Experimental Human Pneumococcal Colonisation in Older Adults is Feasible and Safe, Not Immunogenic

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Running head: Experimental pneumococcal colonisation and aging

Despite low rates of pneumococcal colonisation detected in older people in previous community surveys, experimental colonisation was established in 39% of volunteers aged ≥ 50 years. The serological and functional immune responses of older adults to challenge and colonisation with live pneumococci were markedly different to those of young adults. Experimental human pneumococcal colonisation was safe, supporting the use of this methodology in clinical trials of pneumococcal vaccines for older people.

MeSH keywords: *Streptococcus pneumoniae*; human challenge models; immunity; elderly; aged; pneumococcal disease; vaccination

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Some of the results of these studies have been previously reported in the form of a preprint (medRxiv, 29 April 2020; https://doi.org/10.1101/2020.04.23.20077073)

At a Glance

What is the current scientific knowledge on this subject?

Colonisation is rarely detected in older adults, despite high rates of pneumococcal disease. We hypothesise that the susceptibility of elderly participants to pneumococcal infection could be associated with their lack of immune boosting to pneumococcal carriage.

What does this study add to the field?

We have shown that pneumococcal carriage is possible in older adults and carriage rates are not different to young adults. However, different to what we have previously reported in young adults, carriage is not an immunising event in older adults.

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Abstract

Rationale: Pneumococcal colonisation is key to the pathogenesis of invasive disease, but is also immunogenic in young adults, protecting against re-colonisation. Colonisation is rarely detected in older adults, despite high rates of pneumococcal disease.

Objectives: To establish experimental human pneumococcal colonisation in healthy adults aged 50—84 years, to measure the immune response to pneumococcal challenge, and to assess the protective effect of prior colonisation against autologous strain rechallenge.

Methods: Sixty-four participants were inoculated with *Streptococcus pneumoniae* (serotype 6B, 80,000CFU in each nostril). Colonisation was determined by bacterial culture of nasal wash, and humoral immune responses were assessed by anti-capsular and anti-protein IgG levels.

Measurements and Main Results: Experimental colonisation was established in 39% of participants (25/64) with no adverse events. Colonisation occurred in 47% (9/19) of participants aged 50—59 compared with 21% (3/14) in those aged ≥70 years. Previous pneumococcal polysaccharide vaccination did not protect against colonisation. Colonisation did not confer serotype-specific immune boosting: geometric mean titre (95% CI) 2.7μg/mL (1.9—3.8) pre-challenge versus 3.0 (1.9—4.7) four weeks post-colonisation (p = 0.53). Furthermore, pneumococcal challenge without colonisation led to a drop in specific antibody levels from 2.8μg/mL (2.0—3.9) to 2.2μg/mL (1.6—3.0) post-challenge (p = 0.006). Anti-protein antibody levels increased following successful colonisation. Rechallenge with the same strain after a median of 8.5 months (IQR 6.7—10.1) led to recolonisation in 5/16 (31%).

Conclusions: In older adults, experimental pneumococcal colonisation is feasible and safe, but demonstrates different immunological outcomes compared with younger adults in previous studies.

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Introduction

Older adults have high rates of morbidity and mortality from pneumococcal disease, including pneumonia and meningitis (1-4). Nasopharyngeal pneumococcal colonisation necessarily precedes pneumococcal disease (5). Paradoxically, colonisation has generally been considered to be infrequent in older adults, with rates ranging from 1.9% to 4.2% in studies employing culture-based methodology (6-8). This may be due to insensitive detection methods or an altered niche of colonisation in older adults (9, 10). Others have suggested that older adults are inherently less susceptible to colonisation, perhaps due to age-related dysregulation of airway inflammatory pathways (11-13). Colonisation is immunogenic in young adults, protecting against future recolonisation with the same serotype (14, 15)., Therefore reduced colonisation or altered post-colonisation immunity in older adults may partially explain their susceptibility to pneumococcal disease. The immune dynamics of colonisation in older adults have not been previously studied.

In adults over 65 years of age, recommendations for pneumococcal polysaccharide vaccine (PPV23) and 13-valent pneumococcal conjugate vaccine (PCV13) administration vary between countries (16, 17). Both vaccines induce anti-capsular polysaccharide antibody production, although the functionality of antibodies produced is reduced in older adults (18, 19). Both vaccines confer a degree of protection against vaccine-serotype invasive pneumococcal disease (20, 21), although a combination of imperfect efficacy and serotype replacement by non-vaccine serotypes have reduced the effectiveness of both vaccines (22). PCV13 confers short-term protection against colonisation (23), most likely via antibody-mediated bacterial agglutination (24), while high-quality prospective data regarding PPV23 and colonisation are lacking (25). Protection against colonisation is a surrogate for protection against disease. Since neither vaccine confers complete protection against pneumococcal disease in older adults, improved vaccines and immunisation strategies are urgently required.

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Controlled human infection models have revolutionised the study of infectious disease pathogenesis (26). Such studies typically recruit healthy, young adults who are at low risk of severe disease or complications. Rhinovirus challenge studies have been performed in older participants, including those with respiratory comorbidities (27), but bacterial challenge studies have not been systematically attempted in older people. There is an unmet need for such research in this population, given the higher age-specific incidence of pathogens such as pneumococcus, and the uncertain generalisability from studies of young adults.

We report the expansion of the experimental human pneumococcal colonisation (EHPC) model into adults aged ≥50 years, aiming to establish the feasibility, susceptibility and safety of EHPC in older adults. We also define the immunogenicity of pneumococcal colonisation in this population measured by antibody responses, and the protection conferred by primary challenge against subsequent re-colonisation by the same strain.

Methods

Healthy adults aged 50—84 years were approached using advertisements, research volunteer databases and primary care patient lists. All participants provided written informed consent and underwent safety screening including review of primary care records, physical examination, electrocardiography, spirometry, complete blood count and biochemical profile before participation (see Supplementary Appendix for full inclusion/exclusion criteria and study methods). In brief, participants were excluded if they had regular close contact with children aged <5 years or immunosuppressed adults, uncontrolled medical comorbidity, recent steroid or antibiotic therapy, significant smoking history, or history of culture-proven pneumococcal disease. Vaccination history was recorded: in the UK, PPV23 is recommended to all 65-year olds, but PCV13 is not routinely offered. The study was overseen by an independent Data Monitoring and Safety Committee.

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Experimental pneumococcal challenge was performed as previously described (15, 28). Baseline nasal wash and serum samples were taken up to seven days before inoculation. Inoculation entailed installation of an estimated 80,000 CFU per nostril of *Streptococcus pneumoniae* serotype 6B (strain BHN418 (29), GenBank accession number ASHP00000000.1) using a micropipette, with the participant in a reclining chair. Participants recorded and communicated their temperature to the research team by SMS every day for the following week. Nasal washes were repeated on days 2, 7, 9, 14, 22 and 29 post-inoculation, with a second serum sample on day 29. After completion, if participants' nasal wash remained culture-positive at day 22 or day 29, they were treated with oral amoxicillin for three days. Those who had been colonised at any time point were invited to return up to one year later for rechallenge using the same pneumococcal strain, dose and procedure. Repeat nasal washes for the re-challenge study were taken at baseline and 2, 7 and 14 days post-inoculation. The timeline is summarised in FIGURE 1.

Nasal wash samples were processed within an hour of collection, and incubated overnight on gentamicin/blood agar at 37°C in 5% carbon dioxide (an aliquot was also plated on non-selective blood agar to ensure accurate estimation of density). Experimental colonisation was defined as the growth of serogroup 6 pneumococcus at any timepoint during the 29 days post-inoculation, identified using standard microbiological criteria (30). Anti-6B capsular polysaccharide (CPS) IgG levels in serum were measured using a modified WHO enzyme-linked immunosorbent assay (ELISA) protocol (see Supplementary Appendix). Serum antibodies against 27 pneumococcal proteins were measured using multiplex electrochemiluminescence (Meso-Scale Discovery, MSD) as previously reported ((15), methodological details in Supplementary Appendix).

Statistical analysis

The primary endpoint was the rate of experimental colonisation in older adults, which we compared with the rate in younger adults in other EHPC studies using the same methodology carried out during the same time period. We did not recruit a designated young control cohort for this study; >200

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young adults took part in other EHPC studies during this time, following the same inoculation protocol.

The typical experimental colonisation rate in young healthy adults is 45% (15) and we hypothesised based on cross-sectional colonisation studies (6) that this would fall to 10% in older adults. A sample size of 64 would detect this difference in rates of experimental colonisation at $\alpha = 0.05$ with a power of 0.80, allowing for a 10% drop-out rate. We did not pre-specify that we would exclude participants who were naturally colonised with pneumococcus at baseline, but performed post-hoc sensitivity analyses excluding such participants from colonisation outcomes.

Secondary microbiological endpoints included colonisation rates stratified by age, colonisation rates in PPV23-vaccinated participants, colonisation density and duration, and adverse events. Immunological endpoints included the association of pre-existing antibodies with probability of colonisation and colonisation density, and the change in antibody titre after challenge. Total bacterial density during the study was defined as the area under the time-density curve (AUC), calculated according to the trapezoid rule using values of $[\log_{10}(\text{bacterial density}+1)]$ for each interval, with all participants assigned a density of 0 CFU/mL on inoculation day. For participants in the rechallenge phase of the study, colonisation densities up to day 14 post-rechallenge were compared with the colonisation densities over the same time period during the primary challenge.

Colonisation rates in different groups were compared using χ^2 or Fisher's exact test. Antibody results were log-transformed and compared between groups using the unpaired *t*-test or ANOVA, or within groups (before and after pneumococcal challenge) using the paired *t*-test, with results presented as geometric mean titres (GMT) and 95% confidence intervals. Correlations between continuous variables were assessed using Pearson's correlation. Non-parametric tests were used for comparisons of untransformed values within the (smaller) rechallenge cohort. The effects of baseline anti-6B antibodies, adjusted for age and sex, on the development of colonisation or on colonisation density AUC were assessed using logistic regression and linear regression, respectively.

