Pneumococcal nasopharyngeal colonisation in adults

Thesis submitted in accordance with the requirements of the Liverpool School of Tropical Medicine for the degree of Doctor of Philosophy by Hugh Adler

April 2019

Table of contents

Declaration

This thesis is the result of my own work and effort. I conceived and developed this project, writing the protocol and documents for ethical submission and presenting the project to the ethics committee meeting. I also obtained co-sponsorship from the Liverpool School of Tropical Medicine and the Royal Liverpool and Broadgreen University Hospitals NHS Trust, and support from the local Clinical Research Network. I coordinated participant recruitment and consent, clinical sampling, safety oversight and laboratory analyses. I was assisted in these latter activities by a team of clinicians, research nurses and laboratory staff based in the two co-sponsoring organisations, all of whom are listed in the Acknowledgments. I performed all polysaccharide ELISAs and antimicrobial susceptibility tests myself, and am responsible for all aspects of data synthesis, analysis and interpretation of the results. For ease of reading, the results are written in the first-person plural to acknowledge the extensive teamwork involved in delivering a clinical research project of this scale.

All data reported in this thesis have been archived in the Liverpool School of Tropical Medicine. Other researchers wishing to access the primary data may discuss such requests with Professor Daniela Ferreira at LSTM.

The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or qualification.

Acknowledgements

The human infection challenge research described in this thesis (Chapters 3—5) would never have been completed without the assistance of my wonderful colleagues in the experimental human pneumococcal colonisation (EHPC) team. I will remain forever grateful to the clinical team: Lisa Cheng, Andrea Collins, Victoria Connor, Caz Hales, Helen Hill, Angela Hyder-Wright, Lepa Lazarova, Cath Lowe, Katy Piddock, Rachel Robinson, Emma Smith, India Wheeler and Seher Zaidi, as well as the laboratory team: Beatriz Carniel, Esther German, Simon Jochems, Elena Mitsi, Elissavet Nikolaou, Sherin Pojar, Jesús Reiné and Carla Solórzano-Gonzalez, for all their expertise and good-natured support during my three years at LSTM. Elena, Elissavet, Esther and Sherin were responsible for experimental inoculum preparation and culture-based colonisation determination (Chapter 3). Special thanks to Esther and Elena for their forbearance and guidance when I was learning how to do polysaccharide ELISA (Chapter 4). Even more special thanks to Esther for the Sisyphean task of completing all the qPCR analyses (Chapter 5). Funding for EHPC programme was obtained from the Medical Research Council and the Bill & Melinda Gates Foundation by Stephen Gordon and Daniela Ferreira. Daniela Ferreira was also responsible for oversight of the project alongside Jamie Rylance (Chief Investigator) and Andrea Collins (Principal Investigator).

The community-acquired colonisation project (Chapter 6) relied on data collected by Jenna Gritzfeld and all of the aforementioned during the first seven years of the EHPC programme. I obtained financial support for this project from the Jean Clayton Fund at the Liverpool School of Tropical Medicine. I am very grateful for the technical assistance of Jason Hinds and Kate Gould at St George's University of London, who performed the molecular serotyping analysis, and to Nick Feasey and Neil French for advice on antibiotic selection. This project would never have been completed without the supremely patient help of Elissavet Nikolaou or the expert input of Adam Roberts.

All of the research reported in this thesis relied on samples donated from healthy volunteers, and words cannot express my gratitude to all of the participants in EHPC trials over the years. In

particular, I will be forever grateful to the volunteers in the “Aging and Immunity” study for their generosity and good will.

I am also particularly grateful to Catherine Molloy and Kelly Convey for all their administrative support, the data safety and monitoring committee (Rob Read and Brian Faragher) and the on-call physician team (Stephen Aston, John Blakey, Hassan Burhan, Neil French, Gareth Jones, Will Kent and Ben Morton). Further thanks to the staff of the Royal Liverpool University Hospital Clinical Research Unit (Richard Fitzgerald, Jen Gibney, Zalina Rashid-Gardner, Stephen Chand, James Morrison and Jack Sanders) as well as the research governance staff of both the Liverpool School of Tropical Medicine (Carl Henry and Brian Slater) and the Royal Liverpool University Hospital (Heather Rogers and Sue Charnock). Many organisations around Merseyside provided valuable assistance with participant recruitment, in particular the Liverpool Central Library and the Claughton Medical Centre in Birkenhead.

While writing this thesis, I benefited from regular reviews by an independent Progress Assessment Panel—Paul McNamara and Britta Urban—whose insight and recommendations greatly improved this final product.

I was lucky to have a trio of engaged and encouraging supervisors during my journey from novice researcher to slightly-more-experienced researcher. Stephen Gordon—thank you for encouraging me to apply for this post in the first place and for all your advice along the way; Daniela Ferreira—you are a great leader and have taught me so much during my time in LSTM; and Jamie Rylance—thank you always having an answer for every question and for exponentially improving my writing skills. (And for all the good jokes.)

Finally, thank you to my parents for always believing in me and for raising me as an insatiable reader, a trait that stood me in good stead during these last three years. And my most profound thanks of all to my wife Rachel, my reason for moving to Liverpool, for your love and support…and for always reminding me that there’s more to life than research.

Glossary

AMR ..... Antimicrobial resistance

ANOVA ..... Analysis of variance

AUC ..... Area under the curve

BTS ..... British Thoracic Society

CAP ..... Community-acquired pneumonia

CAPiTA ..... Community-Acquired Pneumonia Immunization Trial in Adults

CFU ..... Colony-forming unit

CHIM ..... Controlled human infection model

CI ..... Confidence interval

CpG-ODN ..... CpG oligodeoxynucleotides

CPS ..... Capsular polysaccharide

cpsA ..... Capsular polysaccharide gene

CRN ..... Clinical Research Network

CRU ..... Clinical Research Unit

CSF ..... Cerebrospinal fluid

CWPS ..... Cell wall polysaccharide

DMSC ..... Data Monitoring and Safety Committee

ECG ..... Electrocardiogram

ELISA ..... Enzyme-linked immunosorbent assay

EHPC ..... Experimental human pneumococcal colonisation

EUCAST ..... European Committee on Antimicrobial Susceptibility Testing

FDA ..... US Food and Drug Administration

GCP ..... Good Clinical Practice

GP ..... General practitioner

HIV ..... Human immunodeficiency virus

IPD ..... Invasive pneumococcal disease

IQR ..... Interquartile range

IRAS ..... Integrated Research Application System

ISRCTN ..... International Standard Randomised Controlled Trials Number

LSTM ..... Liverpool School of Tropical Medicine

lytA ..... Autolysin gene

MDR ..... Multi-drug resistant

MH-F ..... Mueller-Hinton media for fastidious organisms

MIC ..... Minimum inhibitory concentration

NALT ..... Nasopharyngeal-associated lymphoid tissue

NHS ..... National Health Service

NVT ..... Non-vaccine type

NW ..... Nasal wash

OD ..... Optical density

OPS ..... Oropharyngeal swab

PBS ..... Phosphate-buffered saline

PCV ..... Pneumococcal conjugate vaccine

PCV7 ..... 7-valent pneumococcal conjugate vaccine

PCV13 ..... 13-valent pneumococcal conjugate vaccine

PPV ..... Pneumococcal polysaccharide vaccine

PPV23 ..... 23-valent pneumococcal polysaccharide vaccine

PspA ..... Pneumococcal surface protein A

qPCR ..... Quantitative polymerase chain reaction

RCT ..... Randomised controlled trial

REC ..... Research Ethics Committee

RD&I ..... Research Development and Innovation

RLBUHT ..... Royal Liverpool and Broadgreen University Hospitals Trust

RLUH ..... Royal Liverpool University Hospital

SAE ..... Serious adverse event

SD ..... Standard deviation

STGG ..... Skim milk, tryptone, glucose and glycerin

TMP-SMX ..... Trimethoprim-sulfamethoxazole

VT ..... Vaccine type

WHO ..... World Health Organisation

List of tables

List of figures

Publications and presentations

Publications arising from work within thesis

1. **Hugh Adler,** Daniela M Ferreira, Stephen B Gordon, Jamie Rylance. Pneumococcal capsular polysaccharide immunity in the elderly. *Clinical and Vaccine Immunology* 2017 24;6: e00004-17.
2. **Hugh Adler**, Elissavet Nikolaou, Katherine Gould, Jason Hinds, Andrea M Collins, Victoria Connor, Caz Hales, Helen Hill, Angela D Hyder-Wright, Seher R Zaidi, Esther L German, Jenna F Gritzfeld, Elena Mitsi, Sherin Pojar, Stephen B Gordon, Adam P Roberts, Jamie Rylance, Daniela M Ferreira. Pneumococcal colonization in healthy adult research participants in the conjugate vaccine era, United Kingdom, 2010—2017. *The Journal of Infectious Diseases* 2019 (in press, doi: 10.1093/infdis/jiz034)

Presentations arising from work within thesis

Abstracts based on the "Aging and Immunity" study at various stages of completion have been presented at:

1. The 10th International Symposium on Pneumococci and Pneumococcal Diseases, Glasgow, 2016 (Abstract ISPPD-0173);
2. The British Infection Association Spring Meeting, London, 2017 (Abstract 0010);
3. "Shots of Science", part of the Pint of Science Festival, Liverpool, 2017;
4. Experimental Human Pneumococcal Carriage Mid-Term Programme Grant Review Meeting, Wirral, 2018;
5. Liverpool School of Tropical Medicine Annual Postgraduate Researcher Conference, Liverpool, 2018 (Winner of poster prize in "Clinical Sciences" category).

