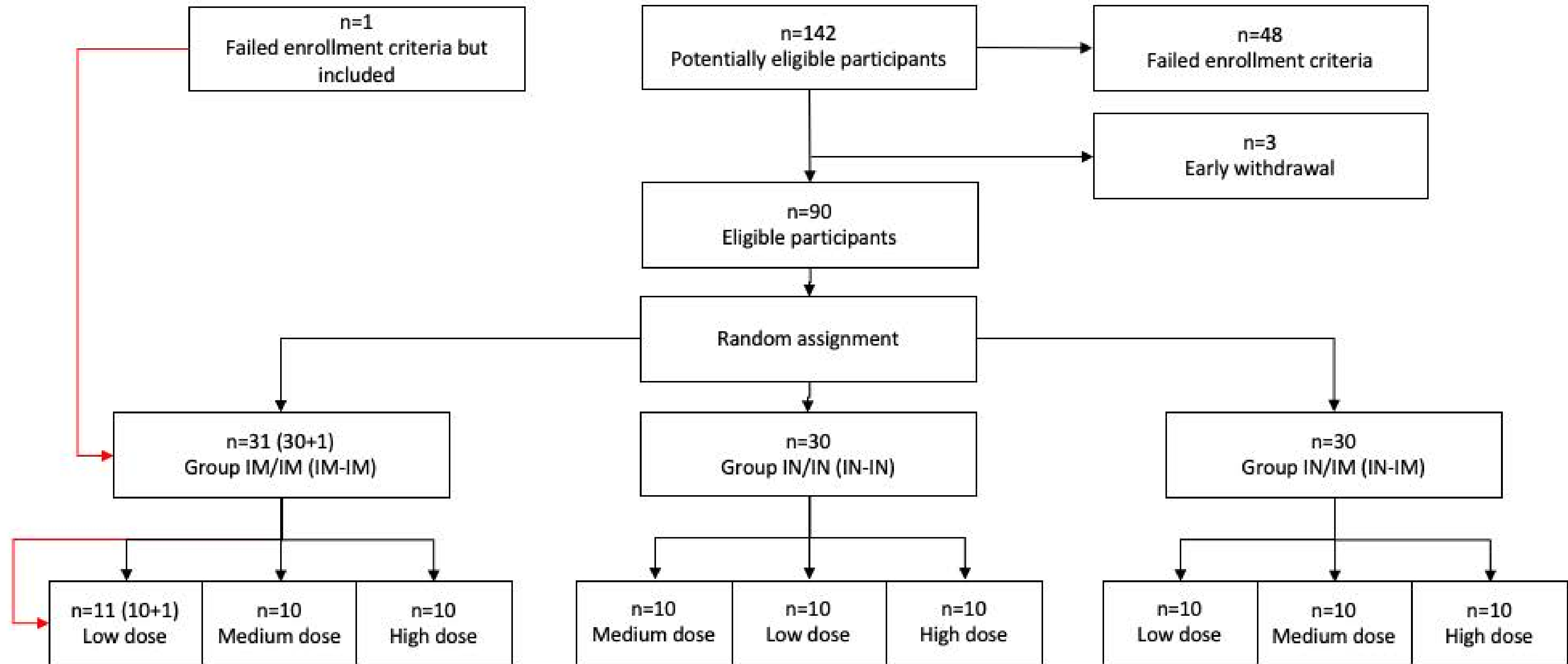
C

Subgroup characteristics and demographic information of participants.