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Fold changes in the 27 anti-protein antibodies between baseline and day 29 were compared between colonised and non-colonised participants using multivariate regression. No imputations were made for missing data. All tests were two-tailed, and a p value <0.05 was considered significant. All analyses were performed using SPSS version 24 (IBM, New York).

Results

Participant characteristics

The oldest participant was aged 80 years, and the median age was 64; the baseline characteristics of the cohort are given in TABLE 1. The recruitment process and screening outcomes are outlined in FIGURE E1 in the Supplementary Appendix. Ten participants were excluded on the basis of abnormal clinical findings at their pre-screening visit, and no participants had previously received pneumococcal conjugate vaccine. Three were naturally colonised with *Streptococcus pneumoniae* at baseline, all of whom remained in the study and underwent experimental inoculation as per protocol.

Experimental colonisation rates

The median inoculation dose was 84,333 CFU/mL (range 69,333—92,833). Experimental colonisation developed in 25 participants (39.1%; 95% CI 28.1—51.3%) (FIGURES 2 and 3, and TABLE E3 in the supplementary appendix). This was not significantly different from the 46.7% achieved in 225 young adults in the comparison group (p = 0.281) (FIGURE 3). Colonisation rates were 32% in men (9/28) versus 44% (16/36) in women (p = 0.32). Colonisation rates within the over-50 cohort did not differ by age decile (χ^2 for trend p = 0.146), FIGURE 3. When baseline natural carriers were excluded, the overall colonisation rate was 37.7% (26.6—50.3%, n = 23/61).

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Experimental colonisation density and duration

Colonisation densities varied substantially within and between participants; the average AUC of density was 34.4 CFU.days/mL (95% CI 19.9—48.9) and a graph of individual participants' colonisation dynamics is in the Supplementary Appendix (FIGURE E2). The median duration of colonisation was 22 days, with 8/25 participants still having detectable colonisation at day 29. Four participants had no detectable colonisation after day 2. AUC of density did not differ with age, either as assessed by age deciles (ANOVA p = 0.84), or by linear correlation with age (r = -0.099, p = 0.64).

Experimental colonisation in PPV23 recipients

Experimental colonisation rates were not different in those who were previously immunised with PPV23 compared with those who were not (8/22 [36.4%] vs 17/42 [40.5%] respectively, p= 0.75). This pattern remained in sensitivity analyses restricted to over-65s and to the most recent vaccine recipients. We did not identify any significant predictors of colonisation using logistic models, having tested PPV23 immunisation status and other clinical and laboratory data (Supplementary Appendix).

Immunological results

Paired serum samples from baseline and 29 days were available for 62/64 participants. There were no differences in baseline antibody anti-capsular polysaccharide (CPS) IgG titres between males and females (GMT 2.82 µg/mL [95% CI 1.91—4.18] and 2.71 µg/mL [1.99—3.69] respectively, p = 0.87). Previous PPV23 was associated with higher baseline antibody levels compared with no prior PPV23 (GMT 4.17 µg/mL [95% CI 2.84—6.12] versus 2.24 µg/mL [1.67—2.99] respectively, p = 0.01). We did not find evidence of a fall in antibody levels over time following vaccination. In fact, there was a moderate positive correlation between PPV23 recipients' baseline antibody levels and time since vaccination (r = 0.46, p = 0.034). Baseline antibody levels increased with age (r = 0.34, p = 0.007), but

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this correlation was not evident when PPV23 recipients were excluded (r = 0.19, p = 0.24; FIGURE E3 in the Supplementary Appendix).

Anticapsular antibodies and experimental colonisation

Higher baseline antibody levels were not associated with protection against experimental colonisation: IgG GMTs in those colonised and non-colonised were 2.71 µg/mL (1.91—3.84) and 2.79 µg/mL (2.0—3.9) respectively (p = 0.90). Adjustment for age and sex using logistic regression did not alter these findings (Supplementary Appendix).

We did not observe an increase in antibody levels following colonisation (FIGURE 4, TABLE E5). Of interest, pneumococcal challenge without colonisation led to diminished antibody levels: the GMT in subjects who did not develop experimental colonisation fell from 2.79 µg/mL (2.9—3.9) at baseline to 2.17 µg/mL (1.57—3.0) at day 29 (p = 0.006). On sensitivity analyses subdivided by PPV23 administration, this finding was significant only in the vaccinated sub-cohort (FIGURE 4; TABLES E6 and E7 in the SUPPLEMENTARY APPENDIX).

When antibody responses in colonised and non-colonised participants within each age decile were compared, the only statistically significant change was the fall in antibody levels post-challenge in non-colonised over-70s (all of whom had received PPV23; TABLE E8 in the SUPPLEMENTARY APPENDIX). However, the statistical significance was borderline (p = 0.047) and we did not correct for multiple comparisons. When antibody responses to colonisation were summarised as fold changes, there was no correlation with age in either colonised (r = -0.025, p = 0.906) or non-colonised participants (r = -0.155, p = 0.352). Higher baseline antibody levels did not correlate with lower colonisation density, nor did higher density correlate with higher day-29 antibody levels or greater fold change in antibody levels (SUPPLEMENTARY APPENDIX).

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Anti-protein antibodies

Serum antibody levels against 27 pneumococcal proteins were measured at baseline and at day 29 post-challenge using MSD multiplex electrochemiluminescence. There were no differences in the baseline levels of any antibody between colonised and non-colonised participants (FIGURE E4). In contrast to the anti-capsular IgG response, antibody titres against several pneumococcal proteins were increased following pneumococcal colonisation, including PspC, PspA-UAB055, RrgA-Tigr4, PiuA and PcpA (all $p<0.001$) (FIGURE 5). In individuals who remained uncolonised, there was no significant change in any anti-protein antibodies.

Mucosal antibodies

Anti-6B antibody levels in nasal wash were similar at baseline between colonised and non-colonised participants, and these levels did not change significantly following bacterial challenge (SUPPLEMENTARY FIGURE E5)

Protection against experimental re-colonisation

Rechallenge was performed in 16 of the 25 participants who originally developed experimental colonisation, after a median interval of 259 days (IQR 205—308 days; Figure S1 in the Supplementary Appendix). One was colonised with serogroup 15 pneumococcus at time of rechallenge, and participated in the study as per protocol. A further participant was colonised with a serotype 6B pneumococcus at time of rechallenge; he could not recall whether he had taken the 3-day course of amoxicillin prescribed following the initial study completion. Community-acquired colonisation with serogroup 6 pneumococci is uncommon in Liverpool (31). The participant was not re-inoculated, but treated with amoxicillin for five days. Subsequent nasal washes at seven and 28 days were negative by both culture and polymerase chain reaction.

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The median age of rechallenge participants was 63.5 years, and 11 (68.7%) were female. Within 14 days of inoculation, colonisation was detected in five participants (31.3%).

Colonisation density in rechallenge participants

Of the five colonised participants, two had detectable colonisation at all three post-inoculation timepoints, while the remainder were only culture-positive at one timepoint each. The mean colonisation density by day 14 in rechallenge participants (as measured by AUC) was 10.0 CFU.days/mL (95% CI 0—21.94). This was non-significantly lower than the mean density in the same five participants during the first 14 days of the primary challenge (31.01 CFU.days/mL, 95% CI 11.08—50.95, p = 0.08 using Wilcoxon's matched pairs test).

Anti-capsular antibodies in rechallenge participants

Antibody levels pre- and 14 days post-rechallenge are shown in FIGURE 6, and in TABLE E11 in the SUPPLEMENTARY APPENDIX, along with the corresponding antibody levels from the same participants in the primary challenge for comparison. One participant did not have serum taken on day 14 post -rechallenge. Antibody levels did not fall significantly during the interval between the end of the primary challenge and the start of the rechallenge. The pre-rechallenge baseline antibody levels were similar between participants who did and did not develop colonisation following rechallenge: respective GMTs (95% CI) 3.72 µg/mL 1.18—11.76) and 1.66 µg/mL (1.17—2.36) (p = 0.07 using the Mann-Whitney U test). Antibody levels fell in participants who remained uncolonised post-rechallenge (GMT at day 14 1.53 µg/mL (95% CI 1.08—2.17), p = 0.041 using Wilcoxon's signed rank test to compare with pre-challenge levels).

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Safety of EHPC in older adults

There were no serious adverse events, and no cases of pneumococcal disease. During active surveillance (the week following inoculation), no participants recorded oral temperatures ≥38°C. During the primary challenge, seven participants developed symptoms which, when reviewed clinically, were deemed unrelated to study procedures by the investigators; details are summarised in the Supplementary Appendix. During the rechallenge phase, one colonised participant developed malaise and unilateral otalgia at day 11, but sought no medical help. They attended routinely at day 14, at which time clinical examination and otoscopy were unremarkable. For precaution, a five-day course of amoxicillin was prescribed to clear colonisation.

Discussion

This is the first human bacterial challenge model in older adults. Pneumococcal colonisation was safely established at similar rates, densities and durations as in younger adults (15, 30). Colonisation rates in the oldest participants in this cohort (ages 70—80 years) were non-significantly lower than those seen in under-50s, but low numbers (n=14) preclude definitive conclusions. The ranges and fluctuations in density and variable durations of colonisation were similar to those seen in young participants following both experimental and natural colonisation (30, 32). PPV23 receipt did not protect against colonisation, and baseline antibody levels were not different in colonised and non-colonised participants, the latter observation consistent with previous findings in young adults (15).

When designing this study, we reviewed data from studies conducted in older adults that showed colonisation rates <5% (6-8). We hypothesised that the older population was somehow less susceptible to pneumococcal colonisation than younger adults, a hypothesis that we now reject based on our experimental colonisation data. After our study was completed, we collaborated on a meta-analysis that combined studies conducted in over-60s with participant-level data on over-60s

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from colonisation studies of the general population (33). This meta-analysis found widely variable point-prevalence rates (0-39%), but an overall rate of 9% (95% CI 6-14%). A similar meta-analysis in younger adults has yet to be conducted, but (for example) one household study in England found pneumococcal colonisation in 8% of adults (14). These findings suggest that older and younger adults become colonised at similar rates, whether in natural or experimental settings, and that older adults' susceptibility to pneumococcal disease is not due to a lack of colonisation-mediated boosting.

