Human Infection Challenge with Serotype 3 Pneumococcus

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RR, MF, KD, DMF - study co-ordination

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At a Glance

What is the current scientific knowledge on this subject?

S. pneumoniae serotype 3 (SPN3) disease in adults has not undergone the reduction in prevalence observed for other vaccine-type serotypes since the widespread introduction of the 13-valent pneumococcal conjugate vaccine into paediatric immunisation programmes. The immune response and mechanism of vaccine escape for this serotype are not well understood, and further studies investigating this are urgently required.

What does this study add to the field?

An SPN3 human infection challenge model can be safety performed with appropriate monitoring in place and provides the ideal platform for investigating the impact of pneumococcal vaccination against pneumococcal colonisation acquisition and density for this important disease-causing serotype.

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Abstract:

Rationale: *Streptococcus pneumoniae* serotype 3 (SPN3) is a cause of invasive pneumococcal disease and associated with low carriage rates. Following the introduction of pediatric 13-valent pneumococcal conjugate vaccine (PCV13) programmes, SPN3 declines are less than other vaccine serotypes and incidence has increased in some populations coincident with a shift in predominant circulating SPN3 clade, from I to II. A human challenge model provides an effective means for assessing the impact of PCV13 on SPN3 in the upper airway.

Objectives: To establish SPN3's ability to colonise the nasopharynx using different inoculum clades and doses and the safety of an SPN3 challenge model.

Methods: In a human challenge study involving three well characterised and antibiotic sensitive SPN3 isolates (PFESP306 [clade Ia], PFESP231 [no clade] and PFESP505 [clade II]), inoculum doses (10,000, 20,000, 80,000, 160,000 CFU/100µL) were escalated until maximal colonisation rates were achieved, with concurrent acceptable safety.

Outcome measures: Presence and density of experimental SPN3 nasopharyngeal colonisation in nasal wash samples, assessed using microbiological culture and molecular methods, on days 2, 7 and 14 post-inoculation.

Results: 96 healthy participants (median age 21, interquartile range 19-25) were inoculated (n=6-10 per dose group, 10 groups). Colonisation rates ranged from 30.0-70.0% varying with dose and isolate. 30.0% (29/96) reported mild symptoms (82.8% sore throat, [24/29]), one developed otitis media requiring antibiotics. No serious adverse events occurred.

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Conclusions: An SPN3 human challenge model is feasible and safe with comparable carriage rates to an established SPN6B human challenge model. SPN3 carriage may cause mild upper respiratory symptoms.

Key words: Pneumococcus, Serotype 3, Challenge model, SPN3

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Introduction:

S. pneumoniae serotype 3 (SPN3) is a frequent cause of community acquired pneumonia (CAP)[1,2] and invasive pneumococcal disease (IPD)[3], even in countries with mature infant pneumococcal conjugate vaccine (PCV) immunisation programmes and is independently associated with a higher incidence of septic shock and mortality[4] in adults. Vaccination with 13-valent PCV (PCV13) provides varying levels of protection against IPD[5,6] and vaccine-type (VT) otitis media[7]. A post-hoc analysis of randomised controlled trial (RCT) data assessing PCV-13 against serotype-specific clinical pneumonia among older adults (at a time when non-SPN3 containing PCV-7 was included in routine paediatric vaccinations) reported an efficacy of 61.5% (95% CI, 17.6-83.4%)[8], with similar (52.5% [95% CI, 6.2-75.9%]) reported in a pooled analysis[9]. Nevertheless, unlike other VT serotypes, national PCV13 paediatric immunisation programmes have not significantly reduced adult SPN3 disease[10,11] possibly because of reduced ability to impact carriage and thus provide indirect protection to unvaccinated age cohorts.

SPN3 differs from other pneumococcal serotypes in its improved ability to evade host-defence mechanisms. It is heavily encapsulated[12,13], leading to distinct colony morphology on blood agar plates; this capsule has been linked to higher virulence in animal studies[14]. SPN3 has a higher capsular shedding rate *in vitro*, allowing the antibody-antigen complex to separate from pneumococcal cells and decrease opsonophagocytic activity (OPA)[15], although the impact during natural infection is unknown. SPN3 surface proteins may inhibit OPA by blocking complement deposition[16]. These features may allow SPN3 to inhibit opsonisation by complement, avoid phagocytosis and resist neutrophil extracellular traps[17,18].

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Most SPN3 isolates are part of clonal complex 180 (CC180)[19-21], which is further subdivided into clades based on variation in accessory genomes[22], two of which are dominant globally, clade I (Ia and Ib) and II[23]. Between 1999-2014 there was a dramatic shift in global SPN3 populations from clade I to II[23]. Clade II emerged in 1999 in Asia before widespread PCV implementation, and the first SPN3 containing PCV; no association with vaccine introduction has been reported. Clade II has distinct antigenic profiles and a higher prevalence of antimicrobial resistance than clade Ia[23].

Understanding immune responses to SPN3-containing vaccines and the continued persistence of SPN3 disease is vital for implementing optimal vaccine strategies. The Experimental Human Pneumococcal Challenge (EHPC) model, in which participants are experimentally inoculated with pneumococccus, allows interrogation of immune responses mimicking "natural" exposure[24]. Previous EHPC studies identified SPN6B as a safe challenge candidate, with a high carriage:disease ratio in natural infection, predictable 50% colonisation rate and density typical of natural colonisation[25]. SPN3 is potentially difficult for EHPC, given a low carriage:disease ratio in natural infection, but offers a unique and essential opportunity to evaluate the efficacy of current and future vaccine candidates against clade-specific SPN3 colonisation. This study aims to identify optimum SPN3 isolates, doses and immune responses induced following challenge. We hypothesise that a safe and reproducible SPN3 model can be developed using appropriate volunteer recruitment, controlled challenge methods, close monitoring and responsive safety protocols. Some of the results of this study have been previously reported in the form of an abstract[26].

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Methods*:*

Trial design and Participants

Healthy participants aged 18-50 were recruited between November 2019- March 2021 (paused March 2020-January 2021 due to the COVID-19 pandemic). All participants provided written informed consent and the study was approved by the North-West NHS Research Ethics Committee (19/NW/0043) and prospectively registered on the ISRCTN database (ISRCTN11306486).

All eligible participants were inoculated with a penicillin-sensitive *S. pneumoniae* serotype 3 (CC180); either strain PFESP306 (clade Ia), PFESP231 (no identifiable clade, referred to as 'no clade') or PFESP505 (clade II). SPN3 isolates were pre-selected from a reference bank (Antimicrobial Testing Leadership and Surveillance database (ATLAS); Pfizer, NCBI BioSample accessions SAMN27406322, SAMN27406323, SAMN27406324) due to their clinical relevance, phylogenesis, clade affiliation, presence of virulence/colonisation genes, and pneumococcal chain length in liquid culture, with the latter correlating with achieved average experimental pneumococcal colonisation rates in an SPN6B model[27]. Serotype, bacterial purity and penicillin sensitivity were confirmed by an independent reference laboratory prospectively (Public Health England, Colindale, UK). Participants were inoculated in groups (allocation dependent on participant availability) with each assigned a pre-defined dose of one of the three chosen isolates.

Detailed methodology for pneumococcal inoculum preparation and participant inoculation has been reported previously[25]. In brief, 100µL of SPN3 inoculum suspended in normal saline at the desired dose is instilled into each nostril and the participant was asked to remain in a semi-recumbent position for 15 minutes. Participants received one inoculation using a pre-

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specified dose, before being followed up for 14 days. A dose escalation format (Figure 1) was used with an initial group receiving 20,000 CFU/100µL per nostril, before either increasing to 80,000 and 160,000 or decreasing to 10,000 CFU/100µL based on nasopharyngeal (NP) colonisation rates and the ongoing safety profile.

Participant monitoring and safety

SPN3 challenge theoretically poses a higher risk of disease than previous EHPC serotypes, therefore robust safety measures were required (online data supplement (ODS) Table E1). A Data Safety Monitoring Committee (DSMC) closely monitored safety procedures including oversight of adverse events and dose escalation. Protocol safety measures included the inclusion of only healthy participants (inclusion/exclusion criteria in ODS Table E2). Participant quarantine post-inoculation was not required as SPN3 is circulating in the community. All experimentally colonised individuals received antibiotics at their last study visit. During the COVID-19 pandemic, study procedures were altered and infection control measures introduced to minimise viral spread risk (ODS figure E1). All serious adverse events (SAE) and adverse events of special interest (AESI, ODS Table E3) were reported to the DSMC and sponsor as per Good Clinical Practice (GCP) guidance.

Detection of experimentally induced pneumococcal colonisation using microbiology culture

Experimental SPN3 colonisation was determined by serial nasal washes (NW) and defined as the detection of SPN3 by culture in NW samples at any time point (days 2, 7 and 14) post-inoculation. NW microbiological culture was performed as described previously[25] and detailed in the ODS. Confirmation of pneumococcal serotype was performed using latex agglutination (Statens Serum Institute, Copenhagen, Denmark).

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Detection of experimentally induced pneumococcal colonisation using molecular methods

DNA extraction from NW samples was performed using a phenol-based methodology utilising magnetic beads. Molecular detection was achieved using sequential qPCR starting with a pneumococcal *LytA* and *SPN3 CPS* genes multiplex and then confirmed with *PiaB* gene[28,29]. Combined detection of *LytA, SPN3 CPS* and *PiaB* was used to enhance test specificity, as the *LytA* gene may also be expressed by non-pneumococcal streptococci.

Detection of viral respiratory pathogens using molecular methods

Oropharygneal swabs were performed at inoculation by rotating a flocked swab over the palatine tonsils and posterior pharyngeal arch ten times. Concurrent nasopharyneal swabs were not performed to avoid impacting on colonisation. Real-time reverse transcription PCR (Panther Fusion®, Hologic, USA) was used to detect ten common respiratory viral pathogens, detailed in ODS Table E4.

NW anti-pneumococcal capsular polysaccharide antibodies

Anti-pneumococcal capsular polysaccharide antibodies (IgG) to SPN3 and SPN6B were measured in participant NW samples using a modified World Health Organization standardized enzyme-linked immunosorbent assay (ELISA) protocol[30], detailed in the ODS.

Luminex analysis of NW samples

The nasal cytokine profile was measured as previously described[31] with samples concentrated x10 following acquisition using a 30-plex magnetic Luminex cytokine kit (ThermoFisher Scientific) and then analysed with an LX200 insrument with xPonent3.1 software, following the manufacturer's instructions. Samples were run in duplicate and standards on all plates.

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Identification and grading of symptoms post-inoculation

Pre-defined AESI are shown in ODS Table E3 and grading system in Table E5. All participants were questioned for the presence and onset of symptoms of interest at each study visit. Symptomatic participants were defined as those self-reporting symptoms during the study follow-up period, further detailed in the ODS.

Endpoints

The primary endpoint was the presence of experimental colonisation at any time point post-inoculation (days 2, 7, 14) by microbiological culture. Secondary endpoints included 1) presence of experimental pneumococcal colonisation at any timepoint by molecular methods 2) density of experimental pneumococcal colonisation at all time points 3) duration of experimental colonisation, defined as time from inoculation to last confirmed positive NW sample using microbiological culture (up to day 14).

Statistical methods and analysis

Demographic data was reported using descriptive statistics and variance assessed using an ANOVA (Kruskal-Wallis test). All analyses (including safety) were performed among subjects with at least a single follow up visit post-inoculation. For the primary outcome analysis, the presence/absence of pneumococcus at any timepoint post-inoculation, was summarised by number (%) with and without colonisation. Fisher's exact test was used to compare the presence of symptoms in those colonised and non-colonised. Multivariable linear regression models to estimate factors associated with day 14 IgG included isolate, dose, colonisation status and symptoms as independent variables. Similar approaches were applied, using logistic regression, to estimate association with symptoms. For non-parametric data, Mann-

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Whitney U or Wilcoxon tests were utilised for unpaired and paired data respectively. For Luminex analysis, where required *P*-values were corrected by applying multiple correction testing (Benjamini-Hochberg). Analyses were performed in SAS® (version 9.4), GraphPad Prism (version 9.20) and R (version 4.0.4).

Results

141 individuals were screened, and 96 healthy volunteers were enrolled and inoculated (39 males, 57 females, median age 21, interquartile range (IQR) 19-25), as shown in CONSORT diagram Figure 2. None had previously received a pneumococcal vaccination. One participant was excluded since he/she was a natural carrier for SPN3 at screen by microbiological culture. Oropharyngeal swabs for respiratory viral PCR performed retrospectively identified 5 participants carrying rhinovirus at inoculation.

All challenge isolates achieved nasopharyngeal colonisation

As per the primary endpoint, colonisation was achieved with each of the three SPN3 isolates, although there were differences in the dose required and the proportion of participants who were successfully colonised. The experimental colonisation rates for all isolates broadly increased with inoculum dose, with the highest overall colonisation rate of 70% using microbiological culture for clade II at 160,000 CFU/100µL (ODS Table E6), shown in Figures 3A-3C. The no-clade isolate achieved colonisation rates of 60% (6/10) at 20,000 CFU/100µL, a higher colonisation level than achieved in an SPN6B model, therefore the decision was made to not pursue higher colonisation rates at further inoculum doses for this isolate. Molecular analysis using sequential multiplex qPCR (*LytA/SPN3 CPS* then *PiaB* gene*)* closely matched the microbiological culture results, shown in Figures 3A-C.

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Duration of colonisation was similar across all three isolates

The duration of colonisation was comparable between each of the isolates and doses. 33 participants (34.4%) were culture positive on day 2 with 7 (21.2%) participants having colonisation terminated early using antibiotics due to reporting upper respiratory symptoms. All those requiring antibiotics were culture negative on subsequent samples collected post-treatment. 88.5% (23/26) of those who had obtained colonisation on day 2, and did not require antibiotics, remained culture positive at day 14. Five participants had undetectable experimental colonisation on day 2, but colonisation was subsequently detected on day 7 (n=4) or 14 (n=1), suggesting the presence of very low-density colonisation at day 2.