Item [unit]	Total (n=91)	Low dose IM/IM (n=11)	Low dose IN/IN (n=10)	Low dose IN/IM (n=10)	Medium dose IM/IM (n=10)	Medium dose IN/IN (n=10)	Medium dose IN/IM (n=10)	High dose IM/IM (n=10)	High dose IN/IN (n=10)	High dose IN/IM (n=10)
Age [years] (S.D.)*	26.8 (6.6)	25.7 (5)	26.7 (5.5)	28.4 (4.8)	33.8 (11.9)	24 (2.5)	24.4 (3.2)	28.2 (8.5)	29.3 (7.7)	23.7 (5.5)
Age range [years]	18-52	21-38	19-39	19-35	20-52	21-28	20-30	19-31	18-46	18-38
Males [n] (%)	44 (48.4)	5	4	4	6	5	3	6	5	6
Females [n] (%)	47 (51.6)	6	6	6	4	5	7	4	5	4
Weight [kg] (S.D.)*	65.5 (11)	65.3 (8.3)	60.8 (7.9)	66.7 (12.3)	69.4 (14)	66.9 (10.1)	59.4 (6.9)	70.7 (12.2)	60.9 (7.6)	70.1 (14.6)
Height [m] (S.D.)*	1.7 (0.1)	1.6 (0.1)	1.6 (0.1)	1.6 (0.1)	1.7 (0.1)	1.7 (0.1)	1.6 (0.1)	1.7 (0.1)	1.6 (0.1)	1.7 (0.1)
Body-mass Index* [kg/m^2] (S.D.)	24 (2.8)	24.6 (2.3)	22.5 (2.4)	25.3 (3.4)	24.9 (2.7)	24.2 (3)	22.5 (2.4)	24 (2.6)	23 (2)	24.6 (3.8)

*Average values reported.

Figure 2



A

Figure 3

Participants that reported local reactions within 7 days after any vaccination

Frequency of participants (%)

100
80
60
40
20
0

Mild
Moderate
Severe

LD MD HD LD MD HD LD MD HD

IM-IM IN-IN IN-IM

Treatment Group

B

Participants that reported systemic reactions after any vaccine administration

Frequency of participants (%)