Indeed, we found that the immunological outcomes following pneumococcal challenge in older adults differ considerably from young adults, in whom anticapsular antibody levels are raised following experimental colonisation remain and unchanged in non-colonised participants (15). Amongst older adults, antibody levels were unchanged following colonisation and actually fell in non-colonised participants. Our statistical power to detect age-related immunological trends within the cohort itself was limited by small numbers in each age decile. During rechallenge we observed no protection against re-acquisition of homologous serotype colonisation in this older cohort in contrast to the universal protection seen in a previous study of participants aged <50 years (0/10 recolonised) (15).

There are a number of potential explanations for the antibody responses we describe in older adults. Serotype-specific memory B cell levels have been shown to fall following revaccination with PPV23 in older adults (34), and one could speculate that memory B-cells may undergo similar terminal differentiation in response to live pneumococcus. Our results may reflect those of vaccine studies which have demonstrated hyporesponsiveness following repeated doses of polysaccharide in the form of PPV23 (35-37). This hypothesis is supported by our finding that antibody levels fell more in PPV23-vaccinated participants than in unvaccinated participants following challenge, although numbers were small in these subgroups, and we did not correct for multiple comparisons. The hyporesponsiveness hypothesis is further supported by the fall in anti-CPS antibodies in non-colonised participants following rechallenge. Alternatively, peripheral antibodies were perhaps

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sequestered in the nose following pneumococcal challenge, leading to a drop in circulating levels. We have previously reported mucosal antibody sequestration by inoculated bacteria in PCV13-vaccinated young adults (preventing colonisation via agglutination), but did not observe an effect on peripheral antibody levels (24). Equally, in the current study we did not identify any changes in nasal wash anti-6B IgG levels at any timepoint post-challenge in colonised or non-colonised participants.

Our finding that PPV23 does not prevent colonisation contrasts with a recent study in US adults aged ≥65 years. The authors followed 100 participants with fortnightly sampling for one year and identified a high period prevalence of colonisation in the cohort as well as significantly lower colonisation in participants who had ever received either pneumococcal vaccine (38). However, most pneumococcal detections were by *lytA* qPCR rather than culture, and serotype confirmation was unavailable for the vast majority of isolates. Longitudinal surveillance of US children suggests that any reduction in vaccine-type pneumococcal colonisation following vaccination is mostly balanced out by serotype replacement (39). It seems unlikely that PPV23 vaccination of older adults disrupts colonisation to such a degree.

Using MSD, we identified boosted antibody levels against a number of pneumococcal proteins following colonisation. These included PspA-UAB055, PspC and PiuA, which were also boosted following colonisation in young adult participants in a previous study, using the same MSD methodology (15). This suggests that these proteins are visible to the immune system during colonisation, and are good markers of exposure. Our finding that baseline anti-protein antibody levels were not different between colonised and non-colonised participants suggests that serum anti-protein immunity may not be a major component of anti-pneumococcal immunity in older adults.

Limitations

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Our participants were carefully screened, which may have resulted in a particularly healthy cohort, characterised by non-smoking and minimal medical comorbidity. While this limits the generalisability of our findings, we felt it was necessary to maximise the safety of participants. We defined colonisation based on culture positive nasal wash samples, rather than molecular testing with typically higher sensitivity (32, 40, 41). Some authors have argued that the oropharyngeal niche has higher yield for detecting colonisation in older adults than our nasal wash method (9). We did not measure functional antibody activity in this study, but IgG levels and serum opsonophagocytic activity are reasonably correlated in older adults (19). The antibody response to colonisation may be serotype dependent: population studies have shown a decline in antibodies against certain serotypes (e.g. serotype 3) with age, but not in other serotypes including 6B (42). By contrast, an observational study in young adults found that 6B colonisation did not elicit as strong an antibody response as other serotypes (14). Future research should study the immune response of older adults to challenge with non-6B serotypes.

Conclusions

New strategies are needed to protect older people against pneumococcal disease. The safety, tolerability and high rates of experimental colonisation seen in this study support the use of EHPC for vaccine testing in this key vulnerable population.

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TABLES and FIGURES

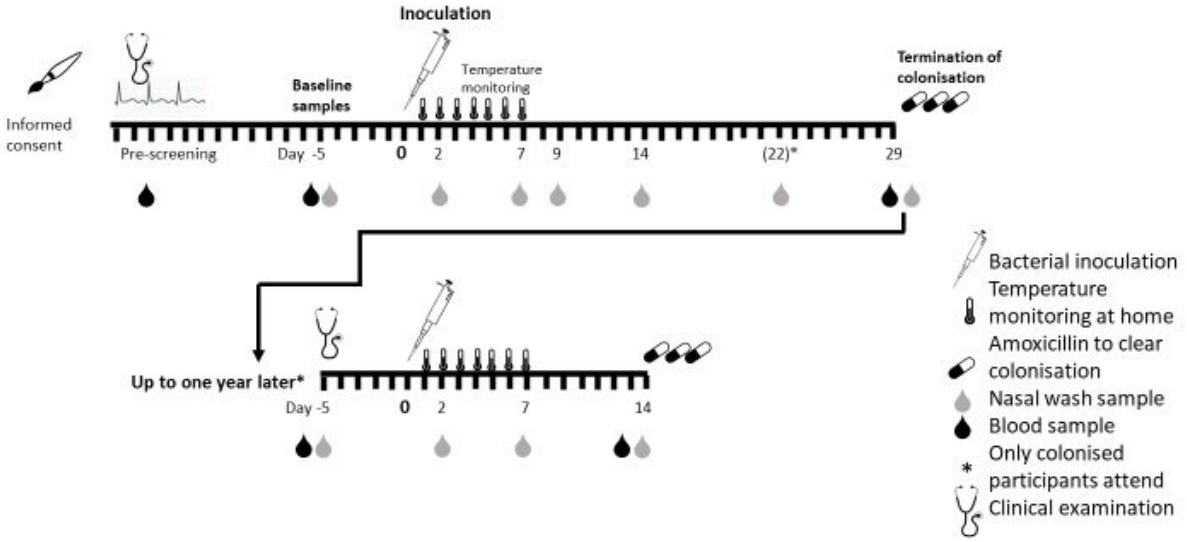

Figure 1: Timeline for the study, including the optional re-challenge (for participants who developed colonisation during the primary study) up to one year later.

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Table 1: Baseline characteristics of all volunteers in the study

	Age-defined risk category			Total
	50-64	*65-74*	*75 – 84*	
Number of participants inoculated	34	25	5	64
Number of females	18 (52.9%)	15 (60%)	3 (60%)	36 (56.3%)
Age at inoculation, median (range)	59 (52—62)	69 (66—70)	78 (78—79)	64 (59—69)
Inoculation dose, CFU/mL, median (range)	83,833 (76,000—90,167)	84,333 (69,333—92,833)	84,750 (73,833—89,167)	84,333 (69,333—92,833)
Number of ex-smokers	10 (29.4%)	11 (44%)	2 (40%)	22 (34.4%)
Pack years smoked, median (IQR)	10 (4.5—10)	5 (2—10)	8 (1—8)	6.5 (3.3—10)
Number with any reported comorbidity*	14 (41.2%)	15 (60%)	3 (60%)	32 (50.0%)
Number prescribed any regular medication	11 (32.4%)	18 (72%)	3 (60%)	32 (50.0%)
Prior pneumococcal polysaccharide vaccine	0	17 (68%)	5 (100%)	22 (34.4%)
Naturally colonised at baseline, n (serogroups)	3 (23, 3, 15)	0	0	3

**Comorbidities reported in more than one participant included the following:*

- *Benign prostatic hyperplasia: 5*
- *Depression: 5*
- *Hiatus hernia: 4*
- *Hypothyroidism: 4*
- *Osteoporosis: 3*
- *Bicuspid aortic valve: 2*
- *Glaucoma: 2*
- *Migraines: 2*
- *Previous malignancy: 2 (1 melanoma, 1 testicular)*

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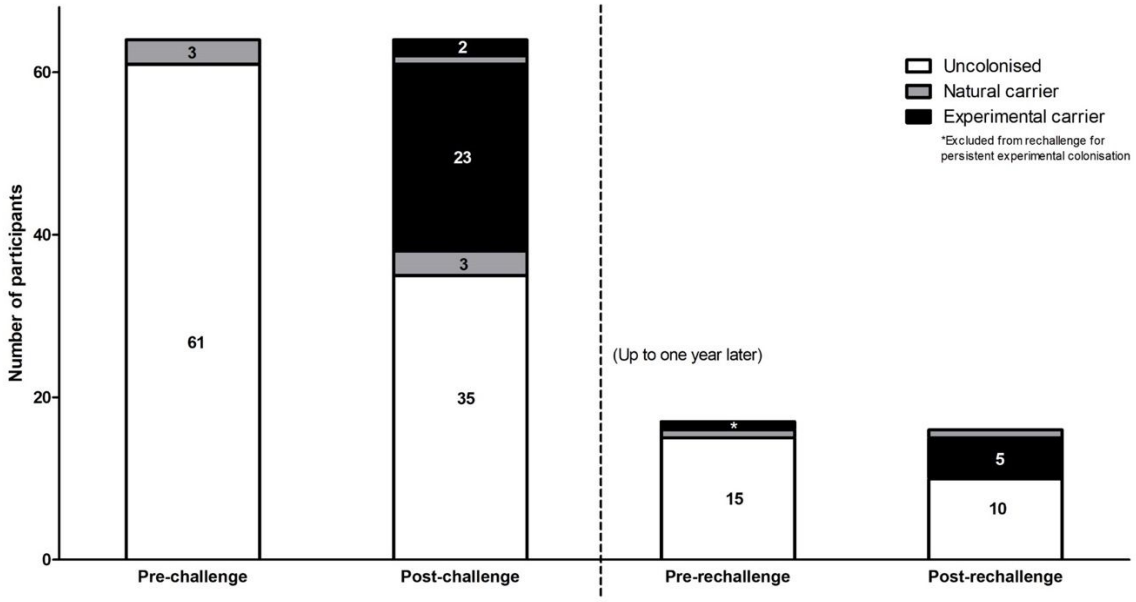

Figure 2: Microbiological status of participants pre- and post-inoculation. Three participants were naturally colonised with non-6B serotypes at baseline, two of whom subsequently developed experimental colonisation following inoculation (see SUPPLEMENTARY APPENDIX for details); 23 previously uncolonised participants developed experimental colonisation, and colonisation with non-experimental strains (serogroups 15, 20 and non-vaccine-type, Statens Serum Institut group G). At screening prior to rechallenge up to one year later, one volunteer was naturally colonised (with serogroup 15) and another was still colonised with the experimental strain; the latter was treated with amoxicillin and excluded from the remainder of the study. Five out of the remaining 16 developed experimental colonisation following rechallenge.