Colonisation density was high for all three isolates

The experimental colonisation density was comparable between the three isolates tested and increased with inoculum dose, up to 80,000 CFU/100µL, with a maximal mean density of 5.15 (SD 0.87) log CFU/mL of NW in the clade II at 80,000 CFU/100µL group, shown in Figure 4. 30% (6/20) of the 160,000 CFU/100µL group began antibiotics before day 7 due to symptoms. All participants commenced on antibiotics had no detectable bacteria in the next NW sample (day 7) using either microbiological culture or qPCR.

Molecular analysis of NW samples followed a similar pattern to microbiologic culture (Figure 4). 80,000 CFU/100µL yielded the highest number of DNA copies/ml of NW for each isolate tested.

The recovered SPN3 NW densities were compared with those of the SPN6B challenge study[25] shown in Figure 4, with densities using microbiological and molecular methods more closely matched than a comparative SPN6B model. On day 2 (prior to antibiotics onset) inoculum

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doses of 20,000 and 80,000 CFU/100µl yielded higher densities than SPN6B inoculation, using microbiological culture. The density kinetics per participant are demonstrated in ODS Figures E2A-C.

Mild post-inoculation symptoms occurred frequently

The frequency of reported symptoms in the SPN3 colonised participants was significantly increased compared to non-colonised participants (SPN3 colonised reporting symptoms=52.6% [20/38], non-colonised reporting symptoms= 15.5% [9/58], Fisher's exact test *P*=<0.001, odds ratio (OR) 6.05, 95% CI 2.2-15.1). 29/96 (30.2%) participants reported symptoms at any timepoint post-inoculation. The majority described sore throat (24/29, 82.8%), with 16 participants experimentally colonised and 8 non-colonised. Although no participants had an SAE, one participant (clade Ia colonised, dose group 10,000 CFU/100µL) developed earache on day 1 post-inoculation, which progressed to otitis media and tympanic perforation. An aural swab of the discharge confirmed experimental SPN3. Amoxicillin was commenced, after which symptoms resolved, and the tympanic perforation healed without sequelae under outpatient ENT review. The study was paused while this was investigated, and to reduce the risk of further episodes, the post-inoculation monitoring period was increased from 3 to 5 days and the clinical threshold for antibiotic usage was lowered. Instigating these changes prevented any progression of participant-reported symptoms following study re-start.

Most reported symptoms started within 7 days post-inoculation, were mild or moderate and, when required, resolved after antibiotics. All but two of the participants reporting symptoms had negative respiratory viral swabs at inoculation (two had rhinovirus but did not report symptoms until 5 and 7 days later*)*. Of those 24 participants reporting a sore throat post-

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inoculation, 17 had extra viral and bacterial throat swabs taken post-inoculation for clinical purposes. Two participants had positive on viral throat swabs (each had detectable rhinovirus and one rhinovirus and human metapneumovirus) and one had a positive bacterial swab for *group C Streptococcus*. Experimental SPN3 was cultured from two throat swabs of symptomatic participants (2/17, 11.8%), both were SPN3 colonised in NW samples. Antibiotics were initiated early for clinical reasons for 7 participants (27.6%, all experimental carriers), with 1 non-colonised participant self-initiating rescue antibiotic treatment without contacting the clinical team.

Nasal cytokine expression differs between challenge serotypes and inoculum doses

To assess whether induction of nasal inflammatory responses differs during colonisation with SPN3 compared with SPN6B, we analysed a subset of SPN3 colonised (n=36) and SPN6B colonised (n=22) participants using matched NW samples at baseline, day 2 and 7. No difference was observed at baseline between serotypes (ODS Figure E3A) with each varying in specific cytokines induced at day 2 or 7 compared to baseline (ODS Figure E3B).

Direct comparison between the two serotypes showed a degree of variation in type and levels of cytokines induced (Figure 6). When the three SPN3 isolates were compared, there was no difference in the cytokine profiles induced (ODS Figure E4).

Subsequent analysis of SPN3 inoculation doses (fold change (FC) to lowest doses) showed the highest doses led to a more pronounced change in cytokine levels, particularly at 160,000 CFU/100µl on day 7 (ODS Figure E5). For SPN6B, a similar comparison did not identify any significant changes in cytokine expression (data not shown).

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Nasal SPN3 anti-capsular IgG levels do not alter with colonisation by day 14

Paired baseline and day 14 NW samples for ELISA were available for 91/96 participants. At baseline, nasal SPN3 anti-capsular antibodies were similar for those who became colonised and non-colonised (median baseline IgG in colonised = 468.5 pg/mL [IQR 253.6-901.7] vs non-colonised= 450.5 pg/mL [IQR 281-708.7], *P*=0.72). Post-inoculation, no difference in IgG was observed between the two groups (median day 14 IgG in colonised =502.9 pg/mL [IQR 356.7-960.7], non-colonised= 514.7 pg/mL [IQR 213.9-902.8], *P*= 0.26). Multivariable linear regression modelling to estimate factors associated with IgG at day 14, (including baseline IgG, inoculum dose, isolate, colonisation status and reported symptoms) found only baseline IgG significantly associated (coefficient for log IgG baseline =0.34 (95% CI 0.15-0.54, *P*= 0.001), ODS Table E10).

No difference was seen in IgG fold change (FC) across all three challenge SPN3 isolates and doses with the exception of colonised individuals at 160,000 CFU/100µl (*P*=0.04), (ODS Figure E6A). Levels were comparable with those induced by SPN6B colonisation in a previous challenge study (Figure 7A and B).

Altered nasal cytokines but not bacterial density associated with symptomatic SPN3 carriage

Recovered bacterial density, cytokine and antibodies levels were compared between those reporting symptoms and those without. There was no difference in bacterial densities (Figure 8B). Expression of IL-6 and TNF-α was significantly increased in symptomatic compared to the asymptomatic participants (Figure 8C). In a multivariable regression model with 'symptoms' as the outcome (ODS table E11), there was an estimated significant negative association with log CPS IgG at baseline (OR=0.24, 95% CI 0.06-0.76, *P*=0.022). Furthermore when adjusting for dose, isolate, age and gender, their colonisation status was also statistically significant (OR

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=5.72, 95% CI 1.86-19.4, *P*=0.003). Day 14 IgG was not significantly associated with symptoms in similar modelling.

Discussion

We have successfully demonstrated that with appropriate safety precautions in place an SPN3 human challenge model can be safely performed and achieve experimental colonisation rates similar to, and at some doses higher, than the previously published SPN6B model[25]. Crucially this allows assessment of vaccine efficacy (VE) against SPN3 colonisation. While upper respiratory symptoms were common, all symptomatic participants were symptom-free (and SPN3 negative by culture) during follow up after completing antibiotics and none had lasting sequelae.

The impact of PCV13 on pneumococcal NP colonisation is not fully understood, however a reduction in colonisation density is considered the main driver of reduced VT pneumococcal transmission in the community and therefore contributes to the protection of unvaccinated persons[32]. This challenge model provides an effective means of investigating VE against SPN3 colonisation acquisition and impact on density using the dominant SPN3 clades circulating globally. An RCT comparing VE of PCV13 and 23-valent polysaccharide pneumococcal vaccination (23vPS/PPV-23, *Pneumovax* II, Sanofi Pasteur MSD) against clade Ia and clade II SPN3 challenge is underway[33].

Previous studies by our group have demonstrated pneumococcal colonisation to be asymptomatic using SPN6B[34]. However, SPN3 colonised participants frequently reported symptoms, particularly sore throats within the first 7 days post-inoculation. Although involving small participant numbers, the limited data available suggests this is related to inoculum dose with antibiotic usage greater in the higher dose inoculum groups. However, as

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these doses were used later in the study, clinician decision-making may have been influenced by an earlier participant developing experimental SPN3 culture-positive otitis media, leading to a lower threshold for antibiotic use.

Of interest, higher inoculum doses of bacteria were associated with greater inflammatory responses in the nose and high incidence of symptoms shortly after colonisation with SPN3, indicating that nasal inflammation is triggered by bacterial density in a dose-dependent manner, which was not previously observed in a SPN6B model[35].

S. pneumoniae is not commonly associated with pharyngitis in the literature and is considered part of host natural oral flora[36]. Our data raise the possibility that SPN3 infection is a cause of pharyngitis symptoms. The association of colonisation and symptoms necessary to support SPN3 as the pathogen, is suggested by the two symptomatic participants who had SPN3 identified from throat swabs without any other potential causative pathogen (con-current respiratory viral swabs negative). Non-colonised individuals also reported symptoms, however significantly less often. It is possible that these individuals were also SPN3 colonised but cleared the bacteria before samples were obtained, or symptoms were due to an undetected viral infection. An alternative hypothesis is that the challenge strain may alter the upper airway microbiome and induce a previously commensal organism to become pathogenic. The nature of the symptomology after SPN3 inoculation requires further investigation, with emphasis on the development of pharyngeal symptoms within 7 days post-inoculation and full description of the viral and bacterial microbiome before, during and after these episodes, in addition to comparison with placebo inoculation. Assessing whether prior PCV vaccination, which has been shown to reduce respiratory tract infection (RTI) symptoms

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in children[37], reduces possible experimental colonisation-related symptoms also warrants further research.

The pneumococcal densities detected in participants after inoculation of SPN3 isolates were comparable with those detected using an SPN6B model with similar inoculum doses[38]. Further, the microbiological and molecular results were more closely matched than a SPN6B model. The limited day 7 and 14 data at 160,000 CFU/100uL groups restrict interpretation of the highest inoculum dosages, however high carriage densities were noted at both 20,000 and 80,000 CFU/100uL. The latter dose provided consistently high colonisation densities, suggesting this is the optimum dose for future SPN3 challenge studies. Reliable colonisation with detectable bacterial density is important for the model, as observing a reduction in colonisation density will be a crucial indicator of VE in future studies.

We have previously showed that experimental SPN6B colonisation is an immunising event[39,40]. No increase in nasal IgG levels to capsular polyssacharide was observed post-SPN3 colonisation. This could be due to sample timing, being only 14 days after colonisation, sample type, or a difference in the immunising potential of SPN3, which has been already reported in the context of vaccination[41]. A high baseline IgG level was associated with fewer reported symptoms.

Small participant groups reduce the ability to compare doses directly and to apply narrow confidence intervals to the measured experimental colonisation rates. In addition, the study population is younger than one representative of the most at risk population from SPN3 disease would be, however this was necessary for this initial study for safety reasons. The early onset of participant symptoms at higher dose ranges meant antibiotics were initiated during experimental carriage, thus terminating colonisation and therefore impacting on

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duration and density of colonisation. The relatively short follow up duration post-inoculation limits the conclusions that can be drawn from the SPN3 anti-CPS IgG response to colonisation. Mucosal IgA was not measured in this study as IgA1 (predominant in nasal secretions) is known to be cleaved by pneumococcal proteases[42]. IgM was also not measured, as it is a short-lived, low affinity antibody with low titres in the nasal mucosa[43]. However, quantification of these antibodies post-inoculation could be informative about the wider host immune response. The immune response to SPN3 challenge following PCV-13 is not explored in this study and requires further investigation. As this is an exploratory study, no formal sample size calculation was performed, and the results are therefore exploratory and require confirmation in a larger definitive trial. The frequency of post-inoculation pharyngeal symptoms was not predicted at onset from our prior knowledge of SPN6B challenge, and will be fully described in follow-on studies.

Conclusions

We have successfully developed a safe SPN3 human infection challenge model, utilising clinically relevant isolates and obtaining colonisation rates comparable to established SPN6B challenge. Both colonisation rates and pneumococcal density achieved are influenced by the experimental inoculum dose. Mild symptoms occurred frequently but resolved with antibiotics in those with confirmed experimental colonisation. Further research is, however, required to understand why pneumococcal densities differ between challenge serotypes and to explore what factors and mechanisms are associated with progression from SPN3 colonisation to disease. This model provides an important platform for investigating vaccine efficacy against SPN3 acquisition and impact on colonisation density for this clinically important disease-causing serotype.

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Table 1: Study demographics

	Clade Ia	No clade	Clade II	All groups	ANOVA p-value
No of participants	40	20	36	96	-
Median age (IQR)-yr	22 (20-25)	20(19-22)	21 (19-28)	21 (19-25)	0.37
Mean age (SD)- yr	22.85(4.22)	21.55(4.52)	23.36(5.19)	22.77 (4.66)	0.37
Female-no.(%)	25(62.5%)	10 (50.0%)	22 (61.1%)	57 (59.4%)	0.63
Ethnicity *White ethnic group*	30 (75.0%)	15 (75.0%)	22 (61.1%)	67 (69.8%)	-
Black ethnic group	1 (2.5%)	2 (10.0%)	4 (11.1%)	7 (7.3%)	-
People of mixed ethnicity	3 (7.5%)	1 (5.0%)	0 (0.0%)	4 (4.2%)	-
All other ethnicities	6 (15.0%)	2 (10.0%)	10 (27.8%)	18 (18.8%)	-

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Figure legends

Figure 1: Isolate dose ranging format and timeline of the study A) Flow diagram demonstrating the dose escalation format. Specific safety criteria used for the ongoing safety assessment during the dose escalation process are included and stopping criteria are stated. All participants received one experimental inoculation and were then followed up for 14 days. Each dose ranging group (10,000 CFU, 20,000 CFU, 80,000 CFU, 160,000 CFU/100uL per naris) had 10 participants with the exception of the clade II isolate at 10,000 CFU/100uL per naris (n=6). Dose escalation did not take place until day 7 safety data was assessed. If the challenge isolate failed to achieve a nasopharyngeal carriage rate (identified using classical microbiology methods) of 40% in a group of 10 participants by day 7 then a dose increase was considered. All participants that were colonised with experimental SPN3 received antibiotics on Day 14 to terminate colonisation. B) Schematic demonstrating the participant journey and sampling schedule. 96 participants were inoculated and attended for day 2 samples, 95 participants attended for day 7 and day 14 samples.