Publications arising during course of PhD

1. Simon P Jochems, Fernando Marcon, Beatriz F Carniel, Mark Holloway, Elena Mitsi, Emma Smith, Jenna F Gritzfeld, Carla Solórzano, Jesús Reiné, Sherin Pojar, Elissavet Nikolaou, Esther L German, Angela Hyder-Wright, Helen Hill, Caz Hales, Wouter AA de Steenhuijsen Piters, Debby Bogaert, **Hugh Adler**, Seher Zaidi, Victoria Connor, Stephen B Gordon, Jamie Rylance, Helder Nakaya, Daniela M Ferreira. Inflammation induced by influenza virus impairs human innate immune control of pneumococcus. *Nature Immunology*. 2018 (in press).
2. Victoria Connor, Esther German, Sherin Pojar, Elena Mitsi, Caz Hales, Elissavet Nikolaou, Angela Hyder-Wright, **Hugh Adler**, Seher Zaidi, Helen Hill, Simon P Jochems, Hassan Burhan, Neil French, Tim Tobery, Jamie Rylance, Daniela M. Ferreira. Hands are Vehicles for Transmission of *Streptococcus pneumoniae* in Novel Controlled Human Infection Study. *European Respiratory Journal*. 2018 (in press).
3. **Hugh Adler**, Moherndran Archary, Prasha Mahabeer, Philip LaRussa, Razia A Bobat. Tuberculosis in HIV-infected South African Children with Severe Acute Malnutrition. *International Journal of Tuberculosis and Lung Disease* 2017 Apr 1;21(4):438-445.
4. Simon P Jochems, Katherine Piddock, Jamie Rylance, **Hugh Adler**, Beatriz Carniel, Andrea Collins, Jemma F Gritzfeld, Carole Hancock, Helen Hill, Jesús Reiné, Alex Seddon, Carla Solórzano-Gonsalez, Syba Sunny, Ashleigh Trimble, Angela D Wright, Seher Zaidi, Stephen B Gordon, Daniela M Ferreira. Novel Analysis of Immune Cells from Nasal Microbiopsy Demonstrates Reliable, Reproducible Data for Immune Populations, and Superior Cytokine Detection Compared to Nasal Wash. *PLoS One*. 2017 Jan 20;12(1):e0169805.

Pneumococcal nasopharyngeal colonisation in adults

The “p” is silent.

Wodehouse: “Leave it to Psmith!” (1923)

Abstract

Background: Pneumococcal colonisation, although usually asymptomatic, is key to the pathogenesis of invasive disease. Colonisation is infrequently detected in older adults, despite their high rates of pneumococcal disease; this may relate to sampling from the wrong site or reliance on culture-based rather than molecular diagnostic methods. Anti-pneumococcal immunity (including responses to vaccination) declines with age, but the immunogenicity of colonisation in older adults has not been studied. Alongside innate immunity, three specific defence mechanisms exist against pneumococcal disease: herd immunity, adaptive immunity (conferred by natural or vaccine-induced antibodies) and antibiotics. Current pneumococcal vaccines confer incomplete protection against pneumococcal disease in older adults.

Research Questions:

1. Can experimental human pneumococcal colonisation be safely established in older adults?
2. What is the rate of experimental colonisation in older adults?
3. Do anti-pneumococcal antibodies (natural or post-vaccination) prevent experimental colonisation in older adults?
4. Is experimental colonisation immunogenic in older adults?
5. What is the optimal sampling site and diagnostic methodology for detecting colonisation in older adults?
6. What do colonisation studies of the general adult population reveal about herd immunity and pneumococcal antimicrobial resistance in the community?

Findings: Experimental colonisation was established in 39% of volunteers (n = 25/64) with no adverse events. Experimental colonisation was unaffected by previous pneumococcal polysaccharide vaccination or baseline anti-capsular antibody levels. Although the rate of experimental colonisation was similar to that of younger volunteers in previous studies, the immune responses were markedly different: older adults did not demonstrate serotype-specific immune boosting following pneumococcal colonisation. Furthermore, pneumococcal challenge without colonisation led to a drop in specific antibody levels. Nasal wash culture appeared to be the optimal detection strategy compared with molecular testing of nasal wash, oropharyngeal swab or saliva.
In a review of all prospective experimental human pneumococcal colonisation volunteers between 2010 and 2017, community-acquired colonisation was identified at baseline in 6.5% (n = 52/795) using nasal wash culture. The commonest serotype was 3 (a vaccine serotype), but otherwise non-vaccine serotypes predominated. There were no changes in serotype distribution over time. 15% of isolates (n = 8/52, all identified in 2015 or later) were resistant to at least one of the antibiotics tested.
Conclusions: Experimental pneumococcal colonisation is feasible and safe in older people. However, the immunological effects are different to those identified in younger adults in previous studies. We found no evidence that the niche of colonisation changes with age. Nasal wash culture detected higher-than-expected rates of community-acquired colonisation in young adults, with the dominance of non-vaccine serotypes suggesting that the limits of herd immunity have been reached. New pneumococcal vaccines are needed for older adults, and the experimental colonisation model could be used in early phase testing of candidate vaccines. If such vaccines continue to rely on serotype-specific protection, then community colonisation studies could inform the serotype composition.

1 Introduction

Streptococcus pneumoniae (the pneumococcus), a gram-positive, encapsulated, aerobic diplococcal bacterium, is responsible for high levels of morbidity and mortality across the globe. Throughout history, humans have suffered from pneumococcal disease and the pneumococcus has evolved in parallel with us (1). Over 90 different pneumococcal serotypes, defined by capsular polysaccharide (CPS) have been reported, and these vary substantially in their propensities to cause colonisation and/or disease (2, 3). Serotype distributions vary substantially across populations, age-groups and time periods (4, 5). This Introduction comprises an overview of pneumococcal disease and colonisation, followed by our three main defences against pneumococcal disease: natural immunity, vaccination and antibiotics.

1.1 The burden of pneumococcal disease in adults

1.1.1 Pneumococcal pneumonia

Community-acquired pneumonia (CAP) is the sixth biggest cause of death in the UK; it kills 29,000 people and leads to over 200,000 hospitalizations and 2.3 million bed days each year (6). The pneumococcus is the single commonest identifiable bacterial cause of CAP, accounting for 40% of cases in the UK (7). In resource-limited settings, the burden of pneumococcal CAP is even higher (8, 9). Pneumococcal infections are particularly problematic for people aged over 65, who have up to a five-fold increase in the incidence and mortality of pneumococcal CAP relative to those aged under 65 (10, 11). In the United States, an estimated 600,000 episodes of pneumococcal CAP occur annually, with a total cost to society of US$4.85bn (12); hospitalizations for pneumococcal CAP are predicted to increase by nearly 100% by the year 2040, with 87% of this increase accounted for by the older people (13).

1.1.2 Pneumococcal meningitis

Pneumococcal meningitis frequently results in death or long-term sequelae, with mortality rates exceeding 30% in some settings (14). Although pneumococcal meningitis is a global disease affecting all age-groups, it has substantially higher mortality in older people (15). In resource-rich settings, the pneumococcus has become the commonest cause of adult bacterial meningitis (particularly in older people), following the reduction in meningococcal disease due to childhood vaccination campaigns (16, 17). In the African meningitis belt, outbreaks of pneumococcal meningitis occur in parallel with seasonal outbreaks of meningococcal disease in teenagers and young adults (18). With the successful roll-out of effective serogroup A meningococcal vaccines, there are concerns that the pneumococcus will become the predominant causative organism of meningitis in Africa as well (19).

1.1.3 Pneumococcal bacteraemia

Pneumococcal bacteraemia is associated with substantial mortality whether in isolation or when associated with confirmed organ infection, and is associated with increased incidence and mortality in older people (20, 21).

1.2 The importance of pneumococcal colonisation

The link between pneumococcal nasopharyngeal colonisation (or carriage) and the subsequent development of all forms of pneumococcal disease is generally accepted. This is supported by both experimental data (murine models of meningitis) and epidemiologic studies (children with otitis media and adults with pneumonia) (22-24). Children aged under two years have high rates (over 60%) of nasopharyngeal pneumococcal colonisation (25, 26). Up to 15% of colonisation episodes progress to clinical disease (particularly otitis media) before an immune response can clear the pathogen, which could be explained by the lack of a robust anti-polysaccharide immune response in young children (23, 27, 28).

Colonisation rates fall with increasing age, particularly in well-resourced settings, with rates in young adults generally <10% (29, 30).

Colonised children are a reservoir of pneumococci, which can then spread to other children and adults in their household (31, 32). This mechanism has been postulated as the main source of pneumococcal disease in communities; hence, childhood vaccination programmes that reduce colonisation are said to induce "herd immunity" (33). For these reasons, epidemiologic and vaccine impact studies focus on children and their families rather than a random population sample (32, 34). However, adults also contribute to pneumococcal transmission (31), and excluding the general adult population from colonisation studies can miss important trends and reservoirs of colonisation (35).

1.2.1 The definition and determination of colonisation

A large pan-European study recently reported a pneumococcal carriage prevalence of 1.9% in individuals aged >10 years, with a prevalence of 1.2% in UK adults (30). However, this study utilized nasal rather than naso*pharyngeal* swabs, in order to improve participant uptake. Deep nasopharyngeal swabs are recommended to identify pneumococcal colonisation (36). In adults, the addition of oropharyngeal swabs may increase diagnostic sensitivity—a World Health Organisation (WHO) working group concluded that pairing oral swabs with nasopharyngeal samples was desirable but not mandatory (36). Nasal wash (instillation and retrieval of sterile saline via the nasal cavity) is another acceptable method for pneumococcal detection, with equivalent or superior sensitivity to nasopharyngeal swabs (36, 37). There is no consensus regarding the relevance of samples from other niches and the diagnostic methods applied to such samples.

1.2.2 Pneumococcal colonisation in older adults: a controversial topic

Table 1.1 lists examples of studies that attempted to define the rate of pneumococcal colonisation in older adults (defined as either >60 or >65 years in different studies) . Much

of the variation between these studies can be explained by the different sampling sites—nasopharyngeal, oropharyngeal or saliva—and detection methods—classical culture, quantitative polymerase chain reaction (qPCR) or some combination of the two.