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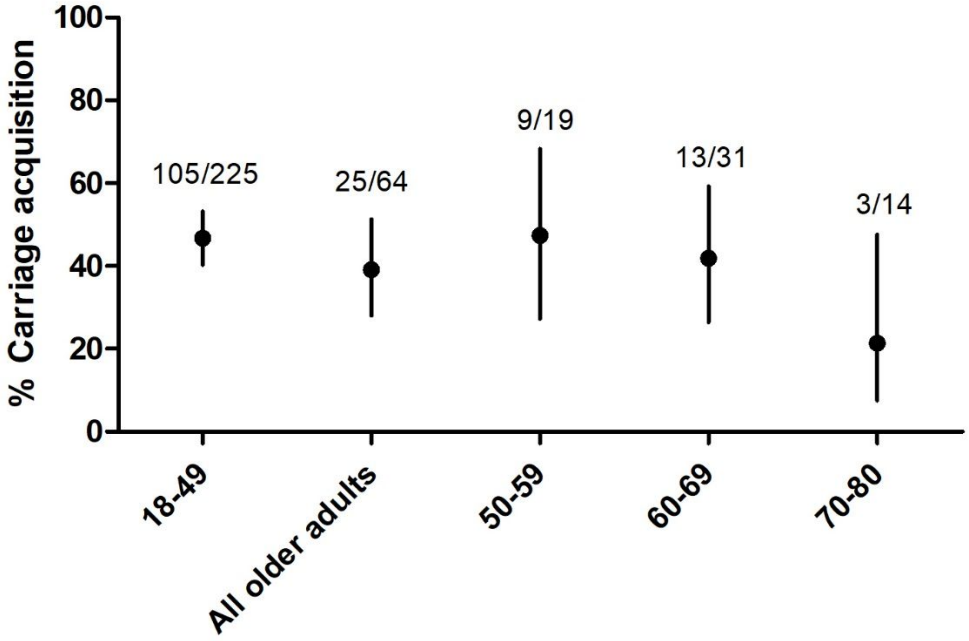

Figure 3: Experimental colonisation rates in older adults (defined by positive pneumococcal culture in nasal wash at any timepoint post-inoculation) compared with a young adult cohort (from similar studies conducted during the same time period), and broken down by age decile within the older cohort. Numbers denote (number colonised)/(total number in that age category). Error bars represent 95% CI. There were no statistically significant differences between the older age category or sub-categories and the younger volunteers.

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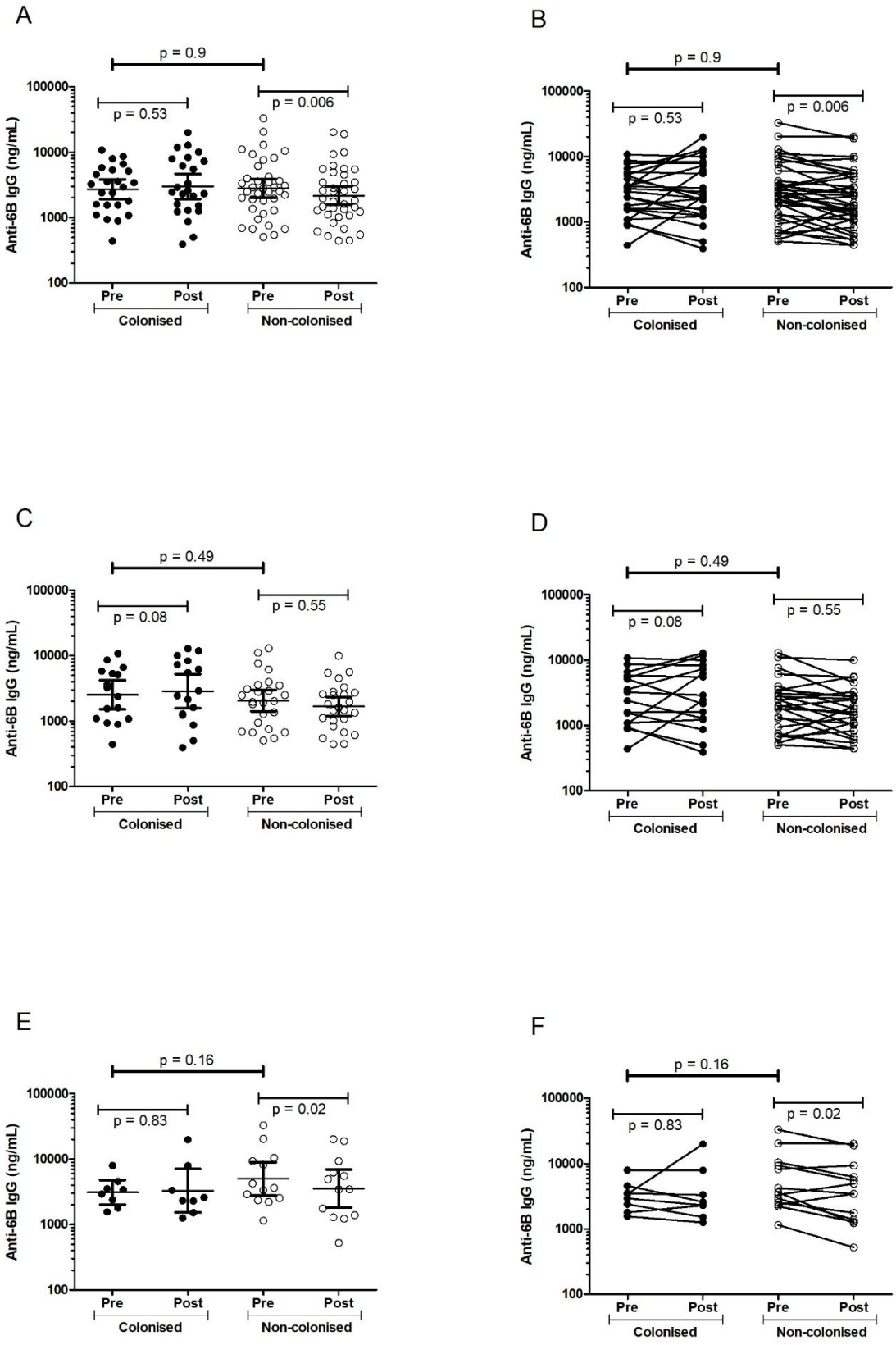

Figure 4: Anti-6B CPS IgG levels at baseline and day 29 following inoculation, in the full cohort (A, B), participants who had never received PPV23 (C, D) and PPV23-vaccinated participants (E, F).
Each symbol represents a single participant. The lines and error bars in A, C and E represent geometric mean (95% CI); the lines in B, D and F connect the baseline and day 29 values for each participant.

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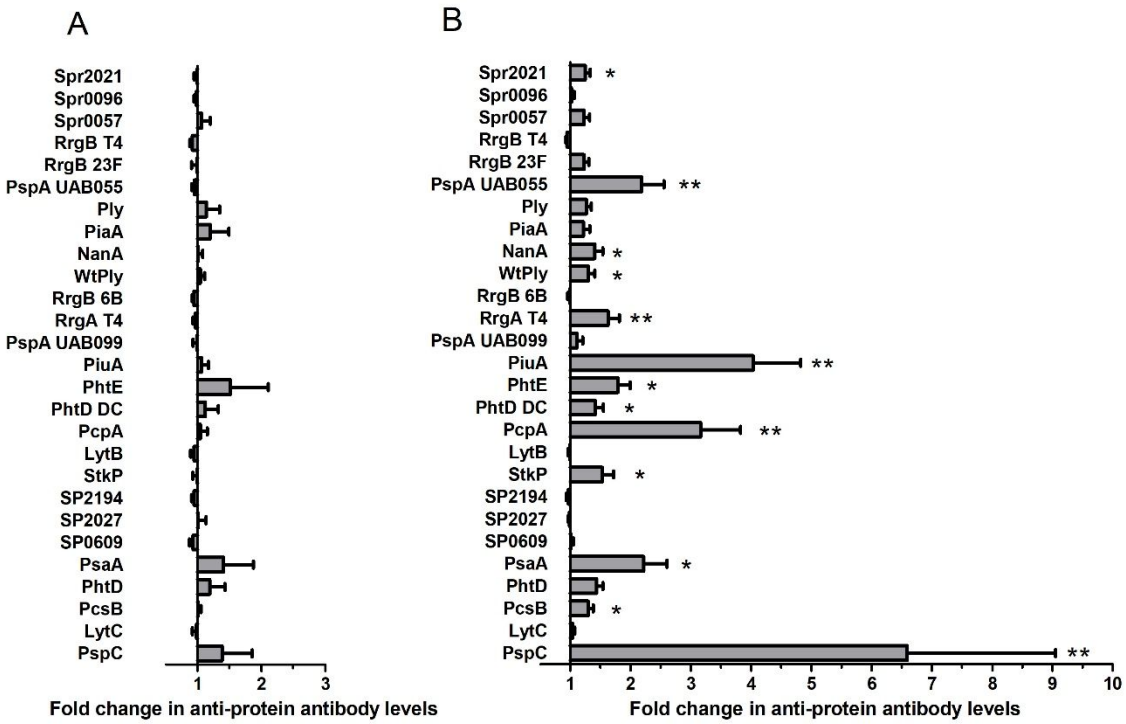