Figure 2: CONSORT diagram. 39 participants declined to participate following consent. In this group 21 decided not to particpate following a pause for the COVID-19 pandemic, 9 declined for personal reasons, and 9 gave no specific reason. 1 participant who received the intervention (SPN3 inoculation) then withdrew after their day 2 appointment for personal reasons.

Figure 3: Rates of experimentally induced pneumococcal colonisation following dose ranging inoculation with SPN3 using microbiological culture and molecular methods. Bar chart showing the experimental colonisation rates of A) clade Ia, B) no-clade isolate and C) clade II in each dose ranging group (n=10) during the follow up period (day 2, day 7, day 14

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and any day [cumulative]) by microbiological culture and qPCR methods. The number of participants inoculated at each dose is stated below the dose group.* 3 participants in the clade Ia at 160,000 CFU/100uL per naris group, 1 participant in the no-clade isolate group at 20,000 CFU/100uL per naris and 4 participants in the clade II at 160,000 CFU/100uL per naris group were excluded from the analysis on days 7 and 14 due to colonisation being terminated by commencing antibiotics for safety reasons.

Figure 4: Mean SPN3 bacterial densities (SD) using microbiological culture and molecular methods (quantity of SPN3 DNA copies/ml) recovered in NW samples by dose in all SPN3 isolates from day 2 to day 14 and comparison with SPN6B challenge model data from a previous study[25]. Line graph of the arithmetic mean bacterial densities and bacterial DNA copies/ml of experimental SPN3 in retrieved nasal wash (with standard deviations) from participants for each challenge dose, obtained at days 2, 7 and 14. In the SPN3 isolates, quantity of bacterial DNA copies was calculated using the *PiaB* gene and a relative threshold. For SPN6B, quantity of bacterial DNA copies was calculated using the *LytA* gene and a relative threshold, data utilised from a previously published EHPC study[25]. This study utilised the same follow up period and dose range. Limited 6B data were available at 10,000 (n=1) and 20,000 (n=2) CFU/100uL per naris due low colonisation levels at these doses. The no-clade isolate was not utilised at inoculum doses of 80,000 or 160,000 CFU/100uL per naris due to the high colonisation rates achieved in the lower dose ranges. The clade II isolate is absent at 10,000 CFU/100uL as it did not achieve colonisation at this dose range. 7 participants in the SPN3 160,000 CFU/100uL per naris group started antibiotics for safety reasons, impacting on the recovered density at day 7 and 14.

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Figure 5: Heatmap of symptom onset by SPN3 isolate and table of presenting symptoms post-inoculation. Participant reported symptoms in each isolate and dose with onset and duration during the follow up period. Symptoms are graded from 1-3; 1) an awareness of symptom but tolerated; transient or mild discomfort; little or no medical intervention required 2) Discomfort enough to cause limitation of usual activity (some assistance may be needed); some medical intervention or therapy required 3) Significant interference with daily activity; some assistance usually required; medical intervention/therapy required; hospitalisation possible. Experimental SPN3 carriers are identified in purple and non-carriers in blue, with a dotted line separating the two for each dose. The dose groups (10,000CFU, 20,000CFU, 80,000CFU, 160,000 CFU/100uL per naris) for each isolate are demonstrated. Experimental SPN3 colonisation is defined as SPN3 being identified in the nasopharynx at any timepoint post-inoculation. Five participants were positive for rhinovirus on routine swab taken on the day of inoculation but did not have any symptoms. Two of these participants (SPN3 colonised) developed symptoms at day 5 and 7 post-inoculation respectively. One participant developed otitis media with tympanic perforation, classified as grade 3 symptoms and identified in red on the heatmap. 7 participants had grade 2 symptoms, identified in orange, due to the requirement for early antibiotics due to symptoms. All participants who were positive for SPN3 in study samples and required antibiotics for safety reasons were symptom free after treatment and all subsequent study samples post-antibiotics were negative for experimental SPN3.

Figure 6: Nasal cytokine expression post-SPN3 and SPN6B challenge. A) Heatmap showing the median Log2FC to baseline at each time point post-inoculation for the 2 experimental groups, based on stratification by serotype SPN3 (blue) and SPN6B (orange). Statistical comparisons were applied against baseline for the same strain for each timepoint. ***P*=<

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0.01, *P=<0.05. On day 2 (FC to baseline), RANTES and IL-2 levels were decreased, while EGF levels were increased for the SPN3 group when compared with SPN6B. By day 7, RANTES and IL-2 remain significantly reduced for SPN3, in addition to IL-4 (P=< 0.01, Wilcoxon's test, not significant with Benjamini- Hochberg adjustment) when compared with the same timepoint for the SPN6B group. B) Boxplots showing the Log2FC levels of the significant cytokines between SPN3 (blue) and SPN6B (orange) serotypes on days 2 and 7 post inoculation (non-significant with Benjamini- Hochberg correction applied). 3 different cytokines' log2FC expression to baseline for volunteers challenged with the serotype SPN3 (blue) or SPN6B (orange) are shown on day 2 and day 7. Mann–Whitney U test P-value is indicated. **P*=< 0.05, ***P*=<0.01.

Figure 7: Nasal SPN3 anti-capsular IgG response post SPN3 and SPN6B challenge. A) Grouped scatter plot showing the Log10 fold change in nasal anti-capsular IgG antibodies to SPN3 and SPN6B from baseline to day 14 in recovered NW with participants stratified by challenge serotype and colonisation status. SPN3 challenge participants are shown in red and SPN6B in grey, with the median and 95% CI shown for each. There was no statistically significant difference in IgG levels between the SPN3 and SPN6B groups when a Mann-Whitney U test is performed. B) Grouped scatter plot showing the pre-and post-colonisation Log10 anti-capsular IgG present in NW for SPN3 and SPN6B challenge. There was no significant difference in anti-capsular IgG response from baseline to day 14 for either serotype post-colonisation when a Mann-Whitney U test is performed.

Figure 8: Participants reporting symptoms post SPN3 inoculation, A) Frequency of reported symptoms B) Recovered bacterial density in NW C) Cytokine expression D) Nasal anti-capsular IgG. A) Histogram comparing the number of individuals reporting symptoms

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(red) and those without symptoms (blue) in the SPN3 carrier group and the non-carrier group. A contingency analysis (using Fisher exact testing) demonstrates a statistically significant difference between the two groups (*P*=0.002). B) Comparison of the density of bacterial colonisation (using microbiological methods) in NW samples of those SPN3 colonised with symptoms (red) and without symptoms (blue). Median and 95% CI are shown. There is a trend toward higher colonisation density in the symptomatic group at day 2, with the opposite observed at day 7 and 14. When a Mann-Whitney U test is performed, there is no significant difference in microbiological density between the two groups at any timepoint. C) A box plot of significant changes in nasal cytokine expression in those with symptoms (red) and those without (blue) at day 2 and day 7. Those with symptoms had different cytokine expression to those without, with significantly increased expression of IL-6 (day 2-*P*=0.016, day 7- *P*=0.042) and TNF-α (day 2- *P*=0.037) and decreased expression of RANTES (day 2- *P*=0.019) and Eotaxin (Day 7- *P*=0.0061) using Mann-Whitney U test. D) A grouped scatter plot of Log10 anti-capsular IgG to SPN3 in NW pre- and post-inoculation (day 14) for those reporting symptoms (red) and those without (blue). There is no significant difference in IgG generated between the two groups (Mann-Whitney U test). When fold change in anti-capsular IgG is compared, there is no significant difference in anti-capsular IgG generated for those with symptoms and those without. (Mann-Whitney U test).

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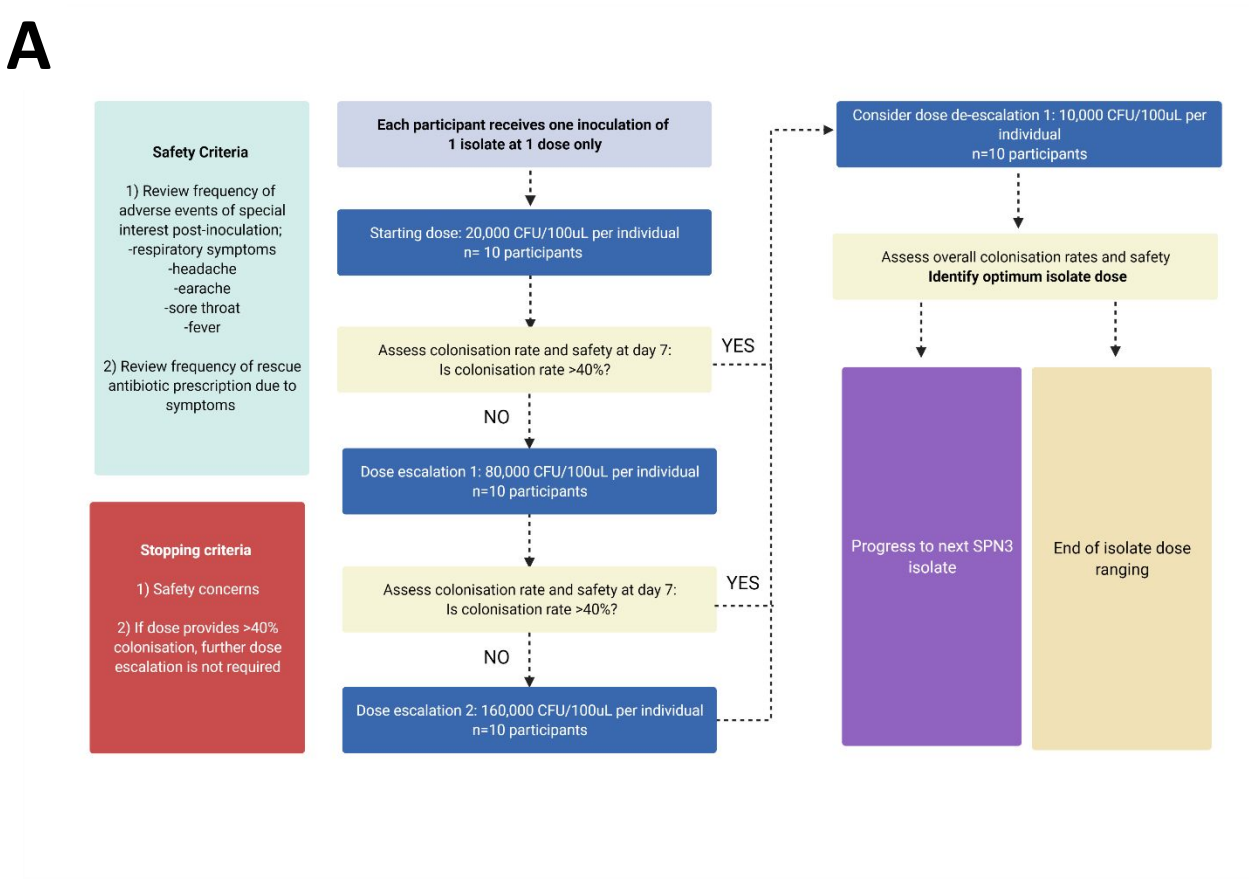
Figure 1