100
80
60
40
20
0

Mild
Moderate
Severe

LD MD HD LD MD HD LD MD HD

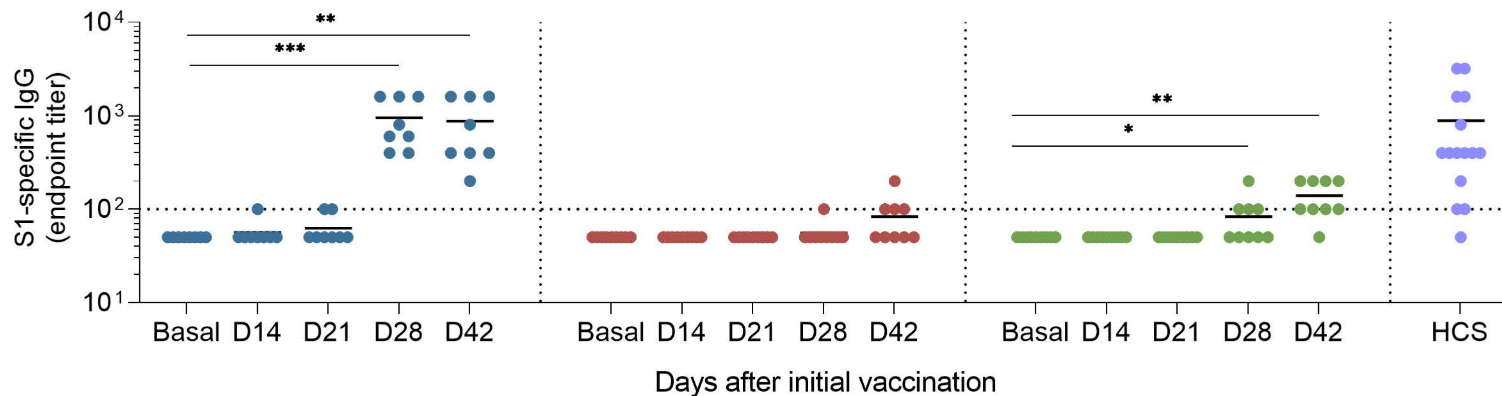
IM-IM IN-IN IN-IM