Figure 5: Changes in antibody levels against 27 different pneumococcal proteins following nasopharyngeal pneumococcal challenge, measured by multiplex electrochemiluminescence (Meso-Scale Discovery), in non-colonised (A) and colonised (B) participants. Antibodies are expressed as fold change between pre-challenge baseline and 29 days post-inoculation with S pneumoniae. *Error bars represent mean (SEM). Significant differences in fold change between carriers and non-carriers were analysed using multivariate regression: * denotes $p < 0.05$, ** $p < 0.001$.*

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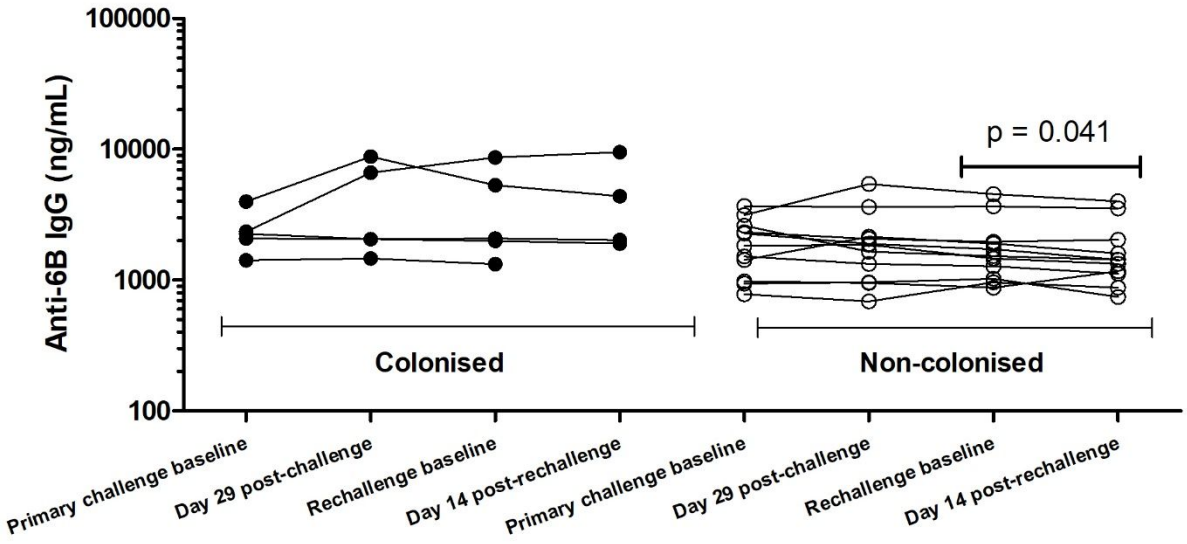

Figure 6: Serum anti-6B capsular antibodies before and after the primary and rechallenge phases of the study. Only participants who were colonised following the primary challenge and returned for rechallenge are shown, subdivided into those who did and didn't become colonised following rechallenge. Each dot represents an individual participant.

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Experimental Human Pneumococcal Colonisation in Older Adults is Feasible and Safe, Not Immunogenic

Hugh Adler, Esther L German, Elena Mitsi, Elissavet Nikolaou, Sherin Pojar, Caz Hales, Rachel Robinson, Victoria Connor, Helen Hill, Angela D Hyder-Wright, Lepa Lazarova, Catherine Lowe, Emma L Smith, India Wheeler, Seher R Zaidi, Simon P Jochems, Dessi Loukov, Jesús Reiné, Carla Solórzano-Gonzalez, Polly de Gorguette d'Argoeuves, Tessa Jones, David Goldblatt, Tao Chen, Stephen J Aston, Neil French, Andrea M Collins, Stephen B Gordon, Daniela M Ferreira, Jamie Rylance

Online Data Supplement

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Inclusion criteria for the "Aging and Immunity" study, with rationales

- Adults aged 50-84 years—previous EHPC studies had used 50 as their upper age limit;
- Fluent spoken English—to ensure a comprehensive understanding of the research project and their proposed involvement;
- WHO performance status 0 (able to carry out all normal activity without restriction) or 1 (restricted in strenuous activity but ambulatory and able to carry out light work)—because attendance at multiple visits were required, and as a surrogate for control of comorbidities
- Access to telephone—to allow safety and timely communication;
- Capacity to give informed consent.

Exclusion criteria for the "Aging and Immunity" study, with rationales

Clinical judgement was used in interpreting these exclusion criteria, with any concerns discussed with the chief investigator before enrolment.

- Caring responsibilities for children aged < 5 years, hospital patients or people with chronic illnesses—to minimise the exposure to potentially pathogenic bacteria in those at high risk;
- History of drug or alcohol abuse—to minimise risk of pneumococcal disease;
- Smoking any cigarettes currently or within the last six months—minimise risk of pneumococcal disease;
- Ex-smoker with a significant smoking history (>20 pack-year history of smoking OR up to 20 pack-year history of smoking but quit less than five years ago OR up to 10 pack-year history of smoking but quit less than six months ago[*])—to minimise the risk of pneumococcal disease, plus ensure that carriage rates were not affected by epithelial changes caused by smoking;

[*] One pack-year is defined as smoking 20 cigarettes per day for one year.

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- Any current treatment for asthma—to avoid excess risk of infection and minimise confounding effect of medications such as corticosteroids;
- Taking daily medications that may affect the immune system e.g. steroids, steroid nasal spray, disease-modifying anti-rheumatoid drugs—to ensure homogeneity of the cohort and minimise risk of pneumococcal disease;
- Taking medication that affects blood clotting e.g. aspirin, clopidogrel, warfarin or other oral or injectable anticoagulants—to reduce risk of bleeding associated with study procedures, also likely a surrogate marker of underlying systemic illness;
- Significant cardiorespiratory disease (excluding stable hypertension)—to minimise risk of pneumococcal disease or severe complications should pneumococcal disease occur;
- Disease associated with altered immunity, including diabetes, active malignancy, rheumatological conditions—to minimise risk of pneumococcal disease;
- Other uncontrolled comorbidities, as determined by the clinical investigator, which would be expected to increase the risk of pneumococcal disease;
- Any acute illness (new symptoms within preceding 14 days which are unexplained by the known past medical history)—to avoid confounding symptoms and minimise risk of pneumococcal disease (e.g. due to impaired mucosal immunity from concomitant viral infection);
- Having received any antibiotics in the preceding 28 days—to improve chance of carriage acquisition;
- History of culture-proven pneumococcal disease—to reduce confounding of carriage rates and other laboratory measurements by immunological memory of prior pneumococcal exposure;
- Allergy to penicillin— beta-lactam antibiotics are used for termination of carriage in the study protocol;

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- Involved in another clinical trial unless observational or in follow-up (non-interventional) phase—investigational medicinal products could have unanticipated effects on immunity, and multiple blood tests for multiple studies could be risky for participants;
- Prior participation in a clinical trial involving EHPC and bacterial inoculation in the past three years—to improve chance of carriage acquisition.

In addition, specific "Stop criteria" were defined for use during participant assessment, and participants were risk-stratified by age.

Table E1: Stopping criteria for use during participant assessment prior to commencing the study.

Stop criteria	
Clinical history and examination	STOP if unexplained or concerning findings on history or examination
Engagement with research team	STOP if the research team have concerns about volunteer's ability to commit to frequent communication and safety checks
Full blood count	STOP if haemoglobin <10g/L STOP if total white cell count <1.5 x10^9/L STOP if total white cell count >10 x10^9/L STOP if platelets <75 x10^9/L
Renal profile	STOP if sodium is outside reference range STOP if potassium is outside reference range STOP if urea is above reference ULN STOP if creatinine is above reference ULN
ECG	STOP if any evidence of significant conductive or ischaemic defect
Resting oxygen saturation	STOP if < 95%
Spirometry	STOP if FEV_1 <LLN

FEV_1 Forced Expiratory Volume in one second; LLN Lower Limit of Normal; ULN Upper Limit of Normal

Risk stratification

Table E2: Age-defined risk categories for participants.

Risk categories			
0	**1**	**2**	**3**
Young healthy volunteers – not included in this study	Age 50-64	Age 65-74	Age 75 - 84

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The rationale for the age groups related to published pneumococcal disease rates (Centres for Disease Control and Prevention (1) and clinical guidance for general use of PPV23 (age ≥ 65).

The study protocol required safety to be demonstrated in Category 1 before moving onto Category 2, and in Category 2 before moving onto Category 3. Safety was defined as per practice in previous EHPC studies: at least six uneventful inoculations per group and no reservations among the clinical team and Data Monitoring and Safety Committee (DMSC) before proceeding.

Safety briefing

Participants were given a post-inoculation advice sheet, including emergency contact details and "red flag" symptoms. They were given a thermometer and requested to send their temperature to the research team by text message daily for the first week post-inoculation. If they became unwell they were advised to check their temperature and contact the research team, who would advise them to seek healthcare, take amoxicillin and/or attend the research facility the next day for a review.

Nasal wash methodology

A modified Naclerio method was used for nasal wash in all EHPC studies (2). This has been validated by our team as being at least as sensitive as, and better tolerated than the WHO gold standard nasopharyngeal swab for the detection of pneumococci in adults (3, 4). In the Naclerio method, 5mL of normal saline is introduced using a syringe and held for a few seconds in the nose before being expelled in to a sterile container. The participant is advised to occlude their pharynx, either by pressing their tongue against their hard palate, or by "holding a swallow mid-way through". The procedure is repeated twice in each nostril, thus using 20mL saline in total. In the event of nasal wash loss (e.g. through inadvertent swallowing) the procedure may then be repeated to obtain an adequate specimen (defined as ≥10mL saline recaptured) using up to an additional 10mL of saline.

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Nasal wash samples were kept at ambient temperature and transported to the laboratory within one hour of collection.