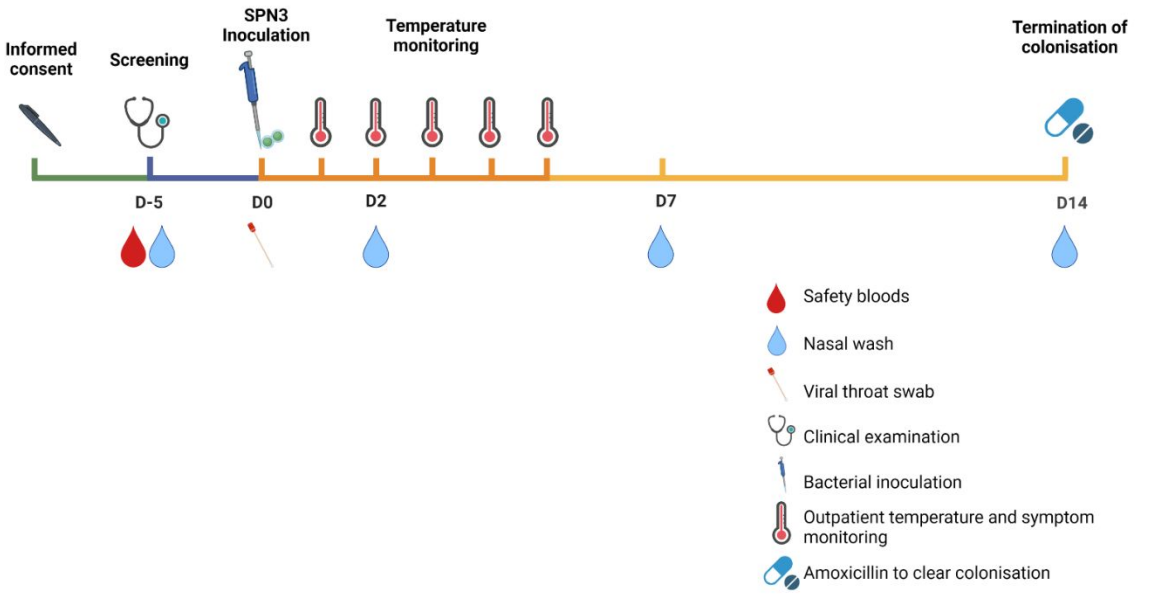


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Figure 2

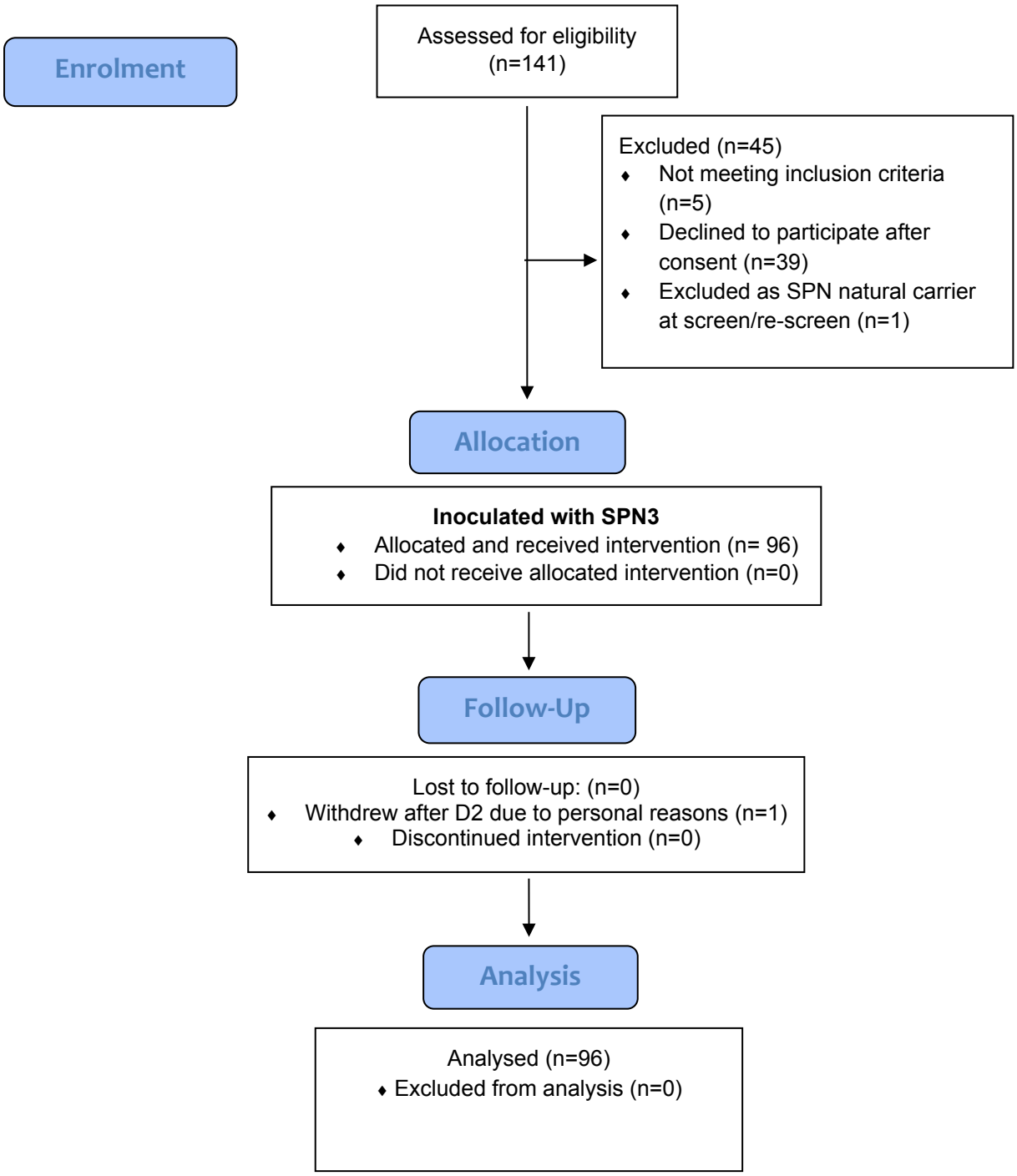

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Figure 3

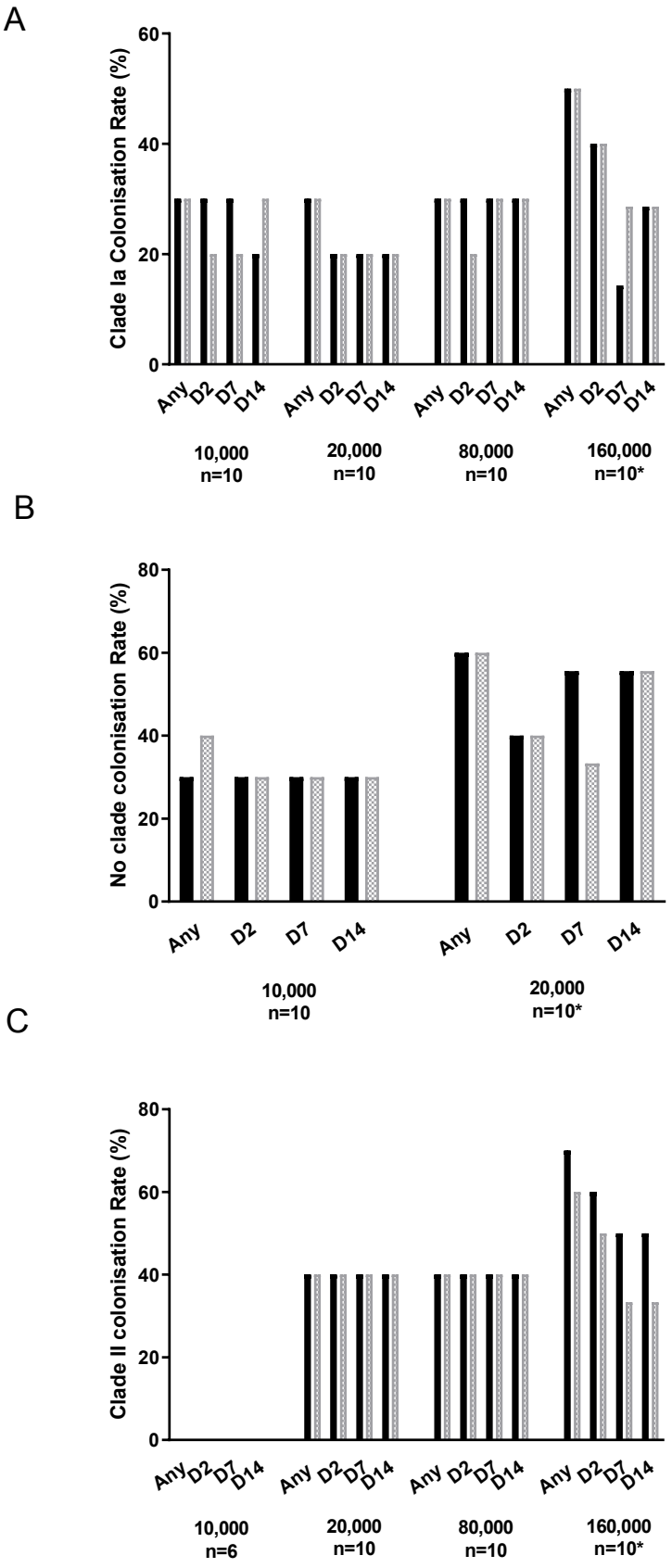

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Figure 4

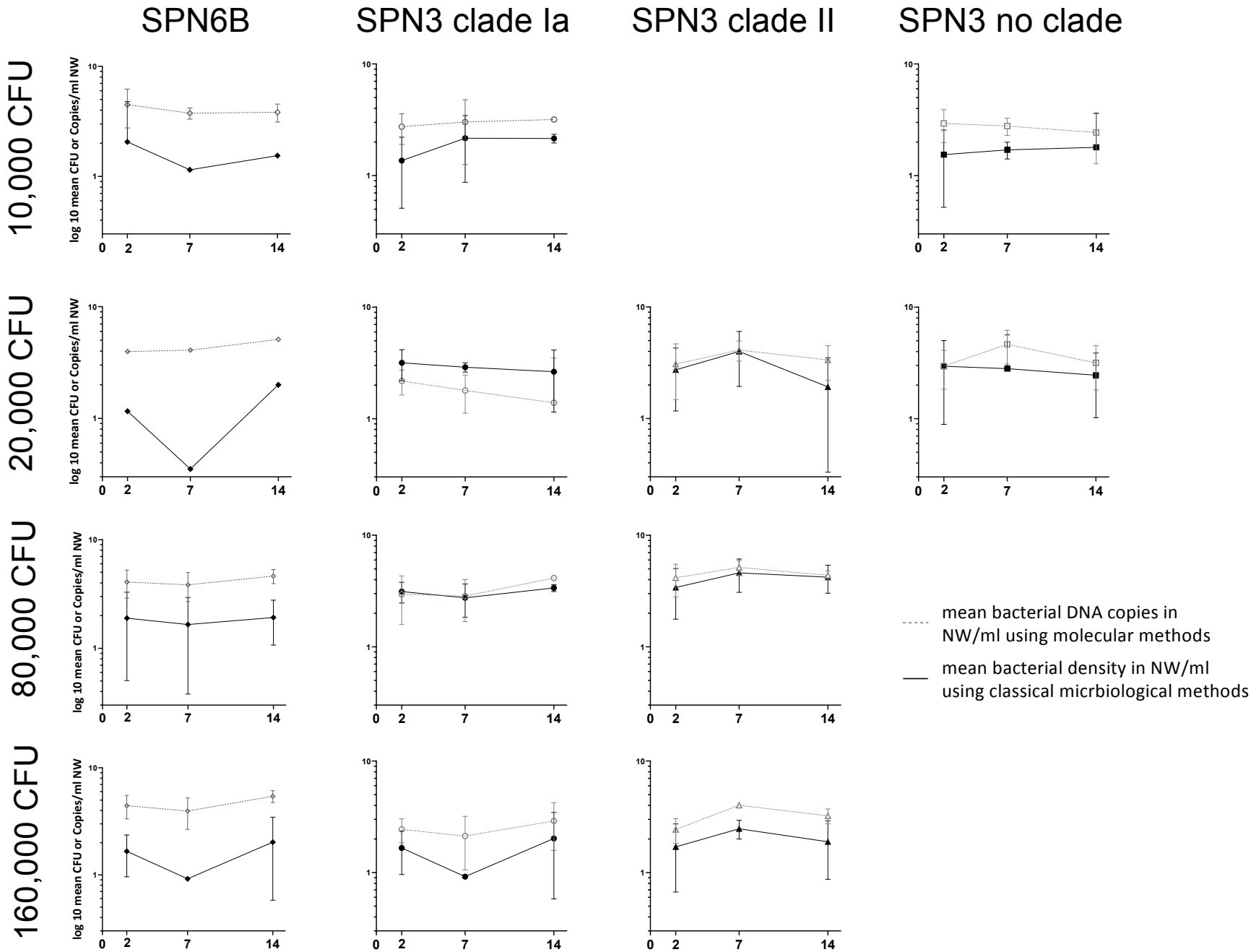

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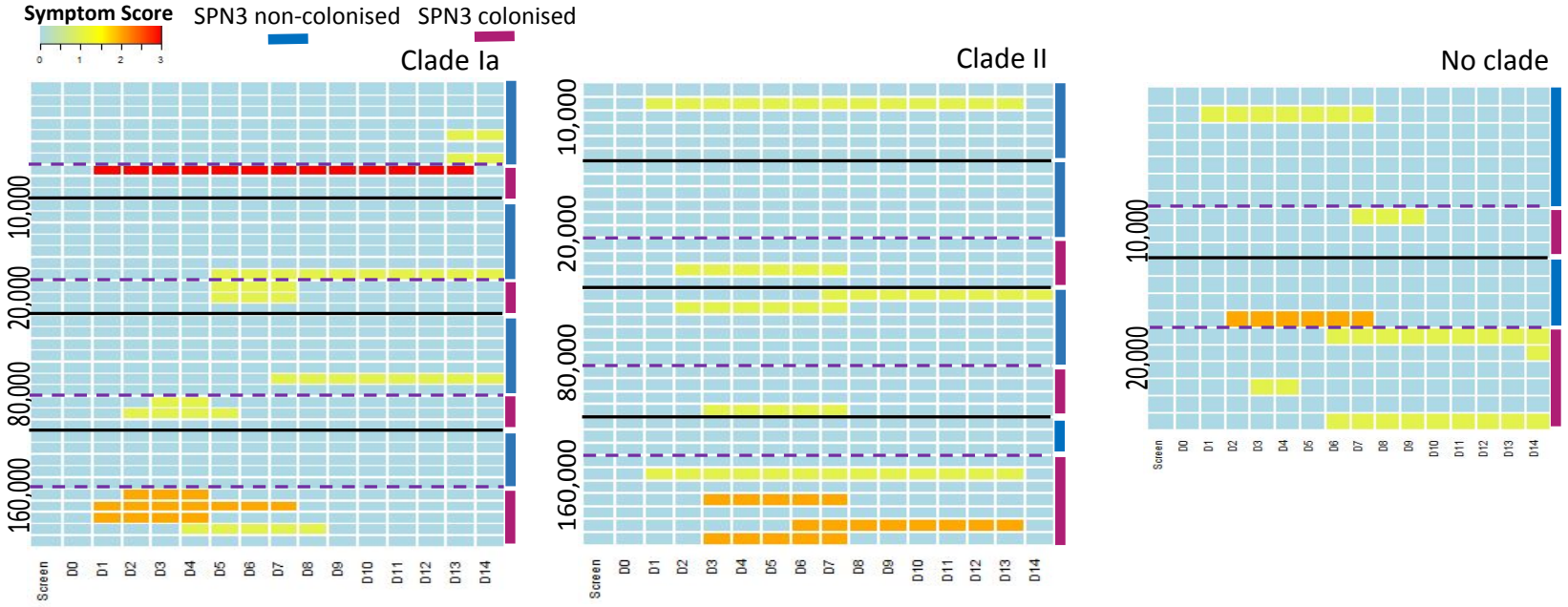
Figure 5

A



B

Reported symptoms	10,000	20,000	80,000	160,000	10,000	20,000	10,000	20,000	80,000	160,000
	Clade Ia				**No clade**		**Clade II**			
No. of participants	10	10	10	10	10	10	6	10	10	10
Respiratory symptoms	0	0	0	0	0	2	0	0	1	0
Headache	0	0	1	0	0	0	0	0	0	0
Sore throat	2	3	2	4	2	3	1	1	2	4
Earache	1	0	0	0	0	0	0	0	0	0
Required antibiotics	1	0	0	3	0	1	0	0	0	3