Treatment Group

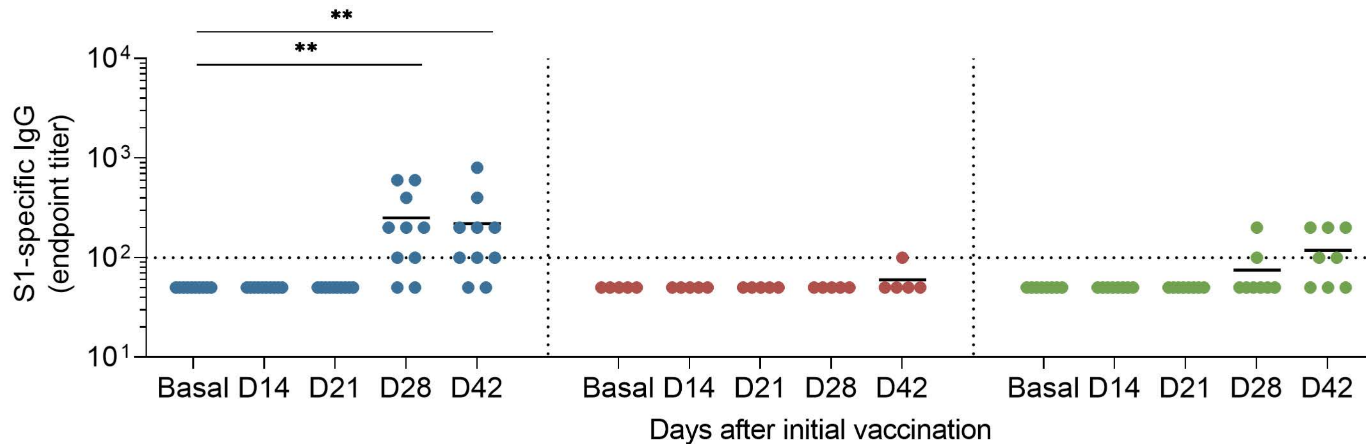
Figure 5

IM-IM IN-IN IN-IM

High dose



Medium dose



Low dose