Laboratory procedures

Experimental bacterial inoculum preparation

An isolate of serotype 6B pneumococcus (strain BHN418, GenBank accession number ASHP00000000.1) was provided by Prof PW Hermans (Radboud University, Nijmegen, The Netherlands). The reference laboratory at Public Health England tested the isolate and found it to be fully sensitive to all standard antibiotics, including penicillin. Genome sequencing for purity of each inoculum batch was performed by the Wellcome Sanger Institute (Hinxton, UK). A mid-log culture was frozen at -80°C in aliquots of 20% glycerol-enriched Vegitone broth (Sigma-Aldrich, Dorset, UK). On experimental inoculation days, an aliquot was thawed, washed twice, and re-suspended in 500µL normal saline before being diluted to achieve the target dose of 80,000 CFU/100µL. The actual inoculated dose achieved on the day was calculated by plating the inoculum prior to transport to the clinical research facility, and again upon return. Three 10µL dots were plated in parallel lines on blood agar, and colonies counted using the Miles and Misra method after overnight incubation (5). The mean of the colony counts on the pre- and post-dose plates was accepted as the true inoculated dose. A variation of half or double of the target dose was considered acceptable, as it had previously been demonstrated in dose-ranging studies that this range is safe and leads to similar colonisation outcomes (6).

Bacterial culture

Nasal wash samples were centrifuged for ten minutes at 3,345g, and the supernatant separated from the residual pellet. The pellet was resuspended in 100µL STGG, 20µL of which was streaked on a gentamicin/blood agar plate and incubated overnight at 37°C in 5% carbon dioxide. (The gentamicin suppresses competing respiratory pathogens, thus maximising the chance of

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pneumococcal detection.) Experimental colonisation was defined as the identification of serogroup 6 pneumococcus on the plate the following day. Pneumococci were defined using classical microbiological criteria:

1. Typical draughtsman-like colony morphology on agar;
2. The presence of α-haemolysis;
3. Optochin sensitivity;
4. Solubility in bile salts;

Serogroup was confirmed by a commercially available latex agglutination test (PneumoLatex, Statens Serum Institut, Copenhagen, Denmark); more detailed sero*typing* was not deemed necessary for experimental colonisation, as pneumococci from serogroup 6 in general are rare in the UK (7). Therefore, the detection of serogroup 6 pneumococci in a volunteer recently exposed to serotype 6B could reasonably be assumed to represent experimental colonisation. Natural colonisation was defined as identification of pneumococci that belonged to any other serogroup from a nasal wash at any timepoint.

Pneumococcal colonisation density determination

The STGG nasal wash pellet suspension was serially diluted on blood agar and incubated overnight as described above. The following day, colonies were counted and multiplied by the dilution factor and pellet volume to determine the density in CFU/µL. This was divided by the volume of nasal wash returned by the participant, leading to a result reported in CFU/mL of nasal wash. The remaining pellet suspension was stored at -80°C pending molecular testing.

Anti-capsular polysaccharide IgG ELISA

We measured anti-6B CPS IgG titres using a modification of the WHO enzyme-linked immunosorbent assay (ELISA) protocol. Serum samples were depleted of cell wall polysaccharide (CWPS) antibodies by incubating for 30 minutes in phosphate-buffered saline (PBS) blocked with heat-inactivated foetal

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bovine serum (ThermoFisher, Basingstoke UK) and 10µg/mL solution of CWPS (Statens Serum Institut, Copenhagen, Denmark). These pre-absorbed serum samples were then transferred to a 96-well plate (Maxisorp microtiter, Nunc, Roskilde, Denmark) that had been coated overnight at 4°C with 5µg/mL purified pneumococcal 6B capsular polysaccharide (Statens Serum Institut). The samples were serially diluted in the microtiter plates, then incubated for two hours at room temperature. Reference serum 89SF (US Food and Drug Administration (FDA)) was pre-absorbed and serially diluted in a similar fashion, with one row left blank, as a standard curve. The samples were washed three times with PBS containing 0.05% Tween (PBS-T), and then the secondary antibody (goat anti-human IgG conjugated to alkaline phosphatase; Sigma-Aldrich Corporation, Dorset, UK) was added and incubated for 90 minutes. The wells were washed three times again prior to incubation with p-nitrophenylphosphate (Sigma-Aldrich Corporation) for 15-20 minutes at room temperature for colour development. Antibody detection was performed using a FLUOstar Omega plate reader (BMG Labtech GmbH, Ortenberg, Germany), with optical densities read at 405nm. The antibody concentrations were determined by comparing the fluorescence in each sample well against the standard curve generated from the serially diluted 98SF reference serum, and are reported in ng/mL. All samples were analysed in duplicate. For each serum sample, we selected the dilution that gave a blank-corrected optical density (OD) closest to 0.1 in both replicates, as these fell along the linear part of the standard dilution curve. We excluded replicates whose covariance (i.e. $\frac{\text{Standard deviation}}{\text{Mean}}$) was ≥25%.

IgG in nasal wash samples was measured using the same methodology, but the lower antibody levels in nasal wash fluid necessitated lower dilutions at each stage of the process.

Anti-protein antibody measurement

We measured IgG antibodies against 27 different pneumococcal proteins using Meso-Scale Discovery (MSD, Rockville, Maryland) methodology. This platform utilises electrochemiluminescence (ECL) to quantify antibodies that have been marked with a Sulfo-tag

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label, and has previously been used to measure anti-CPS and anti-protein antibodies in young adults (6, 8). Each spot was pre-coated with 25 µg/mL of pneumococcal protein. The wells were blocked with 5% bovine serum albumin (BSA) in PBS, shaking for 1 hour at room temperature. Plates were washed 3 times with PBS-T between each step. CPS and CWPS antibodies were depleted by diluting serum samples (pre- and post-challenge samples and two quality control samples) in PBS 1% BSA containing 5µg/mL 22F CPS (ATCC, USA) and 1µg/mL CWPS (Statens Serum Institut). Reference serum 007sp (US FDA) was diluted in PBS 1% BSA containing 10µg/mL CWPS and used as a standard in each plate. Diluted/adsorbed samples were incubated for 30 minutes at ambient temperature on a shaker platform before being transferred to the pre-blocked plates (25µL per well) and incubated for 1 hour at ambient temperature on a shaker platform. Bound antibodies were detected using an MSD Sulfo-tag-labelled goat anti-human IgG antibody. Plates were washed and 150µL of MSD read 6 buffer T (4x, with surfactant), diluted 1:4 in water was added to each well. The plates were read using an MSD Sector imager, model 2400. The antibody concentrations in test samples were determined by referencing their ECL responses against the 007sp standard curve, expressed as units per millilitre (U/mL).

Results

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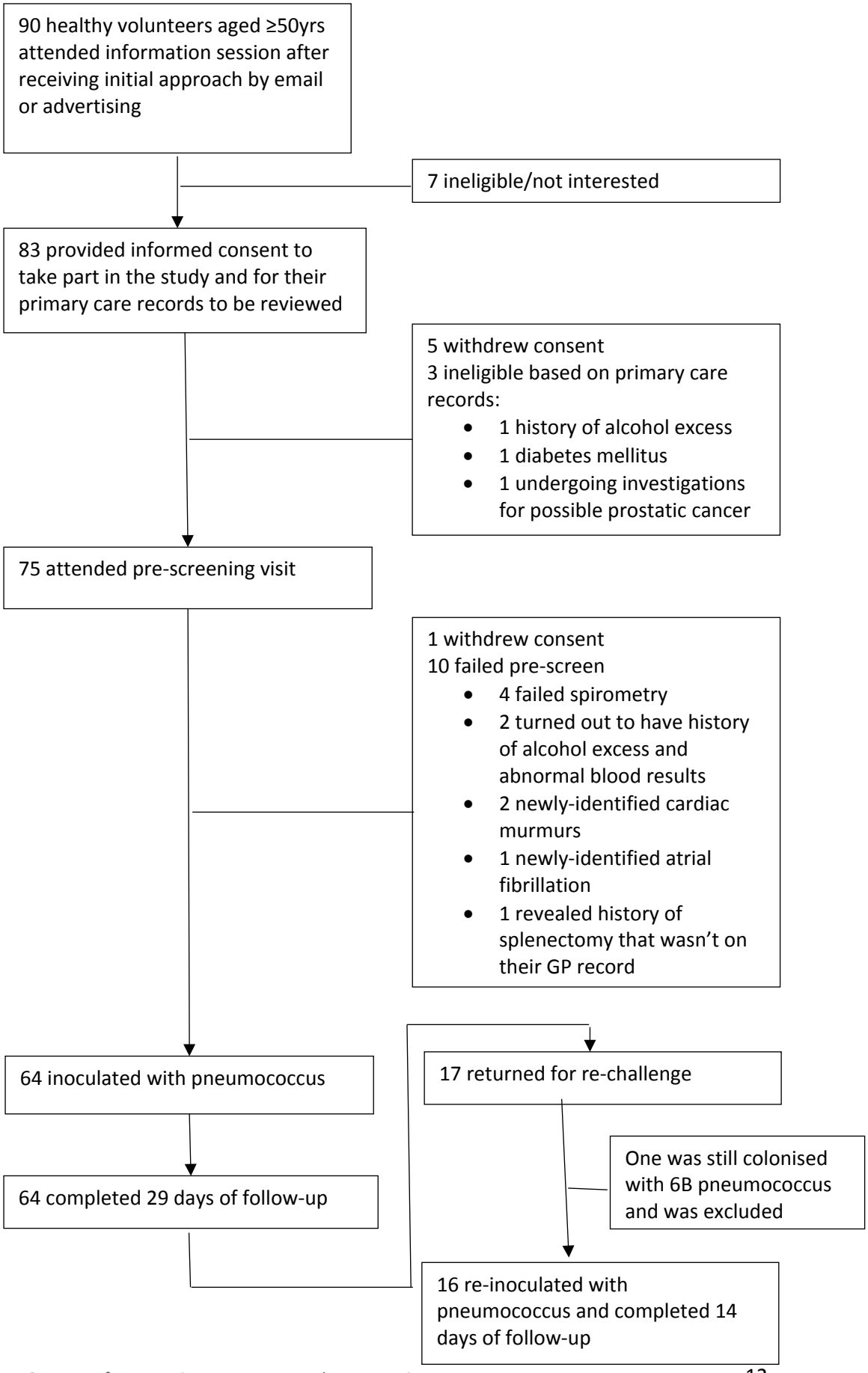