Table 1.1: Examples of studies attempting to define the rate of pneumococcal colonization in older people

Reference	Year	Country	Number sampled	Age (years)	Site	Analysis	Rate of detection of pneumococci, n (%)
(32)	2015	UK	599	NR	NP	Classical microbiology	13 **(2.2%)**
(38)	2015	USA	210	81.4 (6.3)*	NP	Classical microbiology	**4 (1.9%)**
(39)	2014	Portugal	3,361	74.56 (8.2)*	NP	Classical microbiology with qPCR confirmation of positive specimens	61 (1.8%)
					OP		15 (0.4%)
					Overall		**76 (2.3%)**
(40)	2012	Belgium	503	80.3 (10.0)*	NP	Classical microbiology	**21 (4.2%)**
(41)	2016	Italy	417	73.97 (6.66)*	OP	qPCR	**41 (9.8%)**
(42)	2013	Italy	283	NR	NP	Culture-enriched qPCR	**53 (18.7%)**
(43)	2016	Nether-lands	330	72.7 (68.7—79.0)†	NP	Classical microbiology	16 (5%)
						qPCR	32 (10%)
					OP	Classical microbiology	16 (5%)
						qPCR	58 (18%)
					Overall		**75 (23%)**
(44)	2015	Nether-lands	270**	69 (NR)*	NP	Culture-enriched qPCR	13 (5%)
					OP		31 (11%)
					Saliva		76 (28%)
					Overall		**91 (34%)**††

All studies enrolled community-dwelling adults
NP: Nasopharyngeal; NR: Not reported; OP: Oropharyngeal; qPCR: quantitative polymerase chain reaction
** Mean (SD)*
† Median (IQR)
***135 subjects, sampled both pre and post influenza-like illness. At a participant level, 65/135 (48%) tested positive on at least one occasion.*
††Subjects could test positive by one or both methods, hence "overall" does not equal the sum total of the separate methods

Our understanding of pneumococcal colonisation, disease susceptibility and natural immunity in children, young adults and murine models derives from traditional bacterial culture methods in nasopharyngeal specimens (23, 45). For example, salivary qPCR in children can suggest rates of colonisation approaching 100% (46), but this has yet to be correlated with immunological endpoints (such as the generation or boosting of anti-capsular antibodies), incidence of clinical disease or protection against future acquisition.

While studies of nasopharyngeal swab cultures from older adults have shown lower rates of colonisation than in children (1.8—4.2%) (38-40), the addition of oral swabs and the combination of traditional culture and qPCR can estimate rates of colonisation (if defined as ≥1 sample from any site testing positive by any method) to as high as 23% in an older population (43), or 34% if saliva is also sampled (44). False positive qPCR results from other oral streptococci are also a concern, although steps have been taken to increase the test specificity in recent studies, for example by performing qPCR against two rather than one pneumococcal gene target (47).

Thus, although classical microbiological analysis on nasopharyngeal samples from older adults does not have as high a yield as molecular analysis of oral or salivary specimens, it has the advantage of allowing a more direct comparison with previous studies. The choice of diagnostic sampling method must always be guided by the research question. One cannot simply state that qPCR is “more sensitive” than culture, as the clinicopathological significance of qPCR-positive, culture-negative colonisation may not be equivalent to that of culture-positive colonisation. For example, a qPCR-positive, culture-negative result might imply low-density pneumococcal colonisation (48), which could have lower potential to cause disease or spread to other hosts (24). Similarly, the detection of pneumococcal DNA in the oropharynx may not represent the presence of viable pneumococci.

Most importantly, high nasopharyngeal colonisation rates in older people (23%, as defined by classical culture) have been demonstrated during an outbreak in a nursing home (49), suggesting that culture-positive nasopharyngeal colonisation may be a clinically relevant measurement in this population.

In this thesis, for the reasons outlined above and to be consistent when comparing studies of children, adults, older adults and mice, we define colonisation as the isolation of pneumococci from the nasopharynx by culture-based methods unless otherwise stated.

1.2.3 Risk factors for pneumococcal colonisation

Studies in industrialised countries have consistently identified regular contact with children aged <5 years as the biggest risk factor for pneumococcal colonisation (30, 32). Other factors, (including sex, crowding, environment and cigarette smoking) have been inconsistently associated with colonisation in different studies (4, 50-52). Similarly, reports differ regarding whether antibiotic therapy in the weeks preceding sampling is associated with reduced risk of pneumococcal colonisation (50, 53), although recent antibiotics have been associated with increased risk of colonisation by antibiotic-resistant pneumococci in children (54-56).

1.3 The relationship between colonisation and immunity

Pneumococcal colonisation may be a necessary evil: exposure to pneumococcal antigens via repeated episodes of colonisation is key to acquiring and sustaining anti-pneumococcal immunity.

Throughout childhood, adolescence and early adulthood, immunity against pneumococcus improves with age. Colonisation rates fall with increasing age, along with a corresponding reduction in pneumococcal disease (25). It seems that repeated colonisation episodes lead to the development of protective immunity against the most prevalent circulating pneumococcal serotypes (anti-CPS antibodies are specific to a given serotype) (57).

Following the maturation of the immune system and multiple episodes of colonisation, young adults have well-functioning immune systems and established serotype-specific immunologic memory (29).

Naturally-acquired immunity is multifactorial: non-serotype-specific anti-pneumococcal immunity develops alongside serotype-specific immunity in children, through mechanisms that have not been entirely elucidated (58). In young infants with immature anti-CPS responses, epidemiological studies have suggested that non-serotype-specific immunity predominates, while serotype-specific immunity comes to the fore in older children (59) (57). In adulthood, both epidemiologic and controlled human infection studies have suggested that serotype-specific immunity plays a major role in controlling colonisation (29, 60). We hypothesize that anti-pneumococcal immunity in older adults is more akin to that of young adults than to that of infants.

Young adults experience very low morbidity and mortality from pneumococcal disease (e.g. 3.1 cases annually per 100,000 population, versus 38.6 cases per 100,000 population in children aged under one year) (15), and their serotype-specific immunity is boosted by occasional episodes of asymptomatic colonisation (29, 31, 60). However, in old age, a paradox emerges: while nasopharyngeal colonisation is less common in older adults (Table 1.1), they are at extremely high risk of pneumococcal disease.

One hypothesis suggests that the same mechanism (immunosenescence) determines increasing disease susceptibility with reduced colonisation: increased circulating levels of pro-inflammatory cytokines ("inflammaging") could lead to clearance of colonisation before a natural boosting of pre-existing immunity could take place (61-63). Equally but oppositely, inflammaging-induced chronic activation of inflammatory pathways can result in impaired upregulation of inflammation in response to pneumococcal challenge: a murine study found that baseline levels of upper respiratory tract pro-inflammatory cytokines (e.g.

toll-like receptor 1 and interleukin 1β) were higher in older than younger mice, but that pneumococcal colonisation resulted in increased levels of these cytokines in younger but not older mice [Krone 2013].

An alternative line of enquiry explores that hypothesis that colonisation is under-detected in older people (e.g. due to poor choice of sampling site and/or method) and that it is a precursor to disease, which cannot be prevented by the senescent immune system. Mucosal immunity may be more durable than systemic humoral immunity (to be discussed in detail later)—this could explain a protection against colonisation but susceptibility to invasive disease. Regardless, older adults are clearly at high risk of pneumococcal disease, and therefore their natural anti-pneumococcal immunity must differ from that of younger adults. Declines in both innate and adaptive immunity combined with increased rates of comorbidities all contribute to this (64), but we will focus here on antibody-mediated immunity.

1.3.1 Naturally-acquired pneumococcal CPS antibodies

Natural immunity arises following episodic colonisation. Colonisation leads to increased serum levels of anti-pneumococcal antibodies, which are detectable in all adults (65, 66). In this section we will discuss their role in the control of pneumococcal disease. Anti-CPS antibodies are the most widely-studied antibodies and are the direct effectors of vaccine-induced protection, and therefore we focus on these. The importance of anti-pneumococcal antibodies is further supported by the first effective treatment for pneumococcal disease: passive immunotherapy, i.e. the transfer of specific immune serum from naturally-immune donors or immunized animals to patients with pneumococcal pneumonia (67).

In addition to antibodies generated by natural colonisation, others have reported on naturally-arising polyvalent antibodies (often IgM) with potent anti-pneumococcal activity

(68)—whether these antibodies are analogous to those that arise following colonisation is unclear. Furthermore, it is possible that these antibodies undergo refinement and increased specification over time, stimulated by antigen presentation (69). In this thesis we define naturally-acquired antibodies as those that arise following pneumococcal exposure.

Anti-CPS antibodies bind to the pneumococcal capsule and opsonise the bacteria, improving phagocytosis and downstream killing. In addition, antibodies can promote an innate immune response by activating the classical complement pathway; in murine models this appears to be the dominant complement pathway in anti-pneumococcal immunity and is mediated via natural IgM rather than IgG (70).

1.3.2 Protective antibodies are a product of nasopharyngeal colonisation

Anti-CPS antibodies are particularly effective in control of bloodstream infections: passive transfer of human antibodies (generated following experimentally-induced colonisation) was protective in a murine model of lethal bacteraemia (60). Passive transfer of pre-colonisation serum from the same human volunteers conferred a lesser survival benefit. In a separate murine lethal challenge model, CD4-deficient knockout mice were able to mount a protective antibody response following experimental colonisation and survive subsequent bacteraemic challenge, whereas antibody-deficient knockout mice had no survival benefit from prior colonisation (71). Experimental colonisation of mice also generated a protective response against subsequent pneumonia (45). However, this experiment found that all arms of the innate and adaptive immune systems were required for protection: depletion of any of B-cells, neutrophils or CD4 T-cells eliminated the protective response. This suggests that the control of mucosal disease is more complex than the control of bloodstream disease. Thus, based on the evidence accumulated from a combination of murine and human challenge models, antibodies induced by pneumococcal

colonisation have been shown to confer protection against bacteraemia and contribute to protection against pneumonia.