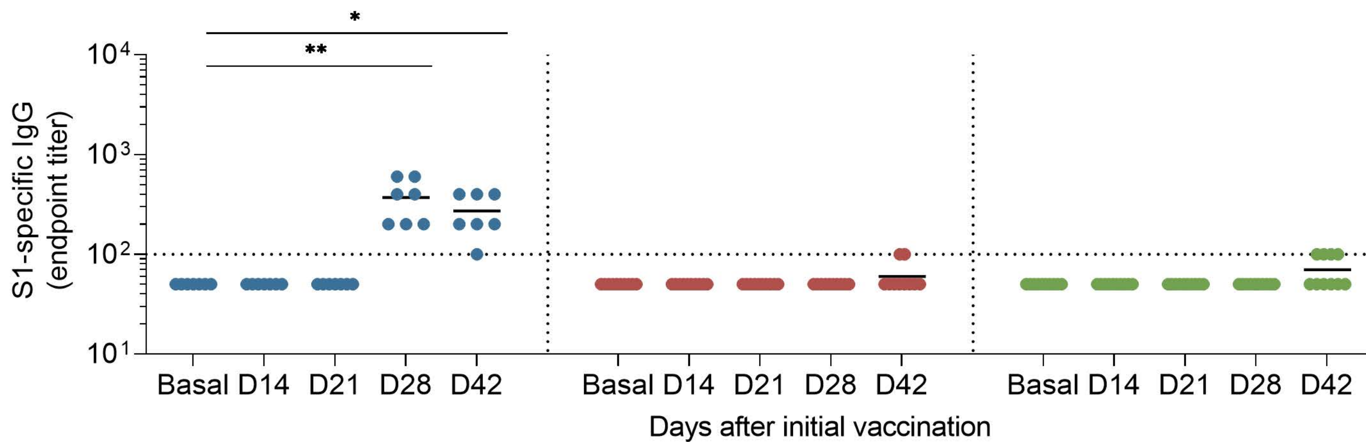


Figure 6

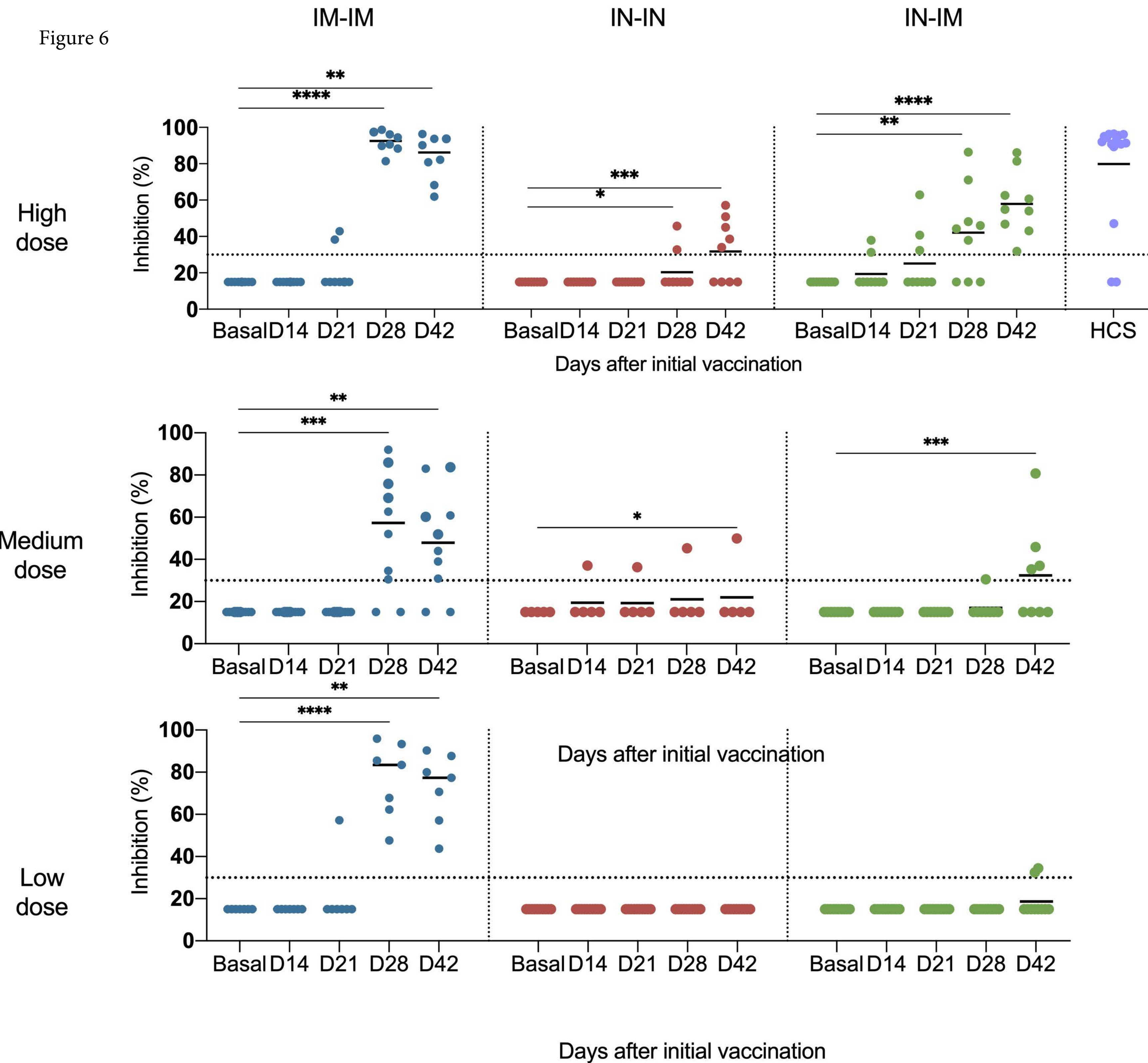
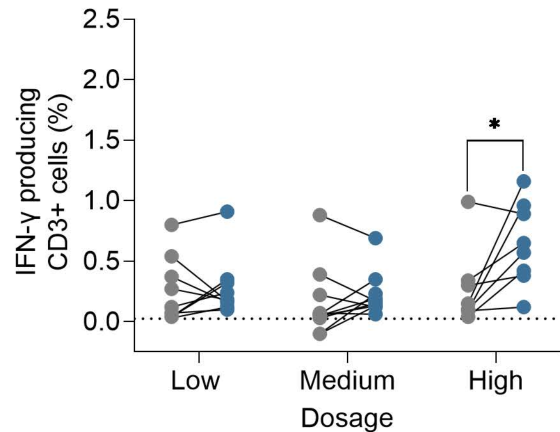