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Figure 6

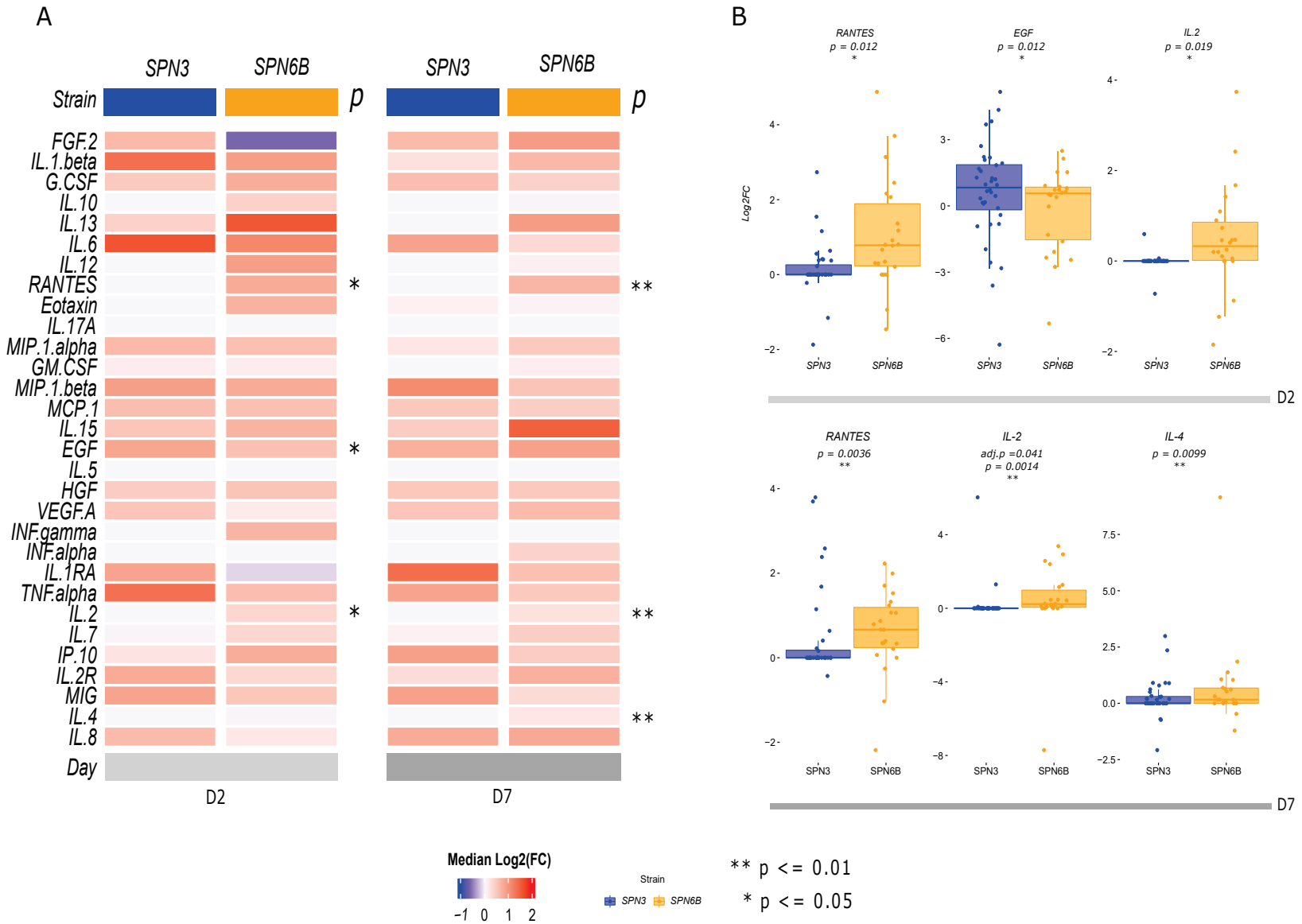

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Figure 7

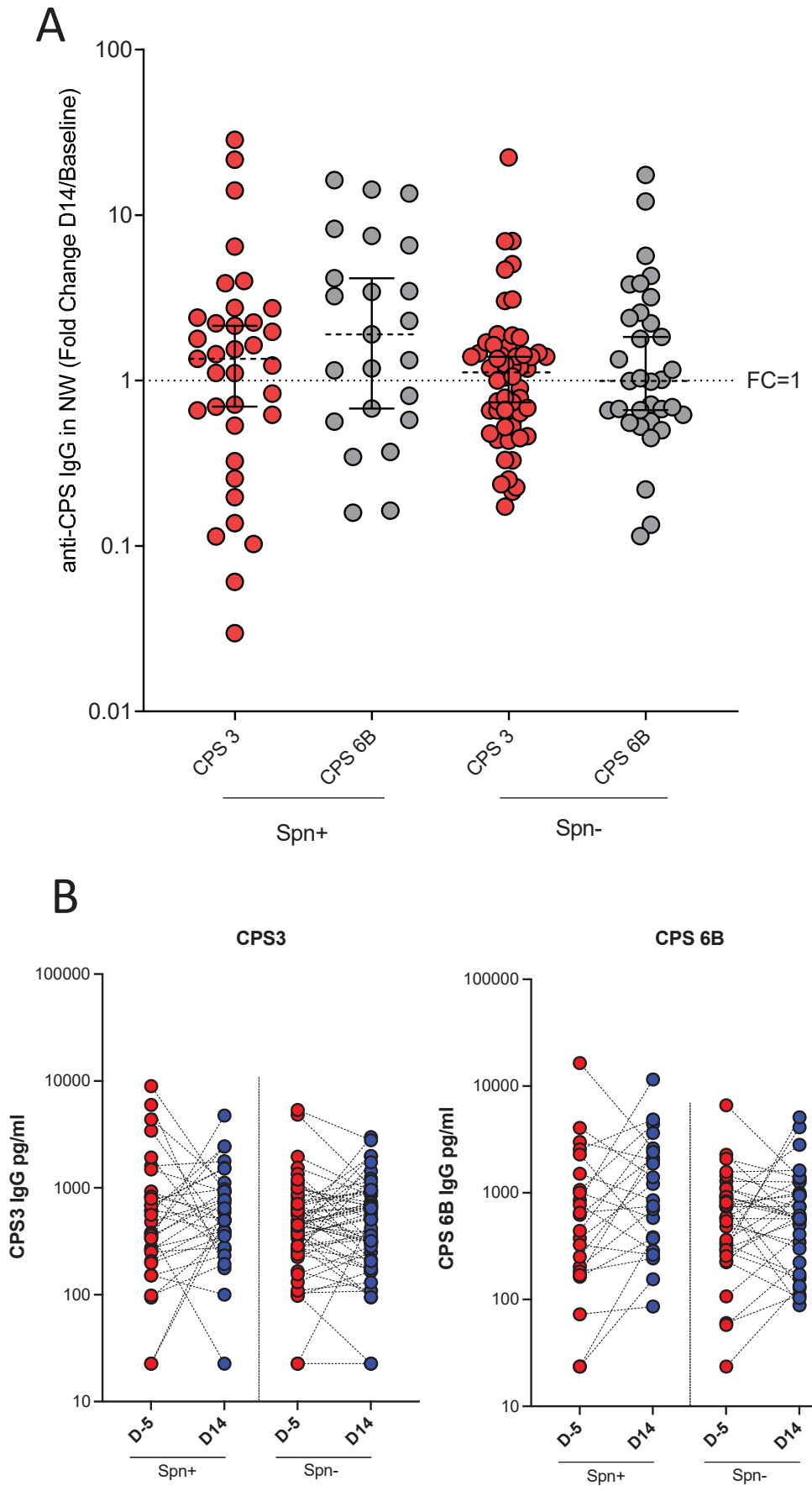

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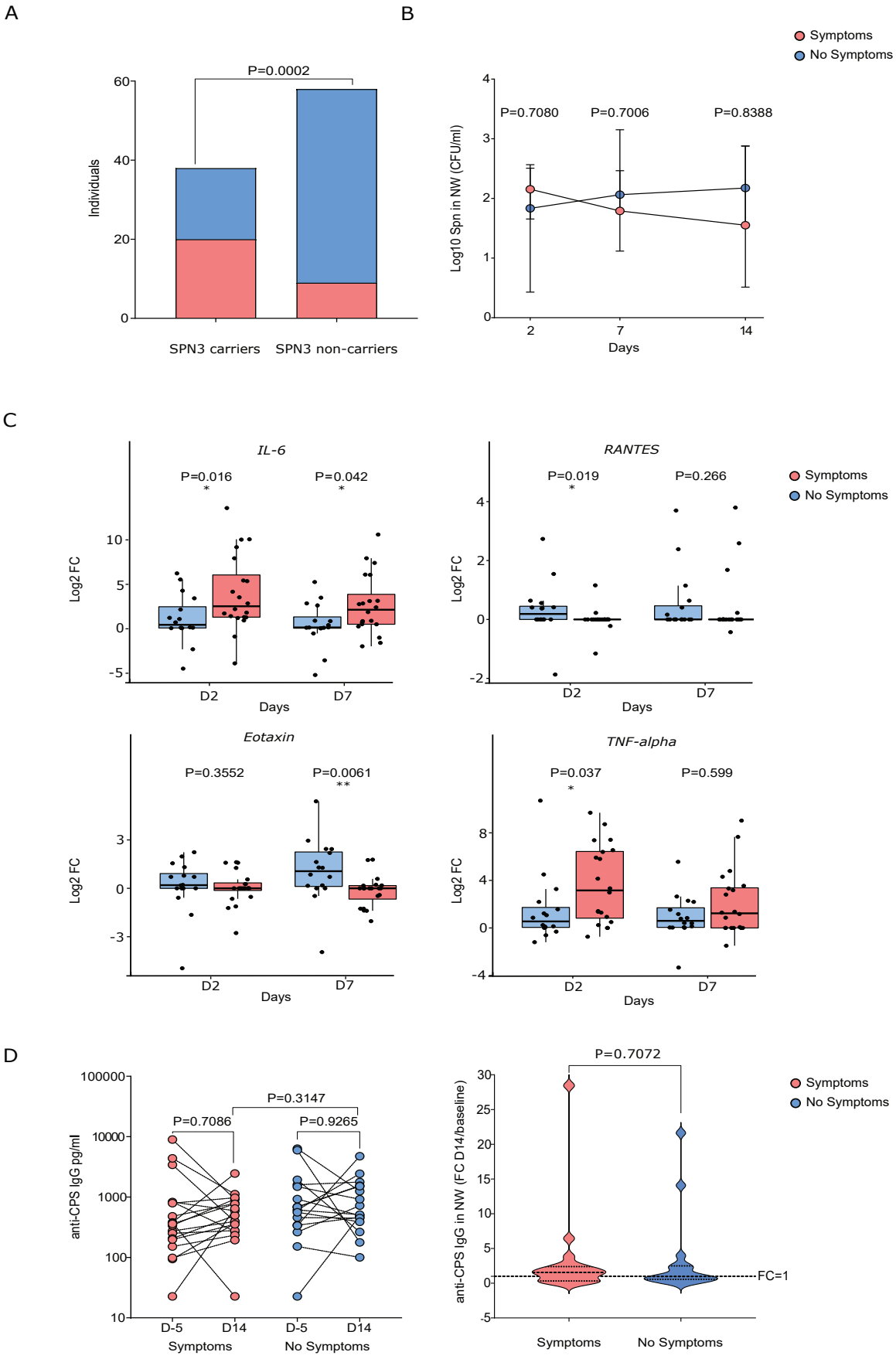
Figure 8



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Human Infection Challenge with Serotype 3 Pneumococcus

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Online Data supplement

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Supplementary Methods

Table E1: Participant safety education and post-inoculation safety procedures

Safety procedures	Detail
Pre-inoculation participant safety education	Advised about important symptoms to observe for, when to contact the study team for help, use of rescue antibiotics and when to utilise the emergency safety phone line
Access to emergency review and health advice	24-hour physician on call for health advice throughout the study with access to inpatient unit if required.
Daily temperature and symptom monitoring	Participant symptoms and temperature monitoring daily for 3-5 days via phone.
Standby antibiotic usage	Rescue antibiotics given pre-emptively to the participant to ensure there is no delay to treatment commencement in the event of worsening symptoms.

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Table E2: Inclusion and exclusion criteria

Inclusion criteria	
Healthy young adults aged 18-50 years (inclusive)	
Fluent spoken English	
Access to their own mobile telephone	
Capacity to give informed consent	
Exclusion criteria	
Research	Currently involved in another study (unless observational or non-interventional except for the EHPC bronchoscopy study)
	Participant in a previous EHPC trial within the last 3 years
Pneumococcal colonisation	Current pneumococcal colonisation at screen
Vaccine	Previous pneumococcal vaccination PPV23, PCV13 or PCV10
Allergies	Allergic to penicillin
Health history	Chronic ill health or higher risk of infection including, immunosuppressive history, diabetes, asthma (on regular medication), recurrent otitis media or other respiratory disease
	Medication that may affect the immune system or clotting e.g. steroids, inflammation altering (eg. nasal steroids, roacutane or aspirin) or disease-modifying anti-rheumatoid drugs
	Recent antibiotics within the last 28 days or long term for known active chronic infection
	Current illness or acute illness within 14 days prior to inoculation
	Major pneumococcal illness during lifetime requiring hospitalisation
	Other conditions considered by the clinical team as a concern for participant safety or integrity of the study
Direct caring role or close contact	With individuals at higher risk of infection:
	Children under 5 years of age
	Chronic ill health or immunosuppressed adults
Smokers	Current or ex-smoker (regular cigarettes, e-cigarettes / vaping, and regular smoking of recreational drugs) in the last 6 months
	Previous significant smoking history – more than 20 cigarettes per day for 20 years or the equivalent (>20 pack years)

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Women of child bearing age	Insufficient or ineffective birth control
	Currently pregnant
History of current drug or alcohol abuse	Frequently drinking alcohol: Men and women should not regularly drink > 3 units/day and 2 units/day respectively - assessed at the discretion of the clinician
Travel	Overseas travel planned in the follow up period
COVID-19	Ongoing COVID-19 symptoms (defined as fever, cough, shortness of breath, anosmia, ageusia) or confirmed current COVID-19 infection
	Any participants with co-morbidities that are known to increase the risk of severe COVID-19 (Diabetes, obesity (BMI>30), cardiovascular disease, pulmonary disease, malignant neoplasm, rheumatological conditions, and conditions/therapies associated with immunosuppression)
	Any household members that are considered high risk for developing severe COVID-19 (elderly or any of the following co-morbidities; Diabetes, obesity (BMI>30), cardiovascular disease, pulmonary disease, malignant neoplasm, rheumatological conditions, and conditions/therapies associated with immunosuppression).