1.3.3 Clearance of colonisation is a multifaceted process

Antibodies have an important role in the protection against becoming colonised. In mice, passive transfer of antibodies led to agglutination of bacteria following intranasal challenge, which caused the bacteria to clump and become more vulnerable to mucociliary clearance (72). Pneumococcal antibody-mediated agglutination has also been demonstrated in humans following vaccination with pneumococcal conjugate vaccine (PCV) (73). In this study, naturally-acquired antibodies were present in the nasopharynx prior to vaccination, but not in sufficient levels to induce agglutination. Murine studies have suggested that the clearance of established colonisation is primarily mediated by CD4 T-cells and interleukin 17 (IL-17), with a possible contribution from anti-protein antibodies (74-76). Thus, it appears that anti-CPS antibodies generated during a colonisation episode do not have a role in its clearance, though they may be protective against the future acquisition of colonisation and subsequent development of disease. This role of anti-CPS antibodies is supported by clinical studies demonstrating a reduction in vaccine-serotype pneumococcal colonisation in vaccinated children, reaching virtual elimination in some countries (77). The functional importance of anti-CPS antibodies is summarized in Figure 1.1.

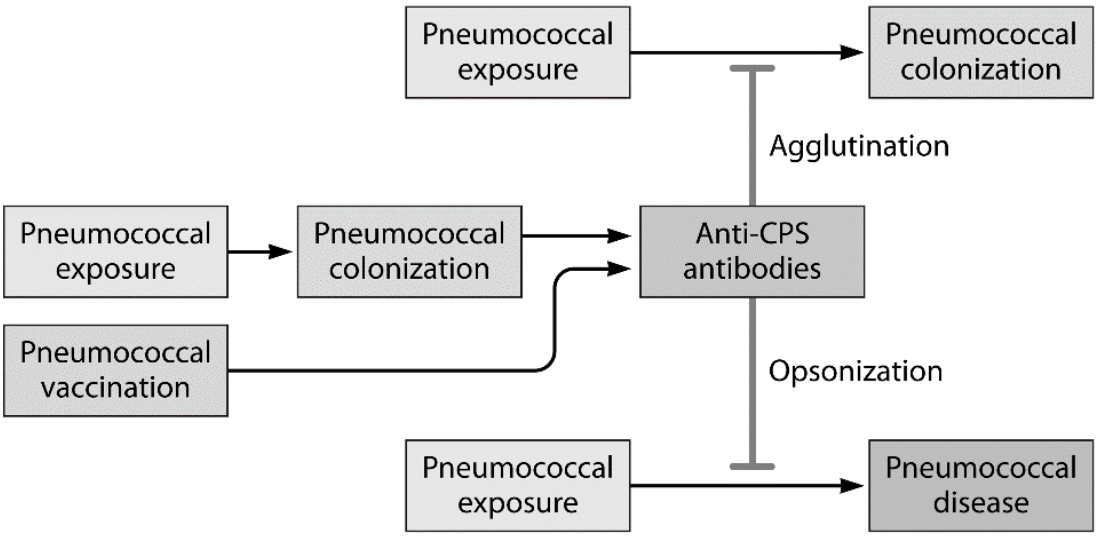

Figure 1.1: Anti-capsular antibodies facilitate pneumococcal killing and can prevent colonisation

(Prior to submission, this figure was published in a review article based on this chapter (78), included in this thesis as Appendix 10)

1.3.4 Antibodies as a marker of pneumococcal exposure

Colonisation-associated boosting of serotype-specific anti-CPS antibodies has been harnessed by researchers as a surrogate marker of pneumococcal colonisation (29, 79). Antibody titres vary markedly between individuals and between serotypes, so "seroincidence" estimation compares titres from two timepoints to assess for pneumococcal colonisation during the intervening period. Seroincidence can only estimate carriage rates for the most common serotypes, as it is generally not practical to measure antibodies against all 96 serotypes. Despite these limitations, seroincidence correlated well with observed colonisation rates in children in one study (79).

1.3.5 Why does lifelong pneumococcal exposure not protect older people?

If pneumococcal colonisation leads to the generation of antibodies, and these antibodies are protective against reacquisition of pneumococcus, then older people should be particularly well protected against pneumococcal disease. Clearly this is not the case, and several explanations have been proposed. Vaccine-induced antipneumococcal antibodies wane over time, and require booster vaccines in order to maintain protective levels.

Perhaps colonisation-induced antibodies may require boosting by regular episodes of colonisation (60), and this is too infrequent in older populations for boosting to occur. Otherwise, the defect in antibody-mediated immunity lies either with the B-cells responsible for secreting the antibodies, or with the antibodies themselves. Although our focus here is on anti-capsular antibodies, other facets of the immune system can also be implicated, including T cell control of B cell responses (64), and alteration in neutrophil function with age (80).

1.3.5.1 B cell populations are altered in older people

IgM memory B-cells, which function in a T cell-independent manner, are a key component of antipneumococcal defences (69). A study found that IgM memory B-cells are less abundant in older than in younger adults (81). In addition, aged IgM memory B-cells were determined to be functionally inferior in this same study, with a reduced capacity for antibody secretion and plasma cell differentiation. Pneumococcal polysaccharide vaccination of the older volunteers led to some improvement in IgM levels and IgM memory B cell percentages, but not to the same degree as in younger subjects. B1 cells are another potential culprit; these cells are responsible for producing naturally-acquired anti-CPS antibodies (while T cell-dependent adaptive antibodies are generated by B2 cells). Levels of B1 cells are reduced in older adults (reviewed in (82)). This is an emerging field, and there is a dearth of human studies relevant to this topic outside of the context of vaccination—we will explore this in a later section.

1.3.5.2 Antibodies decline and lose functional efficacy with age.

Population-based studies have shown that natural anti-CPS IgG and IgM levels fall with age (65, 83, 84). Figure 1.2 shows a schematic of anti-CPS antibody levels and function at different ages relative to rates of pneumococcal colonisation and disease. Antibody function (i.e. opsonic activity, or ability to target pathogens for phagocytosis) can vary

markedly between individuals; populations with high rates of pneumococcal colonisation and disease have higher serum opsonic activity than lower-risk populations, even when matched for age and antibody level (85). For this reason, opsonophagocytic killing activity (measured by exposing pneumococci to antibodies of different research subjects, in the presence of standardised populations of neutrophils and complement) is a stronger correlate of protection than crude antibody levels (86). It is therefore of great importance that the naturally-acquired anti-CPS antibodies of older people have less opsonic activity than those of young people. In one study, the concentration of natural serotype-specific IgG required for opsonophagocytic killing (defined in the study as phagocytosis of 50% of target pneumococci) was up to twice as high in an unvaccinated older population when compared with a young population—differences in IgG function between young and old were even more substantial than differences in concentrations (80). Similar, though less pronounced differences were seen for IgM. The authors noted that serotype-specific IgM concentrations and opsonic activity were poorly correlated, unlike those of IgG. When the decline in antibody level and function are combined, this strongly suggests that antibody defects are responsible for (or at least contribute towards) the age-related increase in vulnerability to pneumococcus.

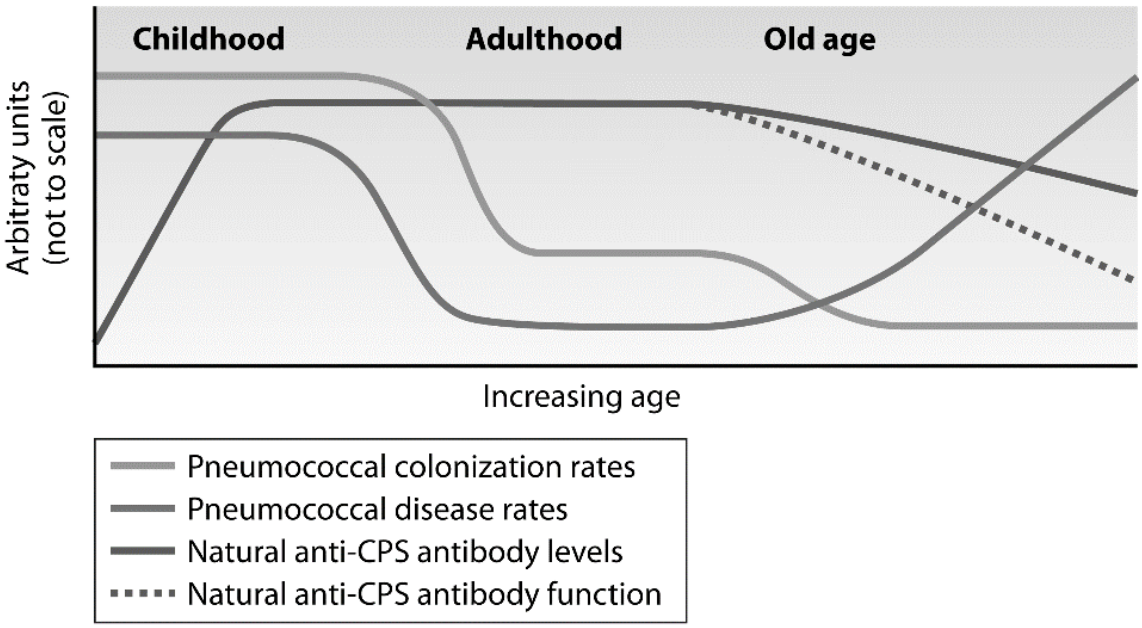

Figure 1.2: Schematic of pneumococcal disease rates, pneumococcal colonisation rates and pneumococcal antibody activity in different age groups.