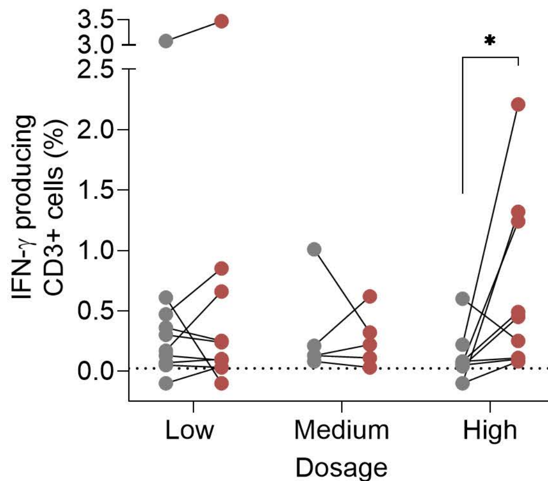
Figure 7

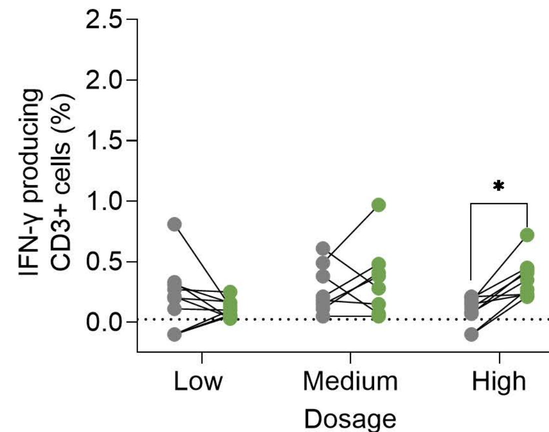
IM-IM IN-IN IN-IM

Basal
D42 IM-IM
D42 IN-IN
D42 IM-IN

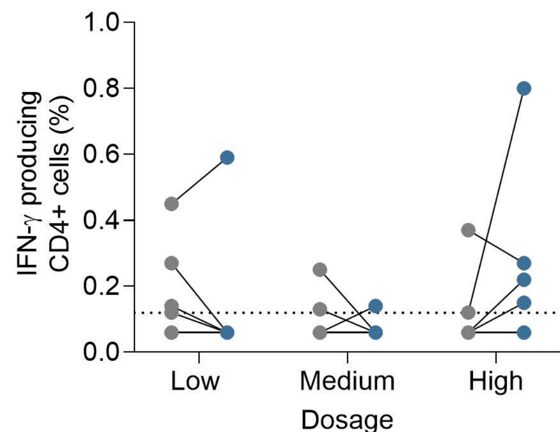
CD3+

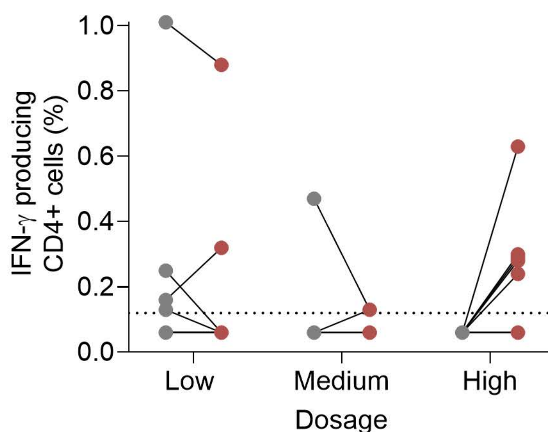