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Table E3: Adverse Events of Special interest

Symptom	**Additional symptom features**
Respiratory symptoms	Productive cough
	wheeze
	Chest pain
	Difficulty breathing
Headache	With pyrexia >38^{0}C
	With changes in vision/photophobia
	With nasal congestion/sinus pain
	With neck stiffness
Earache	With pyrexia >38^{0}C
	With discharge
Sore throat	With pyrexia >38^{0}C
Fever (>38^{0}C)	-

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Table E4: Respiratory viral pathogens detected from oropharyngeal swabs using molecular methods

Viral Pathogen detected
Influenza A
Influenza B
Respiratory syncytial virus
Adenovirus
Human metapneumovirus
Rhinovirus
Human parainfluenzae virus-1
Human parainfluenzae virus-2
Human parainfluenzae virus-3
Human parainfluenzae virus-4

Detection of experimentally induced pneumococcal colonisation using microbiology culture

After undergoing centrifugation, the NW pellet is cultured on Columbia blood agar supplemented with 5% horse blood (OXOID, Basingstoke, UK) and 80µL gentamycin 1mg/mL (Sigma-Aldrich Co, Gillingham, UK) then incubated overnight at 37°C and 5% CO_2. *S. pneumoniae* colonies were confirmed using optochin and bile tests. Serotyping was performed by latex agglutination (SSI Diagnostica, Oxfordshire, UK).

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Detection of nasal wash anti-pneumococcal capsular polysaccharide antibodies

The WHO standardized enzyme-linked immunosorbent assay (ELISA) was utilised[1]. ELISA plates were coated with 5µg/ml of purified capsular polysaccharides (CPS) of either SPN3 or SPN6B (Statens Serum Institute) at 4 °C overnight. Samples were diluted 1:4 in absorption buffer containing 10µg/ml CWPS multi (Statens Serum Institute) and incubated for 30 min at room temperature. Following adsorption samples were transferred to pre-coated plates and incubated for 2hrs at room temperature. Antigen specific antibodies were detected using anti-human biotinylated IgG (1:20,000), HRP-Streptavidin (1:5000) and TMB substrate-based platform. The reaction was stopped using 2N H_2SO_4 and optical density measured at 405 nm using FLUOstar Omega plate reader (BMG Labtech).

Identification and grading of participant symptoms post-inoculation

If inoculated participants reported symptoms, they were recorded and graded by a clinician following clinical assessment using the scale shown in online data supplement Table E5. If the participant reported symptoms between study visits an ad hoc assessment was performed. Clinical samples were obtained if deemed clinically appropriate at review. Any participant reporting symptoms were followed up until resolution. Antibiotics were commenced if symptoms were escalating in severity or significantly troublesome to the participant, as judged by an experienced clinician.

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Table E5: Symptom grading score

Grade	Grading	Definition
1	Mild	Awareness of symptom but tolerated; transient or mild discomfort; little or no medical intervention required
2	Moderate	Discomfort enough to cause limitation of usual activity (some assistance may be needed); some medical intervention or therapy required
3	Severe	Significant interference with daily activity; some assistance usually required; medical intervention/therapy required; hospitalisation possible
4	Potentially Life threatening	Hospitalisation

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Supplementary Results

Table E6: Colonisation identified in NW samples by isolate across all timepoints (microbiological culture)

SPN3 Isolate	Dose (CFU/100uL)	Day 2 colonisation (no.)	%Day 7 colonisation (no.)	%Day 14 colonisation (no.)	%Any day % colonisation rate (no.)
Clade Ia	**10,000**	30.0% (3/10)	30.0% (3/10)	20.0% (2/10)	30.0% (3/10)
	20,000	20.0% (2/10)	20% (2/10)	20% (2/10)	30.0% (3/10)
	80,000	30.0% (3/10)	30.0% (3/10)	30.0% (3/10)	30% (3/10)
	160,000	40.0% (4/10)	14.3% (1/7)	28.6% (2/7)	50.0 % (5/10)
	All doses	30.0 % (12/40)	24.3% (9/37)	24.3% (9/37)	35.0% (14/40)
No clade	**10,000**	30.0% (3/10)	30.0% (3/10)	30.0% (3/10)	30.0% (3/10)
	20,000	40.0% (4/10)	55.6% (5/9)	55.6% (5/9)	60.0% (6/10)
	All doses	35.0% (7/20)	42.1% (8/19)	42.1% (8/19)	45.0% (9/20)
Clade II	**10,000**	0.0% (0/6)	0.0% (0/6)	0.0% (0/6)	0.0% (0/6)
	20,000	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)
	80,000	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)
	160,000	60.0% (6/10)	50.0% (3/6)	50.0% (3/6)	70.0% (7/10)
	All doses	38.9% (14/36)	34.4% (11/32)	34.4% (11/32)	41.6% (15/36)

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Table E7: Colonisation identified in NW samples by isolate across all timepoints (molecular methods)

SPN3 Isolate	Dose (CFU/100uL)	Day 2 colonisation (no.)	%Day 7 colonisation (no.)	%Day 14 colonisation (no.)	%Any day colonisation rate (no.)	%
Clade Ia	**10,000**	20.0% (2/10)	20.0% (2/10)	30.0% (3/10)	30.0% (3/10)	
	20,000	20.0% (2/10)	20.0% (2/10)	20.0% (2/10)	30.0% (3/10)	
	80,000	30.0% (3/10)	20.0% (2/10)	30.0% (3/10)	30.0% (3/10)	
	160,000	40.0% (4/10)	28.6% (2/7)	28.6% (2/7)	50.0% (5/10)	
	All doses	27.5% (11/40)	21.6% (8/37)	27.0% (10/37)	35.0% (14/40)	
No clade	**10,000**	30.0% (3/10)	30.0% (3/10)	30.0% (3/10)	40.0% (4/10)	
	20,000	40.0% (4/10)	33.3% (3/9)	55.6% (5/9)	60.0% (6/10)	
	All doses	35.0% (7/20)	31.5 % (6/19)	42.1% (8/19)	50.0% (10/20)	
Clade II	**10,000**	0.0% (0/6)	0.0% (0/6)	0.0% (0/6)	0.0% (0/6)	
	20,000	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	
	80,000	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	40.0% (4/10)	
	160,000	50.0% (5/10)	33.3% (2/6)	33.3% (2/6)	60.0% (6/10)	
	All doses	36.1% (13/36)	31.2% (10/32)	31.2% (10/32)	38.9% (14/36)	

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Table E8: Returned experimental SPN3 density using microbiological culture in NW samples by isolate at all timepoints

SPN3 Isolate	Dose (CFU/100uL)	D2 median (IQR) density of carriers (median Log10 CFU/mL NW±IQR)	D7 median (IQR) density of carriers (median Log10 CFU/mL NW±IQR)	D14 median (IQR) density of carriers (median Log10 CFU/mL NW±IQR)
Clade Ia	**10,000**	1.76 (1.08 - 1.85)	1.76 (1.44 - 2.68)	2.15 (2.09 - 2.22)
	20,000	2.18 (1.99- 2.37)	1.79 (1.55 - 2.03)	1.39 (0.64 - 2.13)
	80,000	3.33 (2.86 - 3.50)	2.33 (2.23 - 3.06)	3.37 (3.25 - 3.49)
	160,000	1.89 (1.41 -2.14)	0.92	2.02 (1.51 - 2.52)
No clade	**10,000**	1.76 (1.09 - 2.09)	1.63 (1.54- 1.82)	2.80 (1.24- 2.84)
	20,000	2.47 (1.37 -4.05)	2.27 (0.68 -4.14)	1.87 (1.70- 2.47)
Clade II	**10,000**	0.0	0.0	0.0
	20,000	2.86 (2.06 -3.54)	3.63 (2.52 -5.10)	1.79 (1.09 -2.84)
	80,000	3.96 (3.00 - 4.35)	4.25 (3.96 -4.89)	4.66 (4.11 -4.76)
	160,000	2.07 (1.91 - 2.22)	2.46 (2.23 - 2.70)	2.02 (1.41 - 2.43)

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Table E9: Returned experimental SPN3 DNA copies using molecular methods in NW samples by isolate at all timepoints

SPN3 Isolate	Dose (CFU/100uL)	D2 median (IQR) density of carriers (median Log10 DNA copies±IQR)	D7 median (IQR) density of carriers (median Log10 DNA copies±IQR)	D14 median (IQR) density of carriers (median Log10 DNA copies±IQR)
Clade Ia	**10,000**	2.8 (2.5-3.1)	3.3 (2.6-3.9)	3.2 (3.1-3.2)
	20,000	3.2 (2.9-3.6)	2.9 (2.8-3.0)	2.8 (2.3-3.3)
	80,000	3.7 (2.7-4.0)	3.0 (2.6-3.4)	4.4 (4.1-4.1)
	160,000	2.8 (2.3-2.9)	2.1 (1.7-2.8)	3.1 (2.6-3.5)
No clade	**10,000**	3.2 (2.5-3.4)	3.0 (2.5-3.0)	3.6 (2.1-3.3)
	20,000	3.1 (2.2-4.0)	5.3 (4.2-5.7)	2.8 (2.5-3.6)
Clade II	**10,000**	-	-	-
	20,000	3.7 (2.4-4.7)	3.9 (3.5-4.6)	3.3 (2.8-4.0)
	80,000	4.9 (4.1-5.1)	5.5 (4.9-5.7)	4.5 (4.3-4.6)
	160,000	2.6 (1.9-2.7)	4.0 (4.0-4.0)	3.3 (3.1-3.4)

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Table E10: Linear regression models for 'IgG level at day 14' by serotype and carrier subgroups

Model	Variable	Coefficient (95% CI)	p-value
SPN3 IgG	Intercept	1.72 (1.18, 2.27)	2.057
	Dose 20,000	0.08 (-0.15, 0.31)	0.511
	Dose 80,000	0.09 (-0.18, 0.35)	0.528
	Dose 160,000	0.1 (-0.17, 0.37)	0.478
	Baseline IgG	0.34 (0.15, 0.54)	**0.001**
	No clade isolate	-0.02 (-0.27, 0.22)	0.847
	Clade II isolate	-0.12 (-0.31, 0.08)	0.250
	SPN3 carrier	0.1 (-0.1, 0.29)	0.329
	Symptoms	-0.06 (-0.27, 0.16)	0.601
SPN3 non-carrier IgG	Intercept	0.09 (-4.87, 5.4)	0.973
	Dose 20,000	0.42 (-1.05, 1.95)	0.576
	Dose 80,000	0.46 (-1.38, 2.29)	0.615
	dose160,000	1.03 (-0.67, 2.81)	0.238
	Day 14 IgG	-0.87 (-2.13, 0.32)	0.158
	No clade isolate	-0.01 (-1.54, 1.5)	0.991
	Clade II isolate	-0.99 (-2.31, 0.23)	0.123
	Age	0.01 (-0.12, 0.14)	0.828
	Male gender	-0.1 (-1.27, 1.06)	0.865
SPN3 carrier IgG	Intercept	2.75 (1.75, 3.75)	9.776
	Dose 20,000	0.01 (-0.46, 0.47)	0.981
	Dose 80,000	-0.09 (-0.69, 0.5)	0.763
	Dose 160,000	-0.03 (-0.59, 0.53)	0.918
	Baseline IgG	0.09 (-0.22, 0.4)	0.567
	No clade isolate	-0.2 (-0.63, 0.22)	0.358
	Clade II isolate	-0.22 (-0.6, 0.16)	0.269
	Symptoms	-0.2 (-0.56, 0.17)	0.298
SPN6B IgG	Intercept	3.36 (2.64, 4.08)	3.923
	Dose 20,000	-0.26 (-0.79, 0.27)	0.342
	Dose 80,000	-0.3 (-0.7, 0.11)	0.156
	Dose 160,000	0.12 (-0.36, 0.6)	0.622
	Baseline IgG	-0.14 (-0.39, 0.11)	0.291
	SPN6B carrier	0.12 (-0.15, 0.38)	0.382

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Table E11: Logistic regression models with 'occurrence of symptoms' as the outcome, adjusted for baseline IgG, day 14 IgG

Model	Variable	Combined OR (95% CI)	p-value
SPN3 IgG at baseline	Intercept	3.65 (0.04, 566.35)	0.587
	Dose 20,000	2.29 (0.51, 11.19)	0.285
	Dose 80,000	1.39 (0.21, 8.86)	0.724
	Dose 160,000	3.17 (0.52, 21.05)	0.215
	Baseline IgG	0.24 (0.06, 0.76)	**0.022**
	No clade isolate	1.13 (0.24, 5.18)	0.871
	Clade II isolate	0.36 (0.09, 1.3)	0.132
	Age	1.02 (0.89, 1.15)	0.764
	Male gender	0.98 (0.3, 3.17)	0.976
	SPN3 carrier	5.72 (1.86, 19.43)	**0.003**
SPN3 IgG at day 14	Intercept	1.09 (0.01, 220.48)	0.973
	Dose 20,000	1.52 (0.35, 7.04)	0.576
	Dose 80,000	1.59 (0.25, 9.9)	0.615
	Dose 160,000	2.81 (0.51, 16.62)	0.238
	Day 14 IgG	0.42 (0.12, 1.38)	0.158
	No clade isolate	0.99 (0.22, 4.47)	0.991
	Clade II isolate	0.37 (0.1, 1.25)	0.123
	Age	1.01 (0.89, 1.15)	0.828
	Male gender	0.9 (0.28, 2.88)	0.865
	SPN3 carrier	5.66 (1.91, 18.48)	**0.003**

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Online data supplement Figure legends

Online data supplement Figure E1: Changes instigated for participant and staff safety post- COVID-19 pandemic. The study was paused in March 2020 due to increasing COVID-19 case numbers in our locality. After study restart in January 2021, the measures shown were instigated to ensure participants and staff were protected.