(Prior to submission, this figure was published in a review article based on this chapter (78), included in this thesis as Appendix 10)

Impaired opsonic functionality relative to antibody levels is seen in immunosuppression secondary to a wide variety of aetiologies. For example, (although not directly comparable to an aged population), it is notable that anti-CPS IgG levels in human immunodeficiency virus-infected individuals (who have high rates of pneumococcal colonisation as well as disease) have reduced anti-CPS IgG opsonic activity when compared with HIV-uninfected subjects, even though anti-CPS IgG levels are higher in those with HIV (87).

An observational study provides some clinical context and supports the hypothesis that reduced opsonic functionality in anti-CPS antibodies is a risk factor for pneumococcal disease in older people. Sera from patients in the acute and convalescent stages of various types of pneumococcal disease were compared with age-matched controls (88). Only 27% of subjects with pneumococcal disease had IgG to their infecting serotype at time of presentation (compared to 37% of controls and 42% of colonised subjects). Furthermore, acute antibodies from infected subjects had significantly lower opsonic activity than those

of controls or colonised subjects and were less protective via passive transfer in a lethal murine challenge model (20% survival vs 100%). Sixty-two percent of convalescent sera had detectable IgG following pneumococcal disease, which demonstrated good function in >50% of patients. Important limitations of this study include substantial loss to follow-up between the acute and convalescent phases, no reporting of ages, and no pre-disease antibody levels, the last of which means we cannot rule out the possibility of antibody sequestration in diseased tissues as an explanation for low circulating levels.

The functional efficacy of antibodies can also be quantified via antibody avidity. However, avidity does not always correlate with opsonophagocytic activity (89). Studies differ on whether anti-pneumococcal antibody avidity falls with age or rises following vaccination (89)(90)(91). Indeed, one study found that higher levels of antibody avidity correlated with increased severity of pneumonia (91); the authors suggested that the increased avidity reflected greater lifetime exposure to pneumococci (and hence increased affinity maturation over time) in older people who were naturally at risk of severe pneumonia, or alternatively boosted avidity during the initial phase of pneumococcal infection. They concluded that avidity was a poor surrogate for susceptibility to pneumococcal disease.

Most of the more detailed studies of antibody functionality in older people have been conducted in the context of vaccination. Vaccination is an obvious strategy to restore waning natural anti-CPS immunity.

1.4 Vaccines against pneumococcal disease

Vaccination with purified pneumococcal CPS was first described in 1934 (92), with the first report of clinical efficacy emerging following its use to halt an outbreak of serotype 1 pneumococcal pneumonia in Massachusetts in 1938 (93). A 14-valent pneumococcal polysaccharide vaccine (PPV) was formally licensed in 1977, and PPV23 denotes the current 23-valent formulation, licensed in 1983.

Polysaccharide vaccines are not immunogenic in children aged <2 years (94). However, conjugation of the polysaccharide to a protein carrier molecule can overcome this limitation (reviewed in (95)). The pneumococcal protein-conjugated vaccine (PCV) has excellent immunogenicity and efficacy in children: a seven-valent conjugate vaccine was introduced in the USA in 2000 and in the UK in 2006; the most recent formulation is the 13-valent PCV13, introduced in the UK in 2010.

In addition to conferring direct protection against pneumococcal disease in children, childhood vaccination programs generate herd protection by reducing colonisation and thus halting transmission at a population level (33). However, serotype replacement has abrogated much of this benefit in many settings (96, 97). Even without significant levels of serotype replacement, vaccine type disease remains common in older people after childhood vaccination programs are established (98), and residual non-vaccine-type disease will persist as a public health problem (16).

In the USA, current recommendations for adults aged over 65 years advise vaccination with PCV13 followed by PPV23 (99). In the UK, PPV23 is recommended in older adults, but the addition of PCV13 was not deemed to be cost-effective, and the use of PPV23 is to be kept under review (100). Recommendations in other Western European countries vary considerably (101).

1.4.1 Current pneumococcal vaccine strategies provide poor protection in older adults

The discrepancies in national vaccination policies stem from the poor (and disputed) efficacy of these vaccines in older people. A Cochrane review in 2013 concluded that PPV23 effectively prevents pneumococcal bacteraemia and meningitis, including in older adults (102). It has minimal effect at the mucosal level, and thus has not been shown to reduce rates of colonisation. The Cochrane review found no effect of PPV23 on rates of

(non-bacteraemic) pneumococcal CAP or all-cause pneumonia, partially due to the substantial heterogeneity of studies that were included. Nonetheless, some individual studies—including both observational studies and well-conducted randomized controlled trials (RCTs)—have found PPV23 to be efficacious against pneumococcal pneumonia. For example, one double-blind RCT in Japanese nursing home residents (a population expected to have a high incidence of pneumonia, and therefore better positioned to detect a vaccine effect) found a 62% relative risk reduction of pneumococcal pneumonia, and a 39% relative risk reduction of all-cause pneumonia with PPV23 (103). When data from this study were pooled with others for the Cochrane meta-analysis, the effect was no longer significant; however, this does not exclude the possibility of a small protective effect against pneumococcal pneumonia from PPV23, which would be clinically significant in a high-risk population. An important limitation of the Cochrane review is that the many of the studies it included were carried out in a general adult population, with limited data available for age-specific subgroup analyses.

An important study of PPV23 in people aged ≥ 65 years has been published after the Cochrane review (104). This study was observational in nature, but employed a test-negative design: this reduces several biases and has been found to be similar to RCTs in providing estimates of vaccine effectiveness for seasonal influenza vaccines (105). The study, carried out in Japan, found that the effectiveness of PPV23 was 27·4% against all pneumococcal CAP and 33·5% against CAP caused by the 23 vaccine serotypes (104). Effectiveness was not demonstrated against all-cause pneumonia or mortality. Furthermore, it was notable that this effect was only statistically significant for subjects who had been vaccinated within the previous two years.

Conjugated vaccines, while covering fewer serotypes, protect against colonisation in children and young adults (106, 107). In addition to efficacy against vaccine-type

bacteraemia and meningitis, PCV13 has been shown to reduce rates of vaccine-type CAP in a single large RCT in older adults (CAPiTA) (108). However, with vaccine efficacy of 45.6%, this vaccine did not show complete protection against vaccine-type disease. PCV13 efficacy declined with increasing age: In a post-hoc analysis, overall vaccine efficacy against vaccine-type CAP was 65% in 65-year-old subjects but only 40% in 75-year-olds (109). Furthermore, a concomitant increase in non-vaccine type disease was noted, resulting in no effect against pneumococcal pneumonia in general, and all-cause mortality was unaffected (108). A nested study within CAPiTA found that PCV13 reduced rates of vaccine-type pneumococcal colonisation in older adults compared with placebo, but that the effect was only detectable up to six months post-vaccine (110).

1.4.2 Pneumococcal vaccines are variably immunogenic in older people

The clinical findings reported in the previous section are discordant with immunological studies of pneumococcal vaccines in older adults. In a study of 74 older adults, dialysis patients and transplant recipients (i.e. without young healthy controls), PPV23 was found to improve anti-CPS IgG levels against three selected vaccine serotypes (6, 14 and 23) and not only to improve opsonic activity, but to strengthen the correlation between IgG levels and opsonic activity, suggesting that vaccine-induced antibodies are more potent than naturally acquired antibodies (111). A study of 219 adults aged ≥70 years found that PCV7 was more immunogenic (as measured by concentration and function of post-vaccine anti-CPS IgG) than PPV23 for all but one of the PCV7 serotypes (112). However, a larger study (n = 599) of adults aged 50—80 years found that PCV7 and PPV23 were equally immunogenic (as defined by IgG concentrations) at one month and one year following vaccination (84). No functional tests were performed. The reasons for the discrepant results between these two studies remains unclear. A randomized study of nursing home residents aged ≥80 years found that both PPV23 and PCV7 were immunogenic in this population, with the conjugate vaccine resulting in higher IgG levels and opsonic activity for some serotypes,

and both vaccines equally immunogenic for others (113). The effects of single-dose versus boosted vaccination, in various combinations, have been assessed in a number of studies but with conflicting results (reviewed in (114)).

The differential effects of the two vaccines on B-cells have been studied extensively. In a cohort of 348 subjects aged 50—70 years, the antibody responses were similar to previous studies: PCV7 lead to greater anti-CPS IgG concentrations than PPV23 for some but not all serotypes—four out of seven in this case (116). However, serotype-specific memory B cell concentrations increased for all seven serotypes following PCV7 but decreased following PPV23 (117). This is consistent with the T-dependent immunogenicity of PCV7. Importantly, repeated doses of unconjugated polysaccharide vaccines do not result in immune boosting—rather, the antibody response is inferior to that following primary vaccination (hyporesponsiveness) (118). Memory B cell depletion has been implicated in this phenomenon (117), which can be avoided by spacing vaccine administrations by at least five years (119). It is unclear whether repeated natural exposure to pneumococcal antigens is associated with hyporesponsiveness, but this intriguing hypothesis has been proposed as an additional mechanism of pneumococcal immunodeficiency in older people (117) and is an important topic for future research.

The immune responses to PPV23 across an aged population are heterogeneous. One study has suggested that a four-fold increase in IgG concentration from baseline following vaccination is protective against recurrent pneumococcal CAP in older adults (115). This study had a number of limitations (including low rates of confirmed pneumococcal aetiology in cases of CAP) and has not been replicated.