Figure E1: Outcomes from recruitment, consent and pre-screening processes

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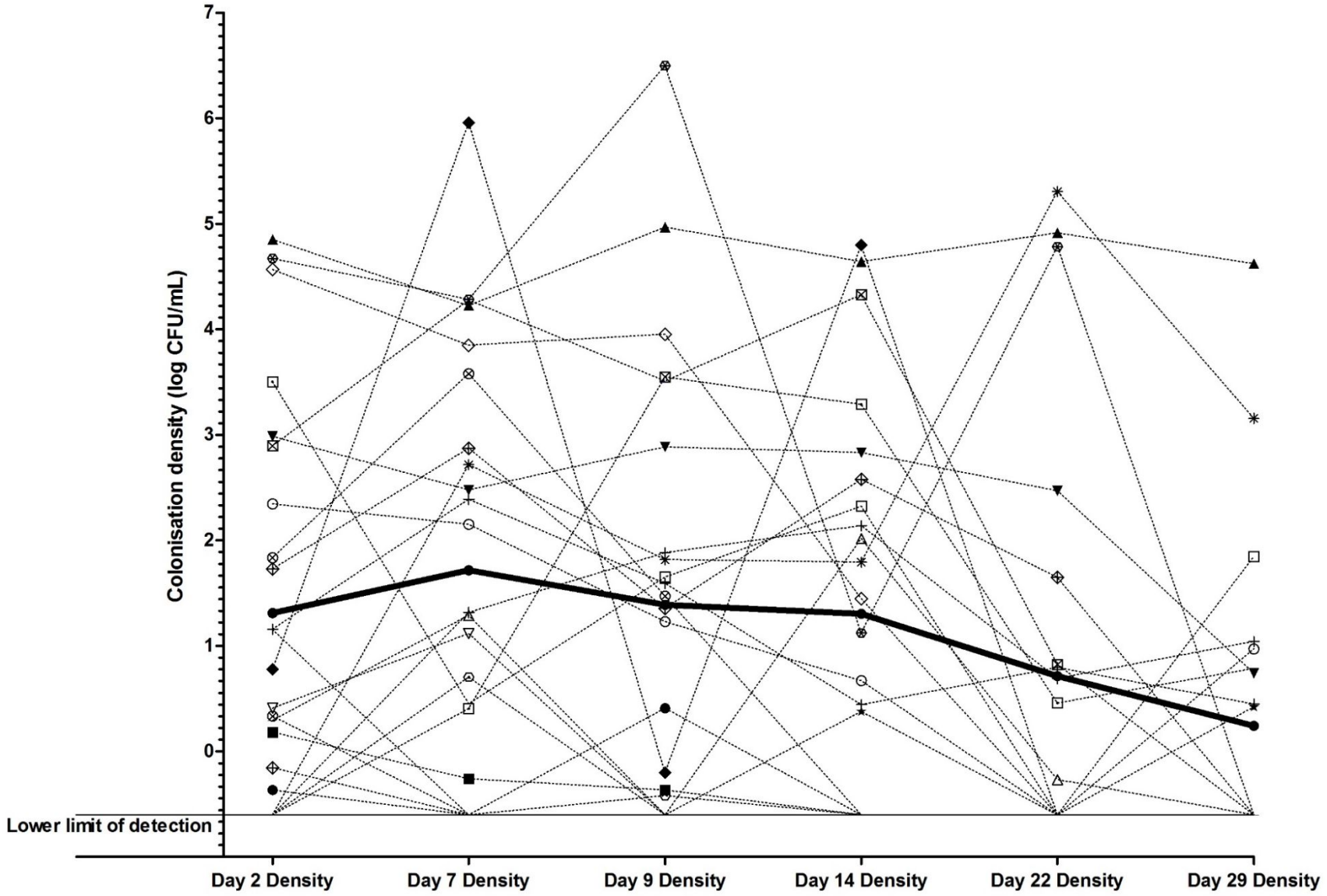

Figure E2: Colonisation density over time in experimental carriers
Each dashed line represents an individual participant; the heavy line represents the average density at each timepoint. The lines for participants identified as carriage-negative at certain time points are shown intersecting with the lower limit of detection for ease of visualisation.

Subgroup analysis: colonisation rates in different age categories

Table E3: Experimental colonisation rates in different age categories

Age category		Number of participants	Number of carriers	Percentage colonisation	P value (versus young adults)
Under 50*		225	105	46.7%	-
50—80		64	25	39.1%	0.28
Age deciles:	50—59	19	9	47.4%	0.95
	60—69	31	13	41.9%	0.62
	70—80	14	3	21.4%	0.095

**The results for "under 50s" were obtained in other studies by the same team between 2015—2017, using the same methodology*

Sensitivity analyses for the effect of PPV23 on colonisation

Experimental colonisation was not affected by prior receipt of PPV23: 36.4% (n = 8/22) of PPV23 recipients developed colonisation versus 40.5% (n = 17/42) of non-recipients. This remained true if the analysis was restricted to over-65s—36.4% (n = 8/22) in vaccine recipients versus 37.5% (n = 3/8). It also remained true when restricting analysis to over-65s who had received PPV23 within the preceding five years—46.2% (n = 6/13) in recent vaccine recipients versus 29.4% (n = 5/17).

Experimental colonisation in prior natural carriers

As shown in Figure 2 of the main paper, three participants were naturally colonised with non-6B pneumococci prior to experimental challenge. The challenge strain displaced the community strain in two cases: one participant remained co-colonised with serogroup 23 at day 2 and then carried serotype 6B alone on days 7, 9 and 14, while another was co-colonised with serogroup 15 on day 2 and then carried serotype 6B alone through to day 29. The third participant was colonised with serotype 3 at screen and every subsequent visit, and never had co-colonisation with serotype 6B detected.

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Regression analysis—experimental colonisation

Table E4: Predictors of experimental colonisation, using univariate logistic regression

	Odds ratio	**95% CI**	**p value (Wald) of predictor**
Age (years)	0.97	0.90—1.03	0.31
Male sex	0.59	0.21—1.66	0.32
PPV23 receipt	0.84	0.29—2.43	0.75
Ex-smoker	0.56	0.19—1.65	0.29
Statin therapy	0.75	0.17—3.32	0.71
Baseline antibody levels (log ng/mL)	0.924	0.26—3.28	0.90

Since none of our predictor variables approached a statistically significant association with the outcome, we did not progress to multivariate regression.

Immunological results

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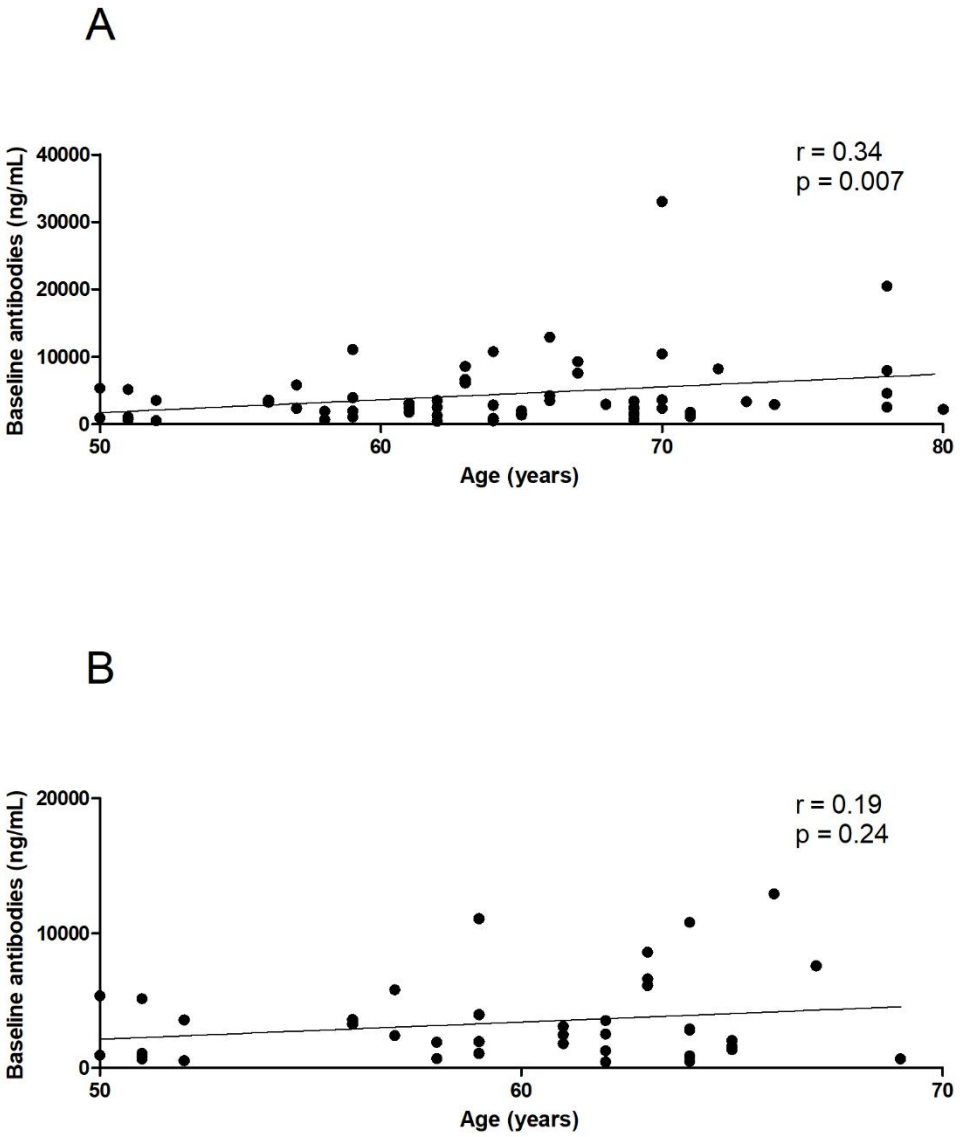

Figure E3: correlations between baseline anti-6B IgG and age in all participants (A) and in participants who had never received the 23-valent pneumococcal vaccine (B). Note the differing scales on the Y axes. Statistical significance was assessed using Pearson's correlation.