Online data supplement Figures E2A,B and C: Density kinetics from day 2-14 for individuals colonised by A) clade Ia B) no-clade and C) clade II. A line graph illustrating the density kinetics of all individuals colonised following inoculation. Red points indicate samples taken after the onset of antibiotics for safety reasons.

Online Data supplement Figure E3: Nasal cytokine expression post –inoculation with SPN3 and SPN6B compared to baseline. A) t-distributed stochastic neighbor embedding (T-SNE) plot. Samples were clustered based on the cytokine levels within the same time point (baseline, Day 2 and Day 7) using stochastic neighbor embedding for SPN3 (blue) or SNP6B (orange). R and *P*-values were calculated for all time points based on analysis of similarity (anosim), but no significance was found. B) Heatmap demonstrating the re-scaled median cytokine levels at each time point for the 2 experimental groups, based on stratification by serotype SPN3 (blue) and SPN6B (orange). Statistical comparisons were applied against the baseline sample for each time point in every group independently. ***P*=< 0.01, **P*=< 0.05. No significant cytokine was found after Wilcoxon's paired test with Benjamini-Hochberg correction for multiple testing. Scale of the data to values between 0 and 1 was done using the function scale from the 'scales R' package.

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Online data supplement Figure E4: Cytokine expression for challenge isolate post-inoculation. A) Heatmap showing the Median Log2FC to baseline at time point day 2 (light gray) and day 7 (dark gray) for the 4 experimental groups, based on stratification by strains of SPN3 (blue) and serotype SPN6B (orange). Statistical comparisons were applied against serotypes at the same time point. ***P*=< 0.01, **P*=< 0.05. No significant cytokine was found after Wilcoxon's paired test with Benjamini-Hochberg correction for multiple testing. B) Heatmap showing the z-score value of the expression of cytokines at baseline for the 2 experimental groups, based on stratification by serotype strain. Upregulation (red) and downregulation (blue) is shown in cytokines' levels from the standard deviation from the mean.

Online data supplement Figure E5: Cytokine expression by challenge dose. Dot plot showing the difference in Median Log_2FC to baseline for the higher SPN3 doses groups; 160,000 and 80,000 CFU/100uL per naris combined (red), 160,000 (green) and 80,000 CFU/100uL per naris (blue) when compared to low doses groups (10,000 and 20,000 CFU/100uL per naris) at day 2 post inoculation (light gray) and day 7 (dark gray). The color represents whether the highest dose is upregulated (red), or downregulated (blue) when compared with the lowest doses. The size of the circles represents the $-\log_{10}$ (Adjusted *P*-value), bigger circles represent lower adjusted *P*-values. The black border around the circles highlights the significant cytokines (Adjusted *P*-value <= 0.05). Circle size > 1.3 represents $-\log_{10}$(Adjusted *P*-value <= 0.05) and circle size >= 3 represents $-\log_{10}$(Adjusted *P*-value <= 0.001). For SPN3, when lower inoculum doses (10,000 and 20,000 CFU/100uL per naris) were compared with the highest dose 13 significant changes in cytokine expression (after multiple comparison correction) were observed on day 2, and 17 cytokines by day 7.

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Online Data Supplement Figure E6: Fold change in nasal SPN3 anti-capsular IgG antibodies generated in NW following inoculation by SPN3 isolate and dose. A) Fold change from baseline to day 14 in nasal anti-capsular IgG antibodies generated by dose, stratified into SPN3 colonised and non-colonised, with the median and 95% CI shown. When Mann-Whitney U test is performed only one dose demonstrated a statistically significant increase in IgG between the colonised and non-colonised, 160,000 CFU/100uL per naris (*P*=0.0259). B) Fold change from baseline to day 14 in nasal anti-capsular IgG antibodies generated by challenge isolate, stratified into SPN3 colonised and non-colonised with the median and 95% CI shown. There was no significant difference in fold change in nasal IgG levels for all three challenge isolates (Mann-Whitney U test). C) Comparison of fold change from baseline to day 14 in nasal anti-capsular IgG antibodies between dose groups. The median and 95% CI is shown. While there is a trend towards high IgG antibodies with increased dose, there was no statistically significant difference between dose groups (Mann-Whitney U test), with the exception of a comparison between 20,000 and 160,000 CFU/100uL per naris (*P*=0.0474).

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References

1. Goldblatt D, Plikaytis BD, Akkoyunlu M, et al. Establishment of a New Human Pneumococcal Standard Reference Serum, 007sp. *Clinical and Vaccine Immunology.* 2011;18(10):1728-1736.