1.4.2.1 The duration of immunity following pneumococcal vaccination is the subject of ongoing research

The above studies based all analyses on blood samples taken up to one month post-vaccination. Another study randomized 252 subjects aged 50—80 years to vaccination with either single-dose PPV23 or PCV7, or PCV boosted with either PPV23 or repeat PCV7, and followed them for two years (120). Surprisingly, there was no significant difference in the quantity of circulating serotype-specific memory B-cells at two years between the four groups. Two-year levels of serotype-specific memory and plasma cells were closely correlated with baseline serotype-specific IgG levels, and not with the IgG levels from 7 or 28 days post-vaccination. The authors concluded that pre-existing natural anti-pneumococcal immunity was a more important driver of the post-vaccine immune response than the type or schedule of vaccine administered. No functional assays were carried out, and there were no young adult control subjects, but this remains an important study. It is unclear why these authors found no difference in memory B cell concentrations between PPV and PCV-vaccinated subjects while other authors found a dramatic difference (117), but different experimental methodologies and sampling timepoints between the various studies are possible explanations.

Although some authors have found durable memory B cell responses following either PPV or PCV, clinical and antibody-based studies are less reassuring. PPV-induced antibody levels decline in older people over five years (119); while they may not decline to the pre-vaccination baseline, clinical data consistently show reduced protective efficacy over time, suggesting that this decline is relevant and clinically significant (104, 121). Similar declines in opsonic function over time were seen in older adults who received PCV13 (110). The immunological properties of PCV13 (T-cell-dependent immunity, leading to lasting immunological memory), suggest that any decline in efficacy would be of a lesser magnitude than that of PPV23; however, immunosenescence may well interfere with this.

In the CAPiTA trial of PCV13 in over-65s, conducted over four years, clinical efficacy did not appear to decline over time (108), although efficacy was lower in the oldest participants (109). This suggests that there an age-related component to the clinical protective response following primary vaccination with PCV13. A longer period of follow-up would be required to determine the duration of protection in older people, but conjugate vaccines do appear to confer longer clinical protection than polysaccharide vaccines.

1.4.3 Pneumococcal vaccines are more immunogenic in young than in older people

While uncontrolled studies have shown an improved antipneumococcal immune response following vaccination in older people, this is far less impressive than the immune response generated by the same vaccine in healthy young people. A study compared anti-CPS antibody levels in 58 volunteers aged >65 years and 44 controls aged <45 years, 28 days after they had received PPV23 (no pre-vaccination levels were taken) (122). For the majority of serotypes, antibody levels did not differ significantly between the two groups. However, opsonic titres against all but one serotype (18C) were markedly higher in the younger subjects. Antibody potency (opsonization titre divided by the antibody concentration) was at least two-fold higher for all serotypes in younger subjects than in older subjects, while the amount of antibody needed to achieve a 1:8 opsonization index (a putative protective level) in young subjects was less than half of that in the older subjects.

We are unaware of any direct comparison studies of the immunogenicity of PCV in older and younger people. Murine studies have explored this question, but the results were markedly different from with what would be expected in human subjects based on the state of current knowledge, and will therefore not be discussed here (123).

1.4.4 Immunoglobulin subsets present a further area for study

Immunoglobulin subsets—for example, IgM versus IgG, or even IgG1 versus IgG2—could differ between older and younger adults. IgG responses can be divided into IgG1

(classically associated with T-cell independent responses to plain polysaccharide vaccination) and IgG2 (classically associated with T-cell dependent responses to PCV in children) (95). However, it is notable that, at least for the meningococcal conjugate vaccine, a study found that vaccination of older children and adolescents generated a predominantly IgG2 response, with IgG1 responses restricted to the youngest children (124). There is a dearth of research into these subsets specifically in older adults. One study of healthy adults of all ages found similar mean concentrations of total IgG1 and IgG2 at all ages, but greater variability of IgG1 and IgG2 concentrations in individuals aged >70 years (125). (124)IgM responses in older adults have been studied in greater detail.

1.4.5 Anti-CPS IgM responses are markedly deficient in older people

A study compared sera from 45 healthy older subjects and 55 healthy young controls, all of whom had been vaccinated four weeks previously with PPV23, and tested the samples against three representative serotypes: 14, 18C and 23F (126). In keeping with previous studies, absolute anti-CPS IgG levels were similar between both groups, but the younger adults had higher opsonic activity and potency than the older adults (albeit not achieving statistical significance for serotype 18C). Young adults commonly demonstrated high levels of opsonic activity even with low levels of antibody (i.e. the correlation between antibody levels and opsonic activity was poor), whereas antibody levels and activity were tightly correlated in the older adults. IgM made a disproportionately significant contribution to opsonic activity: when IgM was removed from the young subjects' samples, their opsonic activity was decreased, with stronger correlation between their IgG levels and opsonic function. When all serum samples were depleted of IgM and reanalyzed, the opsonic activity of the older adults' sera did not decline and the differences in opsonic activity between old and young subjects were no longer statistically significant. The authors

concluded that reduced functionality of IgM rather than IgG was responsible for the reduced opsonic capacity of older adults when compared with younger adults.

The kinetics of IgM could partially explain the above findings: unlike IgG, post-vaccination IgM levels rise more slowly, and to a lower peak, in older adults compared with younger adults (127). All samples in the above study were taken quite soon after vaccination. Little is known regarding the duration of IgM responses in older people beyond 28 days post-vaccination, and thus the relevance of this laboratory-based study to long-term clinical protection is not certain. However, additional research has shown that the underlying IgM B cell responses to vaccination, in addition to IgM activity itself, are also diminished in older people.

A study comparing 14 older adults with young controls examined the immune response against two of the PPV23 serotypes (14 and 23F) and found that serotype 14-specific IgM did not rise significantly following vaccination in the older adults (though anti-23F IgM did) (128). Opsonic activity improved following vaccination in older adults, and this was correlated with IgG levels but not with IgM levels, and was significantly lower than the opsonic activity of young vaccine recipients, consistent with previous studies. Flow cytometric analysis showed differences between young and older subjects in their post-vaccination B cell phenotypes: both absolute and relative numbers of $CD27^{+}IgM^{+}$ (IgM memory) B-cells were reduced in the older adults. The serotype-specific immune response in the older adults was dominated by switched memory B-cells ($CD27^{+}IgM^{-}$). This difference in B cell populations explained the poor IgM response in the older adults, and may provide a key insight into the underlying reasons for poor vaccine-induced clinical protection in this population, but the small numbers (of both subjects and serotypes examined) are an important limitation of this study.

Switched memory B-cells comprise part of a T-cell-dependent immune response while IgM memory B-cells are T-independent (69). Regulatory T cell populations are reduced in older people (129); this has been implicated in altered inflammatory responses and susceptibility to pneumonia in the this population (reviewed in (64)). Therefore, alterations in T-dependent immunity coupled with a reduction in T-independent IgM memory B-cells leaves older people vulnerable on two fronts.

IgM defects are unlikely to be the sole reason for the increased susceptibility of older people to pneumococcal disease. However, by virtue of its pentameric structure, IgM would be expected to agglutinate and opsonize more efficiently than IgG, and thus even small defects in IgM levels or function would be expected to have a disproportionate impact. IgM is also key to activating the complement cascade in response to pneumococcus (70). While the IgM response to PCV has not been widely studied in older people, it is key to the immune response to conjugated vaccines in children (130). Furthermore, PCV-induced IgM antibodies appear to confer cross-protection against some non-vaccine serotypes in children (131)—this has not been demonstrated older people, but could represent another domain in which IgM is of key importance. For now, the above data must be regarded as hypothesis-generating rather than conclusive, but they are intriguing nonetheless.

1.5 Antibodies have mucosal as well as systemic activity

It is generally reported that IgM and IgA are the principal antibodies present at mucosal surfaces (132, 133), although the relative contributions of different globulin fractions to total antibody levels varies markedly between different organ systems (134). IgA-mediated defence against pneumococcus is limited, as all pneumococci synthesize an efficient IgA1 protease, abrogating its protective effect (72). In the final part of this review, we will explore the nature of mucosal anti-pneumococcal immunity and its relationship with age.

There is a degree of overlap between the mucosal and systemic humoral immune systems, and each is capable of influencing the other (133). Antigens from the nasal mucosal surface are presented to nasopharyngeal-associated lymphoid tissue (NALT), leading to both local and systemic immune responses. Germinal centres in NALT are responsible for generating B-cells that secrete IgA and IgM at the mucosal surface. Furthermore, systemic antibodies can be transported from blood to mucosal surfaces.

1.5.1 Systemic exposure to pneumococcal antigens via vaccination can lead to mucosal protection

One study found that PPV leads to an increase in levels of all classes of anti-CPS in secretions (specifically saliva and tears; nasal secretions were not studied) (135). Notably, the fold increases in salivary IgG (4.5-fold) and IgM (4.0-fold) were more pronounced than that of IgA (2.0-fold). However, the functional and clinical effects of these antibodies have not been explored.

In young adults, systemic immunization with PCV13 leads to high serum concentrations of anti-pneumococcal IgG, which spills over into the nasal mucosal compartment and can, by virtue of its agglutinating properties, prevent the development of pneumococcal colonisation (73). This is likely to be the mechanism for the reduction in pneumococcal colonisation following infant vaccination.

1.5.2 Mucosal exposure to pneumococcal antigens can generate both systemic and local responses

As outlined earlier, the upper respiratory mucosa represents humans' first point of contact with the pneumococcus. Transient pneumococcal exposure (in a human challenge model where subjects were inoculated but did not become colonised) resulted in the generation of mucosal anti-protein antibodies but not anti-CPS antibodies, and no change in systemic

antibody levels (136). Prolonged exposure via colonisation leads to increases in functional local and systemic anti-CPS antibodies (60).

Without vaccination, antipneumococcal antibody levels at respiratory mucosal surfaces are too low to prevent colonisation. However, "priming" by experimental pneumococcal colonisation is protective against subsequent colonisation up to one year later (60)—whether this is due specifically to mucosal antibodies, serum antibodies (*à la* vaccination), T-cell immunity or a combination of these remains undetermined.