Table E5: Immunological outcomes following pneumococcal challenge.

	Anti-6B CPS IgG geometric mean µg/mL, (95% CI)		p value
	Baseline	**Day 29**	
Colonised (n = 24)	2.71 (1.91—3.84)	3.0 (1.93—4.66)	0.53
Non-colonised (n = 38)	2.79 (2.0—3.90)	2.17 (1.57—3.0)	**0.006**

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Sensitivity analyses: antibody levels in subgroups

Table E6: Immunological outcomes following pneumococcal challenge, PPV23 recipients only

	Anti-6B CPS IgG geometric mean µg/mL, (95% CI)		p value
	Baseline	**Day 29**	
Colonised (n = 8)	3.10 (2.0—4.81)	3.56 (1.83—6.94)	0.83
Non-colonised (n = 13)	5.0 (2.79—8.96)	3.29(1.53—7.07)	**0.02**

The difference in baseline antibody levels between colonised-and non-colonised participants was not statistically significant (p=0.159)

Table E7: Immunological outcomes following pneumococcal challenge, excluding PPV23 recipients

	Anti-6B CPS IgG geometric mean µg/mL, (95% CI)		p value
	Baseline	**Day 29**	
Colonised (n = 16)	2.53 (1.53—4.20)	2.87 (1.57—5.23)	0.08
Non-colonised (n = 25)	2.06 (1.41—3.01)	1.68 (1.19—2.36)	0.55

The difference in baseline antibody levels between colonised-and non-colonised participants was not statistically significant (p=0.49)

Table E8: Immunological outcomes following pneumococcal challenge, by age decile

		Anti-6B CPS IgG geometric mean µg/mL, (95% CI)		p value
		Baseline	**Day 29**	
Age 50—59	Colonised (n = 9)	2.57 (1.46—4.53)	2.82 (1.23—6.46)	0.75
	Non-colonised (n = 10)	1.59 (0.79—3.23)	1.38 (0.71—2.68)	0.43
Age 60—69	Colonised (n = 12)	2.56 (1.41—4.63)	3.0 (1.44—6.25)	0.51
	Non-colonised (n = 17)	2.74 (1.75—4.28)	2.11 (1.43—3.11)	0.07
Age 70—80	Colonised (n = 3)	4.03 (0.63—26.31)	3.61 (0.66—19.75)	0.70
	Non-colonised (n = 11)	4.80 (2.39—9.63)	3.43 (1.53—7.68)	**0.047**

Antibody responses and colonisation density

AUC of density did not correlate significantly with baseline (r = -0.207, p = 0.33) or day 29 antibody levels (r = -0.89, p = 0.68) or with fold change in antibody levels (r = 0.389, p = 0.06).

Regression analysis: AUC density versus antibody levels

Table E9: Predictors of experimental colonisation density (as defined by AUC), using univariate linear regression

	Unstandardised			**Standardised β**	**p value**
	B	**Standard error**	**95% CI**		
Age (years)	-0.44	0.92	-2.33 — 1.45	-0.1	0.64
Male sex	14.82	14.66	-16.0 — 44.64	0.2	0.34

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Baseline antibody levels (log ng/mL)	-25.6	20.5	-68.2 — 17.03	-0.26	0.23

B denotes the unstandardised regression coefficient, and β the standardised coefficient.

Table E10: Predictors of experimental colonisation density (as defined by AUC), using multivariate linear regression, full model

	Unstandardised			Standardised β	p value
	B	Standard error	95% CI		
Age (years)	-0.46	0.94	-2.41 — 1.49	-0.10	0.63
Male sex	17.30	15.21	-14.43 – 49.02	0.24	0.27
Baseline antibody levels (log ng/mL)	26.64	21.04	-68.52 — 19.23	-0.25	0.26
(Constant)	*140.28*	*86.2*	*39.55 — 320.12*	-	*0.119*

B denotes the unstandardised regression coefficient, and β the standardised coefficient.

Antiprotein antibodies

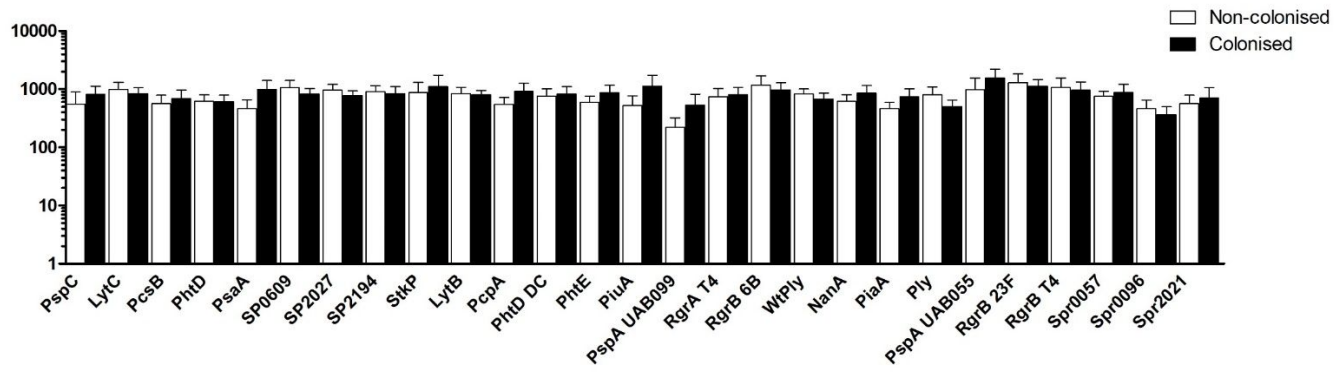

Figure E4: Pre-challenge levels of anti-protein antibodies, as measured by multiplex electrochemiluminescence (Meso-Scale Discovery), in non-colonised (white bars) and colonised (black bars) participants. Data are presented as geometric means; error bars represent 95% CI. There were no differences in baseline levels of any anti-protein antibody between colonised and non-colonised participants when compared using the unpaired t-test without correction for multiple comparisons.

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Mucosal antibodies

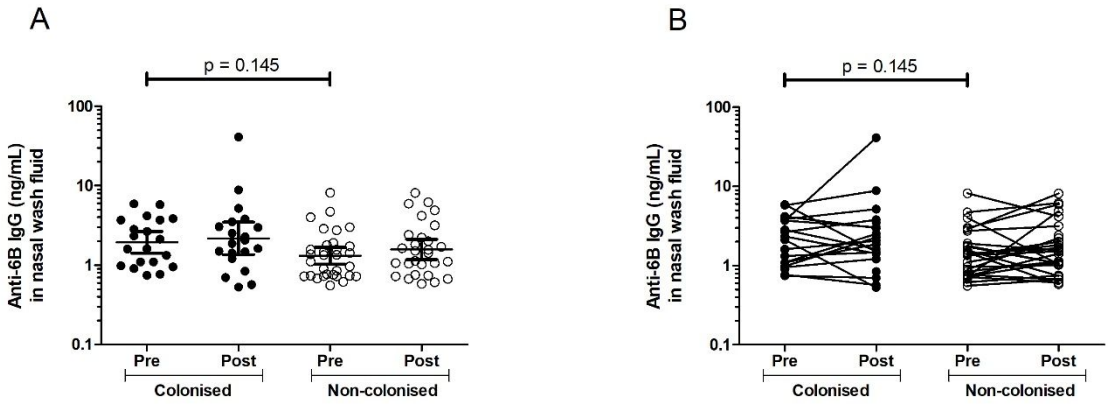

Figure E5: Anti-6B CPS IgG levels in nasal washes at baseline and day 29 following inoculation, in colonised and non-colonised participants. Each symbol represents a single participant. The lines and error bars in (A) represent geometric mean (95% CI); the lines in (B) connect the baseline and day 29 values for each participant. There were no statistically significant differences in pre- and post-challenge antibody levels for colonised or non-colonised participants. Within the subset who returned for rechallenge, the pattern was similar.

Serum levels in the re-challenge phase

Table E11: Serum antibody levels

	Anti-6B CPS IgG geometric mean µg/mL, (95% CI)				p value (for re-challenge)
	Primary challenge		**Rechallenge**		
	Baseline	**Day 29**	**Baseline**	**Day 14**	
Colonised post-rechallenge (n = 5)	2.57 (1.61—4.12)	3.96 (1.17—13.39)	3.72 (1.18—11.76)	3.56 (1.07—11.84)	0.47
Non-colonised (n = 11)	1.74 (1.23—2.46)	1.73 (1.16—2.59)	1.66 (1.17—2.36)	1.53 (1.08—2.17)	0.04

Participants requiring additional clinical reviews during the study

1. One non-colonised participant (age 62) developed sciatica 10 days after inoculation and was advised to take over-the-counter analgesia.

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2. One non-colonised participant (age 58) developed upper respiratory symptoms 20 days after inoculation; clinical examination was normal and a pharyngeal swab was negative for respiratory viruses; the symptoms resolved spontaneously.
3. One colonised participant (age 64) complained of chest discomfort on day 7; ECG and clinical assessment were normal, and a working diagnosis of muscular pain was made; the symptoms resolved spontaneously.
4. One colonised participant (age 64) developed an earache on day 14; otoscopy was normal, and the symptoms gradually resolved spontaneously.
5. One non-colonised participant (aged 65) developed a pruritic abdominal rash on day 7. The rash responded to empirical treatment for tinea corporis with topical miconazole/hydrocortisone.
6. One colonised participant (aged 71) complained of myalgia on day 7 and of an itchy eye on day 22; on both occasions, nothing was identified on clinical examination and the symptoms resolved spontaneously.
7. One non-colonised participant (age 71) attended his primary care physician with a sore throat 20 days after inoculation, and was given an analgesic spray which improved his symptoms.

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