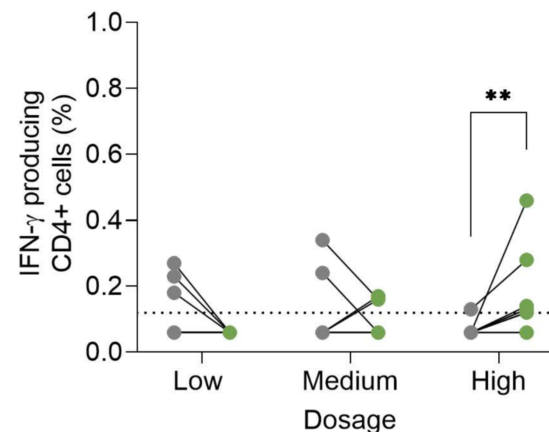




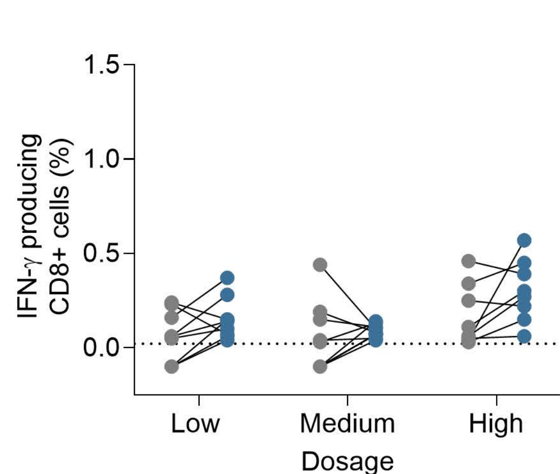
CD4+

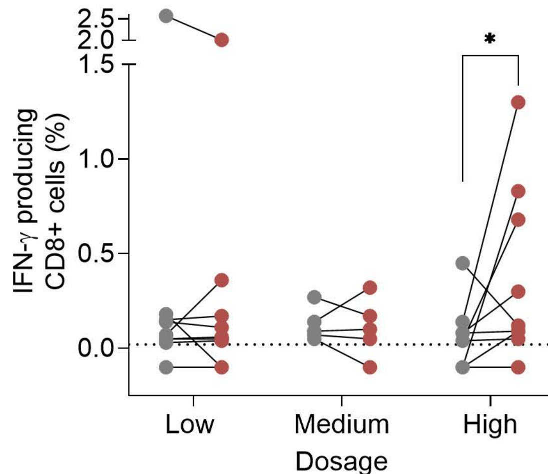


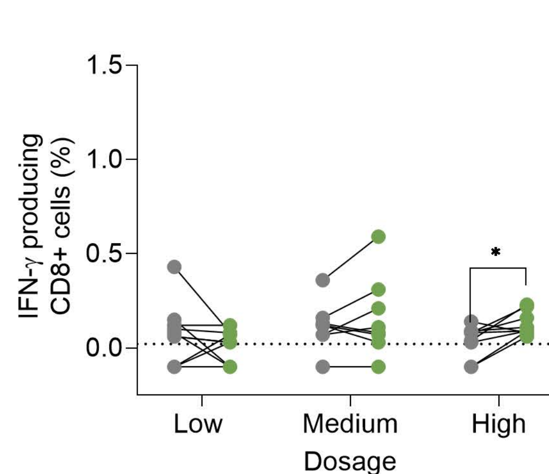




CD8+







S. Fig. 1