In addition to inducing mucosal and systemic antipneumococcal antibodies, human pneumococcal colonisation leads to an increase in the number of pneumococcal-specific memory IL-17A^{+} CD4 T-cells (Th-17 cells) (137). When stimulated by pneumococci *in vitro*, IL-17A secreted by these Th-17 cells enhanced the phagocytic killing of pneumococci by alveolar macrophages. Importantly, this Th-17 increase is seen in both peripheral blood and in the lung itself, thus providing evidence of traffic of acquired immune memory from the upper to the lower respiratory tract. However, an alternative hypothesis is that microaspiration of pneumococci during colonisation directly induces a local T cell infiltration and differentiation within the lungs.

In summary, pneumococci are capable of stimulating a specific immune response at the mucosal surface in addition to generating systemic immunity. The multifaceted mucosal immune response includes both specific antibodies and memory T-cells, and a response in the upper respiratory tract may be echoed in the lower respiratory tract. High concentrations of anti-CPS antibodies at the nasopharyngeal surface can prevent pneumococcal acquisition. A mucosal vaccine against pneumococcus could be a promising strategy to provide protection for the vulnerable older population.

1.5.3 Mucosal anti-pneumococcal immunity is affected by aging

Detailed studies of mucosal immunosenescence in general have only been undertaken in mice: it appears that nasal immune function deteriorates with age, but at a similar rate to systemic immunity, whereas intestinal mucosal immunity "ages" at a faster rate (138). A murine study demonstrated impaired innate antipneumococcal nasal mucosal immunity with increasing age: monocyte/macrophage influx in response to colonisation was delayed in old (18—23 month) mice compared with younger controls, and the older mice had impaired upregulation of innate immune response genes (including Toll-like receptors 1 and 2, nucleotide-binding oligomerization domain-containing protein 2 and interleukin 1β) (139). The effect of aging on nasal antibodies has not been studied in older humans, but salivary antipneumococcal antibodies have been shown to decrease in both concentration and rate of secretion with age (140).

1.5.4 Murine studies of adjuvanted mucosal vaccines have shown promise

Studies of mucosal vaccination strategies against pneumococcus have only been undertaken in murine models (reviewed in (141)) and examined both protein antigens and CPS. The most intriguing findings from these studies have been the effect of novel adjuvants on restoring the immune response in aged mice to both protein and polysaccharide antigens. Addition of CpG oligodeoxynucleotides (CpG-ODN) was found to improve the systemic and mucosal antibody response to conjugated pneumococcal serotype 9V CPS administered nasally to young mice (142). CpG-ODN enhances antibody production through stimulation of type 1 helper T-cells; the underlying mechanism of this remains uncertain (143). This same adjuvant restored the antibody response of aged mice to conjugated serotype 14 CPS administered systemically (144). For nasally-administered pneumococcal surface protein A (PspA), a dual adjuvant strategy of CpG-ODN and plasmid-expressing Flt3 ligand was required to induce similar antibody levels (serum and mucosal

IgG and IgA) in young and old mice (145). This strategy also enhanced PspA-specific CD4 T-cell responses in old mice and was protective against nasopharyngeal colonisation in these mice.

It must be emphasized that mouse IgA, having a different configuration to human IgA, is less susceptible to cleavage by pneumococcal IgA protease. Thus, if the above findings are to have applicability for human vaccination, it will be essential to demonstrate either that antibodies are a dispensable component of the mucosal immune response, or that other immunoglobulins—such as secretory IgM and IgG—are sufficient for protection in humans. If the relative dysfunction of anti-CPS IgM in older humans is indeed of clinical significance, then this may prove to be the Achilles' heel of this vaccination strategy, unless an adjuvant can be identified that can restore the function of IgM in older adults. With this caveat in mind, an appropriately-adjuvanted mucosal vaccine could still have enormous potential for reducing the burden of pneumococcal disease in older adults.

1.6 Alternative antibody targets to polysaccharide

Thus far, we have focused on anti-CPS antibodies. These antibodies are induced by natural exposure to pneumococcus and are also the antigens employed in all currently-licensed pneumococcal vaccines. Furthermore, there is a substantial body of literature comparing anti-CPS immunity in young and older adults. However, the pneumococcus also expresses a variety of surface proteins which are conserved across different serotypes, many of which have been proposed as vaccine candidates (146) and indeed have been explored in mucosal vaccines in animal models as outlined above. Parenteral protein-based vaccines have shown a degree of promise during early-phase studies in young adults, but have yet to achieve their full potential (147). Anti-protein immune responses have been demonstrated following colonisation (60) and may contribute to naturally-acquired protection against colonisation (58) although their mechanistic significance has not been definitively

established (148). In children, studies are conflicting regarding whether anti-protein antibodies confer protection or serve as a marker of exposure and increased risk of disease (149, 150). In a single study of young adults, antibodies against two out of 27 vaccine-candidate proteins were found to be elevated following experimental colonisation with serotype 6B: PiuA and PspA-UAB055, while none were protective against colonisation (60).

1.6.1 Anti-protein antibodies in older people

Anti-protein antibody levels are reduced in older adults (65). Anti-protein antibody levels rise following pneumococcal disease in older adults (151), and there is a suggestion that their functionality may not be adversely affected by aging, though these findings remain preliminary (German E et al, in press). Apart from these, and the above-mentioned murine studies of mucosal anti-protein immunity, we are unaware of any substantial body of work exploring the nature of aging and anti-protein immunity, and this topic must be prioritized in future research.

1.7 Summary of antibody-based pneumococcal immunity

Impaired naturally-acquired CPS immunity leaves older people vulnerable to pneumococcal disease. This may or may not be related to reduced boosting due to infrequent pneumococcal colonisation. The functional antibody responses to current pneumococcal vaccines are also suboptimal in older adults. PCV13 has overcome some, but by no means all of the immunological limitations of PPV23. Reduced antibody functionality combined with limited serotype coverage means that pneumococcal vaccination in older adults does not deliver as substantial a benefit as would be expected.

If anti-CPS antibodies are to remain the mediator of protection, then improvements in the functionality of aged antibodies will need to be induced. A mucosal vaccine, with an appropriate adjuvant, would be an attractive strategy. Vaccination strategies seeking to

exploit non-capsular antigens or T cell-mediated immunity have shown a degree of promise in early-phase studies in young adults, but have yet to achieve their full potential (147). Careful studies of anti-protein immunity in older people would guide the exploration of such a vaccination strategy in older adults. Future studies should investigate the dynamics of colonisation and mechanisms of naturally-acquired immunity in older people in greater detail, as well as exploring the nature of respiratory mucosal immunity in older people, in order to better inform vaccine development for this growing and vulnerable population.

1.8 Controlled human infection models in research

Studies of pneumococcal colonisation are limited by the sensitivity of diagnostic tests, variations in sampling methodology and low prevalence in certain settings, as outlined earlier. Studies of pneumococcal disease have similar limitations. In recent years, research into infectious diseases has been revolutionised by the development of controlled human infection models (CHIMs). CHIMs of a wide variety of pathogens have been carried out throughout history, particularly in the twentieth century, but many early studies were carried out in an ethical vacuum—in the most egregious cases, prisoners were infected without their consent (152). In the decades following the second world war, an ethical framework for human challenge studies has been established (153). CHIMs are now generally accepted as essential to study the pathogenesis and prevention of a number of infectious diseases (154), including cholera (155), malaria (156) and typhoid (157).

1.8.1 Experimental Human Pneumococcal Colonisation

Given that pneumococcal colonisation is key to the pathogenesis of pneumococcal disease as well as natural anti-pneumococcal immunity, a CHIM of pneumococcus can be established by inducing carriage rather than clinical disease (158). The Liverpool School of

Tropical Medicine (LSTM) established an Experimental Human Pneumococcal Colonisation (EHPC) model in 2011. Exclusion criteria typically comprised:

- Allergy to penicillin
- Smoking or ex-smoker with significant history
- Chronic illness, particularly if expected to affect immunity (such as asthma, diabetes)
- Taking steroids (including steroid nasal spray) or other immunosuppressants
- Antibiotic therapy in the preceding four weeks
- Caring responsibilities for children aged < 5 years, hospital patients or people with chronic illnesses
- Pregnancy or lack of adequate contraception (women only)

Initial studies of the model were carried out in volunteers aged between 18 and 50 years, and determined that experimental colonisation is feasible and safe in this population, and that it is reproducible (typically resulting in 45% of subjects becoming colonised), immunogenic and can be used as a vaccine testing platform (60, 107).

1.9 Treatment of pneumococcal disease

In this final section, we discuss the treatment of suspected or confirmed pneumococcal disease, with the former being a more common scenario in clinical practice. Penicillin and other β-lactam drugs (such as cephalosporins) have historically been the mainstay of therapy for pneumococcal disease. Other options include macrolides, tetracyclines and respiratory fluoroquinolones.