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Figure E1

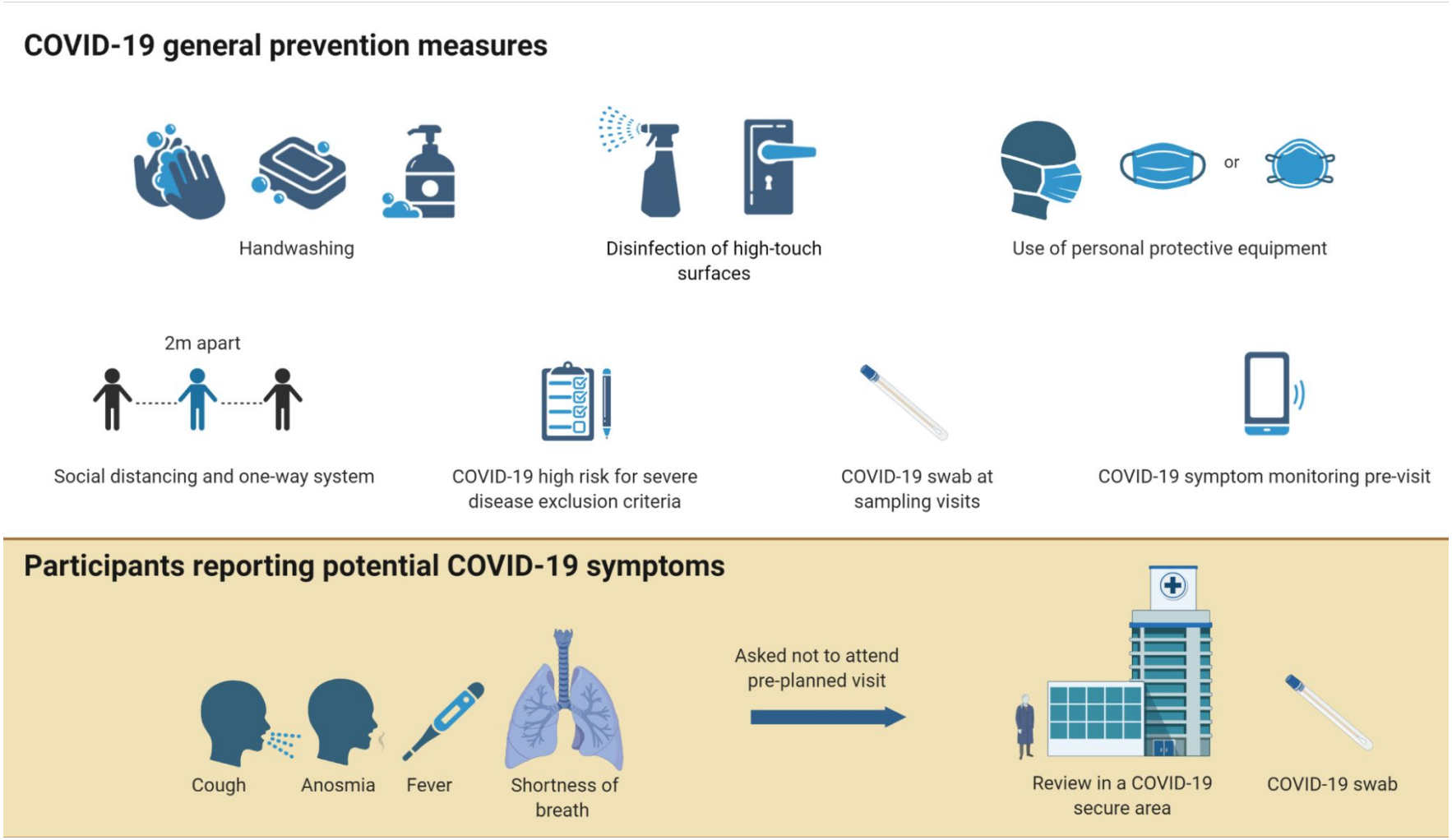

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Figure E2

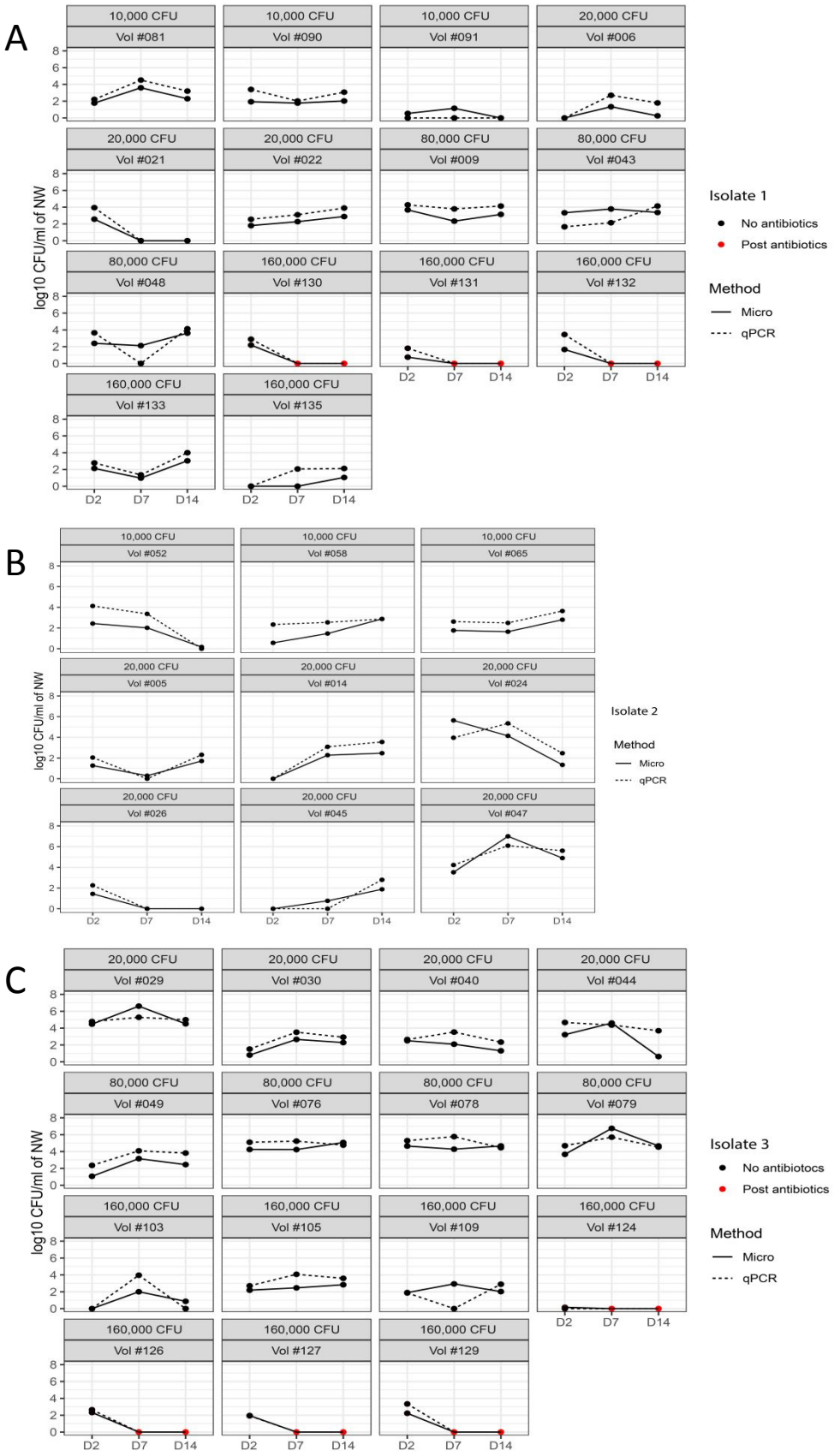

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Figure E3

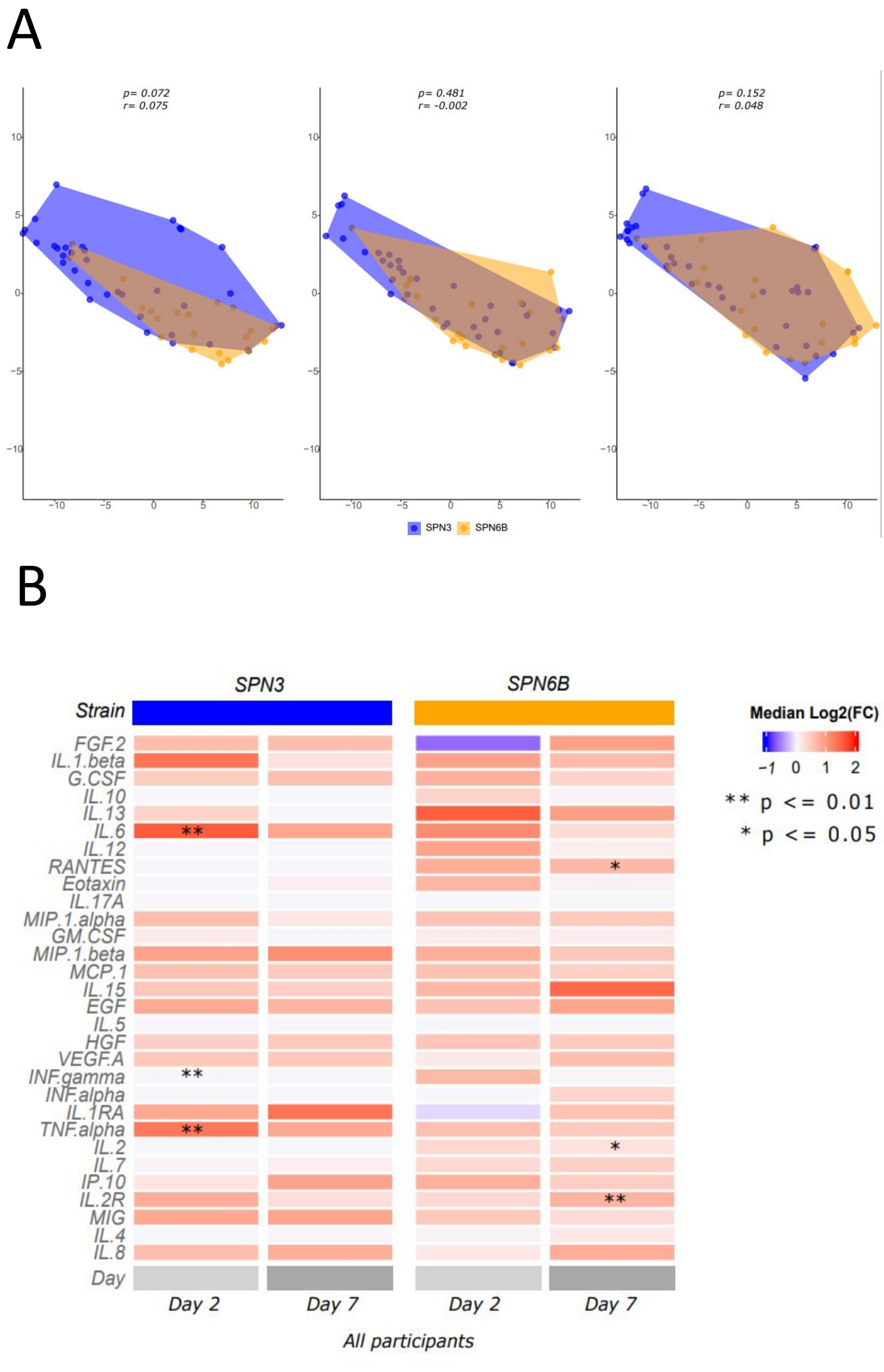

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Figure E4

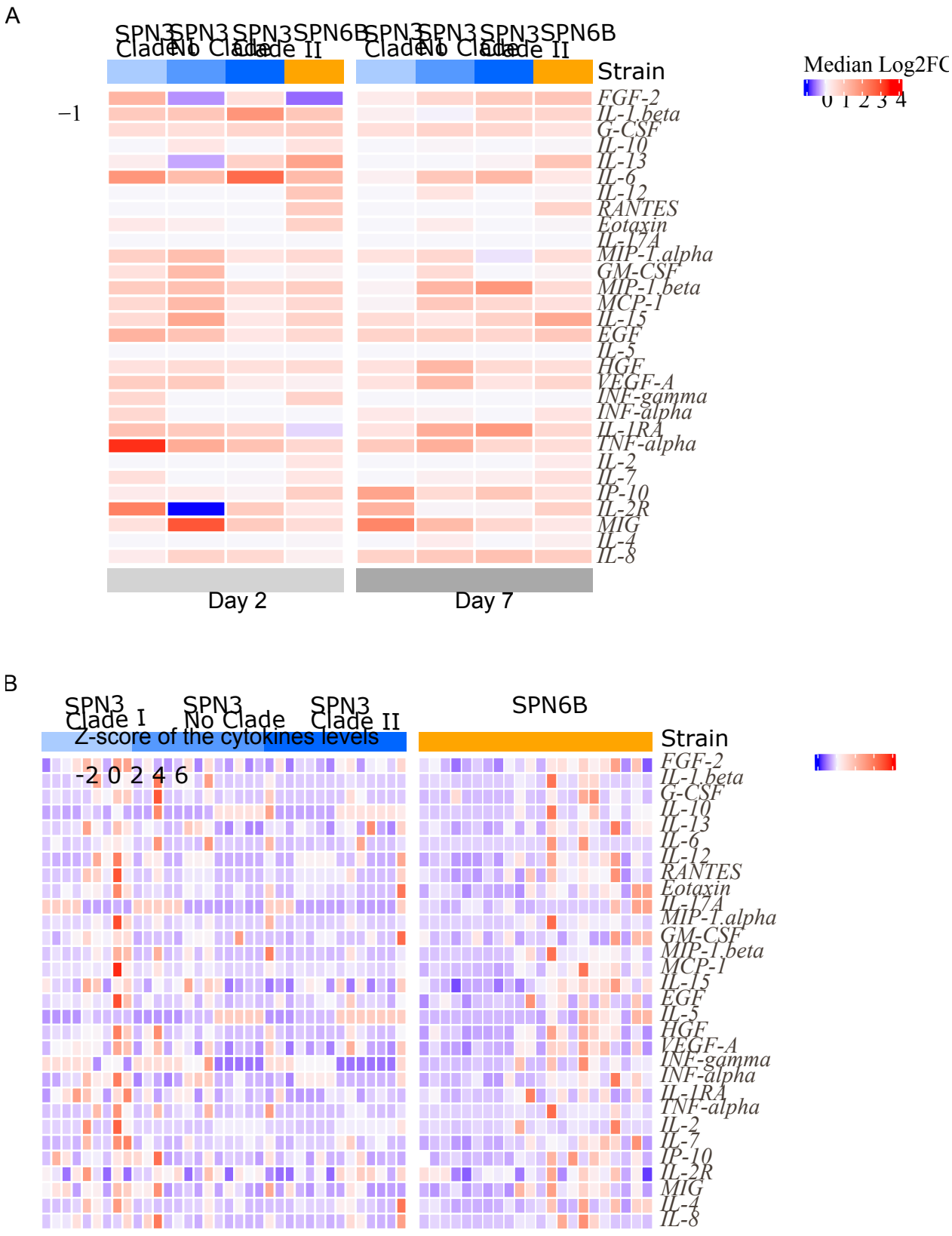

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Figure E5

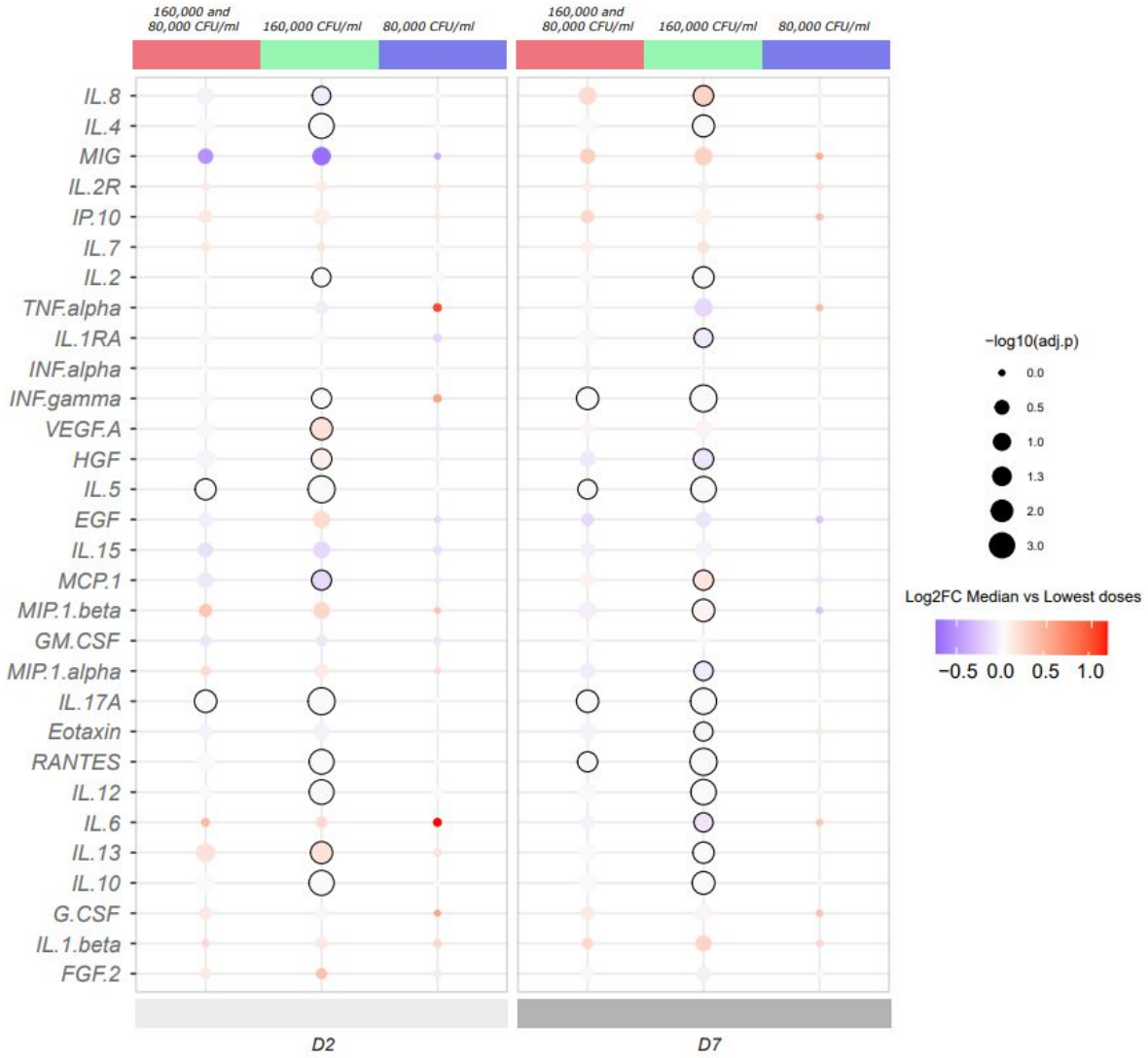

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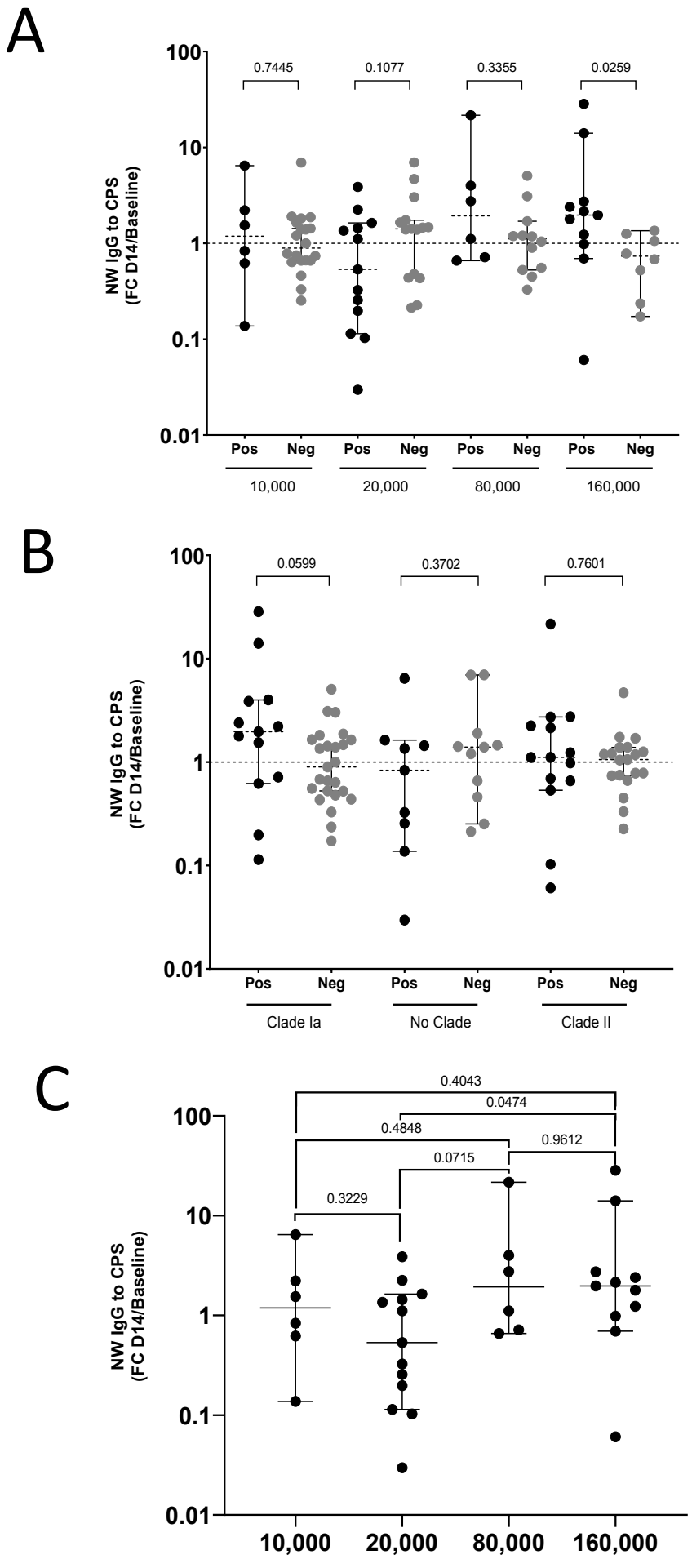
Figure E6



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