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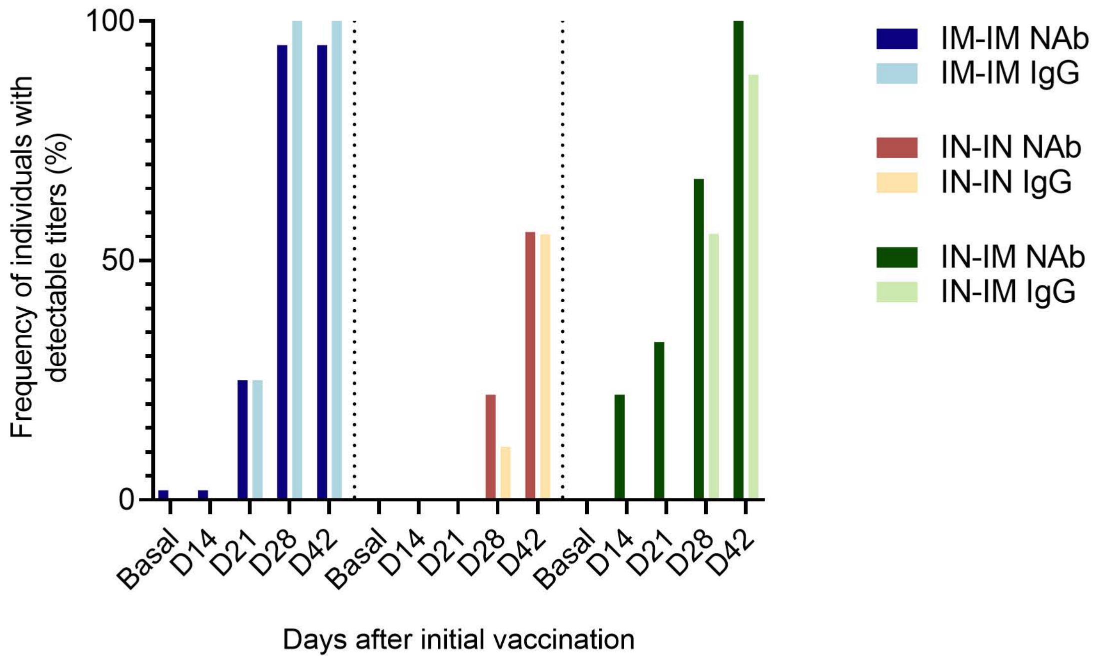
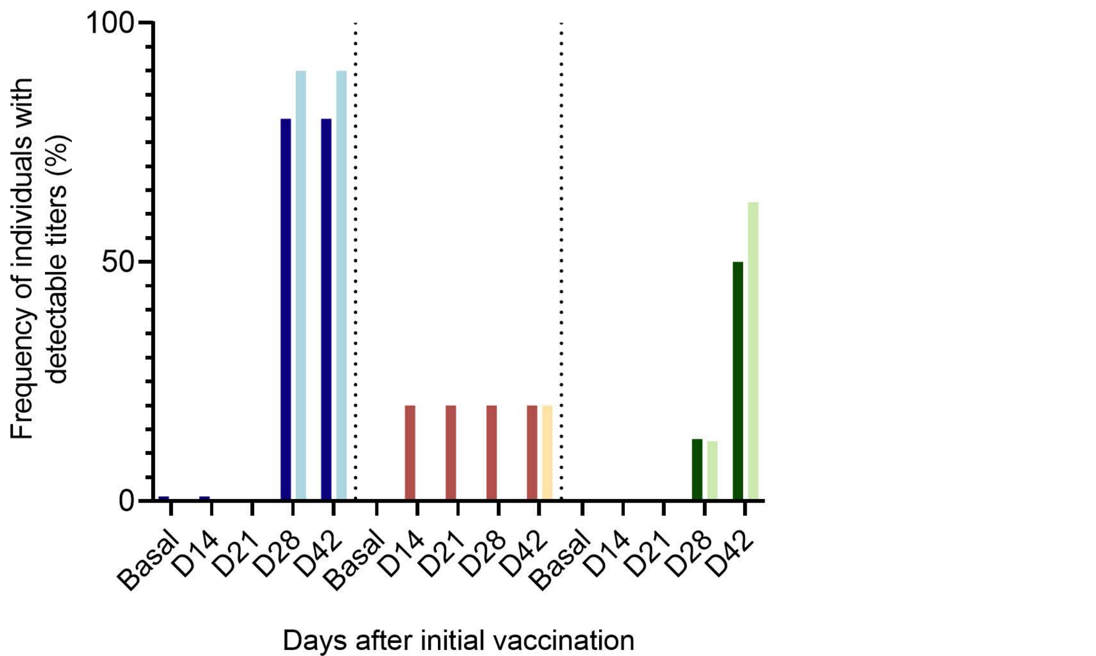
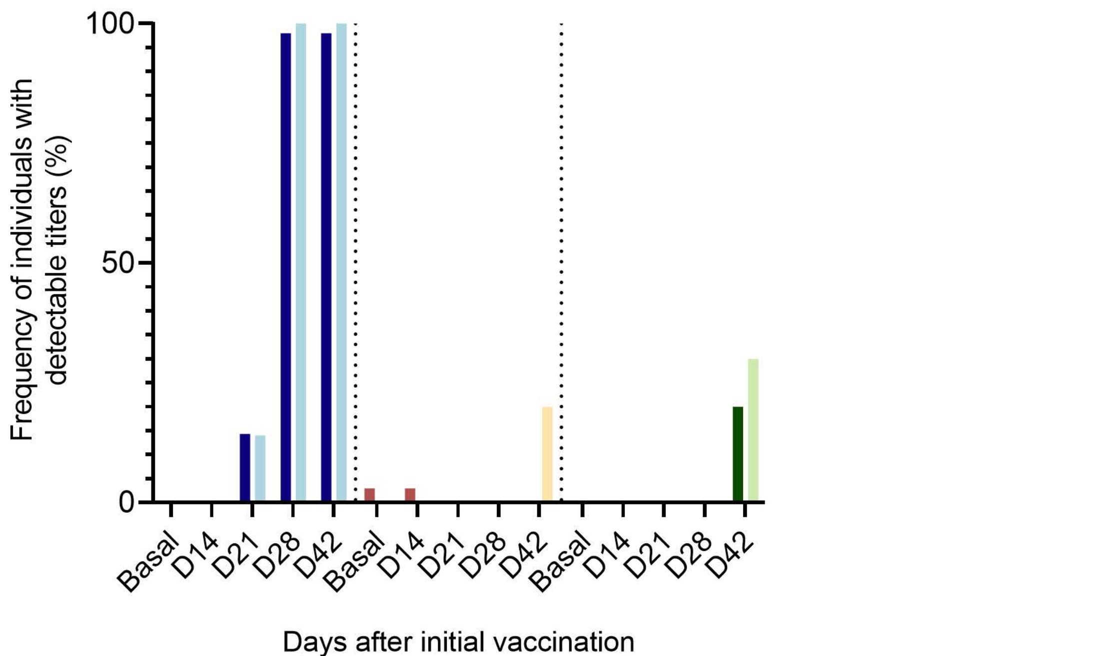
High dose



Medium dose



Low dose



S. Fig. 2