1.9.1 Local treatment guidelines for pneumococcal disease

In Table 1.2 and Table 1.3, we present a review of national guidelines and local formularies (primary and tertiary care) for the recommended treatment of any of:

- Lower respiratory tract infections in healthy adults
- Suspected pneumococcal disease (pneumonia or meningitis)
- Confirmed pneumococcal disease (pneumonia or meningitis)

Table 1.2: Local and national guidelines relevant to the treatment of suspected or confirmed pneumococcal respiratory infection

Guideline	**Clinical scenario**	**First-line antibiotic(s)**		**Alternative antibiotic(s)**	
		Class(es)	*Specific agent(s)*	*Class(es)*	*Specific agent(s)*
BTS (7)	Empiric treatment of mild CAP	β-lactam	Amoxicillin	Macrolide OR tetracycline	Clarithromycin OR Doxycycline
BTS (7)	Empiric treatment of severe CAP	β-lactam PLUS macrolide	Amoxicillin OR Benzylpenicillin OR Amoxicillin-Clavulanate (high severity) PLUS Clarithromycin	Tetracycline OR fluoroquinolone (moderate); OR β-lactam PLUS macrolide (severe)	Doxycycline OR Levofloxacin OR Moxifloxacin (moderate); Cefuroxime OR Cefotaxime OR Ceftriaxone PLUS Clarithromycin (severe)
BTS (7)	Treatment of confirmed pneumococcal CAP	β-lactam	Amoxicillin OR Benzylpenicillin	Macrolide OR β-lactam	Clarithromycin OR Cefuroxine OR Cefotaxime OR Ceftriaxone
Local hospital	Empiric treatment of mild CAP	β-lactam	Amoxicillin	Macrolide	Clarithromycin
Local hospital	Empiric treatment of severe CAP	β-lactam PLUS macrolide	Amoxicillin (moderate) OR Benzylpenicillin (high severity) PLUS Clarithromycin	Tetracycline OR glycopeptide PLUS macrolide	Doxycycline (moderate severity); Teicoplanin PLUS Clarithromycin (high severity)
Local primary care (159)	Empiric treatment of mild CAP	β-lactam	Amoxicillin	Macrolide OR tetracycline	Clarithromycin OR Doxycycline
	Empiric treatment of moderately-severe CAP	β-lactam PLUS macrolide	Amoxicillin PLUS clarithromycin	Tetracycline	Doxycycline
Local primary care (159)	Empiric treatment of acute bronchitis	β-lactam	Amoxicillin	Tetracycline	Doxycycline

CAP: Community-acquired pneumonia; Local hospital: Royal Liverpool University Hospital Antimicrobial Formulary (accessed October 2017); Local primary care: Pan-Mersey Area Prescribing Committee: Antimicrobial Guide and Management of Common Infections in Primary Care, 2017—2019 (159); National: British Thoracic Society Guidelines for the Management of Community Acquired Pneumonia in Adults: update 2009 (7). All guidelines refer to treatment of adults.

Table 1.3: Local and national guidelines relevant to the treatment of suspected or confirmed pneumococcal meningitis.

Guideline	**Clinical scenario**	**First-line antibiotic(s)**		**Alternative**
		Class	*Specific agent(s)*	
National (160)	Treatment of suspected bacterial meningitis	β-lactam (± glycopeptide or rifamycin)	Ceftriaxone OR Cefotaxime; (Add Vancomycin OR Rifampicin if recent travel to a region with high rates of penicillin resistant pneumococci)	Chloramphenicol
National (160)	Treatment of pneumococcal meningitis	β-lactam (± glycopeptide or rifamycin)	Ceftriaxone OR Cefotaxime (Benzylpenicillin acceptable if MIC ≤ 0.06 mg/L confirmed); (Add Vancomycin OR Rifampicin if cephalosporin resistance)	Chloramphenicol
Local hospital	Treatment of suspected bacterial meningitis	β-lactam	Ceftriaxone	Chloramphenicol

Local hospital: Royal Liverpool University Hospital Formulary (accessed October 2017); National: The UK joint specialist societies guideline on the diagnosis and management of acute meningitis, 2016 (160). All guidelines refer to treatment of adults.

1.9.2 Antimicrobial resistance in pneumococci

Antimicrobial resistance (AMR) is a growing global threat. Penicillin and macrolide resistance were identified in pneumococci in the 1960s (161) and today penicillin non-susceptible pneumococci are listed on the WHO's Global priority list of antibiotic-resistant bacteria (162). Data on invasive pneumococcal isolates from the European Centre for Disease Prevention and Control show wide variation in pneumococcal susceptibility profiles between different countries, with (for example) >40% of Romanian isolates non-susceptible to penicillin versus 0.4% of Belgian isolates (163). In recent years there has been a trend towards reduced rates of AMR in the UK; in 2016, 4.9% of invasive isolates were non-susceptible to penicillin, 6.5% non-susceptible to macrolides and 2.6% non-susceptible to both.

Microbiology laboratories define antimicrobial resistance based on the minimum inhibitory concentration (MIC): the lowest concentration of an antibiotic required to completely prevent growth of a given organism (164). MICs are related to predicted concentrations of the given antibiotic (at standard doses) at the site of interest (e.g. in blood or in cerebrospinal fluid (CSF)) to predict whether the organism will be successfully treated by the antibiotic—this determines the "clinical breakpoint". Clinical breakpoints are generally determined by national or supranational bodies rather than individual hospitals; in Europe, they are set by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (165).

1.9.2.1 Penicillin non-susceptibility

Penicillin non-susceptibility in pneumococci derives from mutations in one or more of the six penicillin-binding proteins (166), and may have first been acquired via the "scavenging" of genes from commensal *Streptococcus mitis* (167, 168). The term "penicillin resistance" is rarely employed when discussing pneumococci, as the significance of increased

pneumococcal MICs to β-lactam antibiotics remains controversial (169-171). Low-level penicillin "resistance" (MIC ≤2 mg/L) can be overcome by high-dose intravenous penicillin therapy, as this achieves serum concentrations in excess of the MIC (172, 173). Penicillin reaches lower concentrations in CSF, where its breakpoint is accordingly defined as 0.06 mg/L. Clinical and microbiological failure of penicillin monotherapy for bacteraemic pneumococcal pneumonia is encountered at higher levels of resistance, when penicillin MICs are >2 mg/L (174). Lower-level penicillin resistance is typically associated with alteration of a single penicillin-binding protein, such as PBP-2B, while mutations in multiple genes (e.g. the combination of PBB-2A and PBB-2X in one series) confer higher-level resistance (175). Penicillin non-susceptible isolates are usually sensitive to third-generation cephalosporins (172), which achieve therapeutic concentrations in CSF if administered at maximal doses (176). Given this reliance on cephalosporins to overcome elevated penicillin MICs, it is not surprising that ceftriaxone resistance was associated with mortality from pneumococcal bacteraemia in a recent study (173).

Although the EUCAST guidelines consider non-meningeal infections from pneumococci with an MIC up to 2 mg/L to be susceptible to high-dose benzylpenicillin (165), the doses in question (2.4g x 4—6/day) are in excess of those routinely recommended in the UK (7). Using EUCAST breakpoints, standard doses of benzylpenicillin (1.2g x 4/day) are only recommended for pneumonia caused by pneumococci with MICs up to 0.5 mg/L

1.9.2.2 Macrolide resistance

Resistance to macrolides typically results from either the acquisition of an efflux pump (low-level resistance, via the mef(A) gene) or a mutation in the macrolide binding site in the 23S ribosomal subunit (high-level resistance, via the erm(B) gene) (168). Macrolide resistance has been unequivocally associated with clinical and microbiological failure in cases of both pneumococcal pneumonia and bacteraemia (reviewed in (169)).

Nonetheless, larger-scale studies have suggested that the contribution of macrolide resistance to adverse clinical outcomes remains small (reviewed in (161)).

1.9.2.3 Fluoroquinolone resistance

Fluoroquinolone resistance has also been associated with clinical and microbiological failure in cases of pneumococcal pneumonia ((177), others reviewed in (178)). Prior exposure to the non-respiratory fluoroquinolone ciprofloxacin has been hypothesised to select for low-level resistance mutations in DNA gyrase and topoisomerase IV in colonising pneumococci, with subsequent respiratory fluoroquinolone treatment having the potential to select for higher levels of resistance (177). The lack of large prospective studies prevents us from accurately estimating the impact of fluoroquinolone resistance on treatment outcomes in pneumococcal disease.

1.9.2.4 Resistance to other antimicrobials

Trimethoprim-sulfamethoxazole (TMP-SMX) is not employed against respiratory tract infections in the UK. However, it is widely employed globally as prophylactic therapy in HIV-infected patients, in whom it confers protection against invasive bacterial diseases in addition to its intended target, *Pneumocystis jirovecii*. This has led to high rates (>90%) of pneumococcal resistance to TMP-SMX in regions with high HIV prevalence (179). The clinical significance of TMP-SMX resistance remains undetermined. Similarly, doxycycline is rarely administered to critically-ill patients with community-acquired pneumonia in the UK, which could result in clinical failures being under-noticed and under-reported. One author estimated—based on rates of resistance, likely impact of resistance, and host factors—that there was an 82% likelihood of community-acquired non-meningeal pneumococcal disease responding to doxycycline, and a 65% chance for TMP-SMX, versus 97% for ceftriaxone (168). There are no hard data to back up these assertions. Vancomycin resistance is

exceedingly uncommon—thus, this agent remains the drug of “last resort” for pneumococci.

1.9.3 The significance of colonisation with antibiotic-resistant pneumococci

Given the strong links between pneumococcal colonisation and onward transmission, and between pneumococcal colonisation and disease, it is highly likely that resistance rates in colonising pneumococci will correlate with those of invasive pneumococci. This is supported by epidemiologic studies in children (180). Vaccine impact studies also support this hypothesis: when vaccine-serotype pneumococci are the most likely pneumococci to harbour resistance, pneumococcal vaccination programmes reduce the rates of infection with resistant pneumococci throughout the community (181).

1.10 Summary

1. It is unknown whether older adults are as susceptible to pneumococcal colonisation as younger adults, or even substantially susceptible at all.
2. The niche of pneumococcal colonisation in older people may not be the same as that in young adults.
3. Pneumococcal colonisation may not be an immunising event in older adults.
4. There is a substantial unmet need for an improved pneumococcal vaccination in older adults.
5. Currently, herd immunity from paediatric vaccination is key to protecting older adults from pneumococcal disease, but this is vulnerable to serotype replacement
6. As pneumococcal colonisation is key determinant of pneumococcal transmission within communities, surveillance for antimicrobial resistance among colonising isolates is warranted.

Items 1—4 could be answered by expanding Liverpool’s existing controlled human infection model of *S pneumoniae* into an older cohort than has previously been recruited. This

would provide data to support, refute or generate hypotheses regarding susceptibility, site and immunogenicity of pneumococcal colonisation in this population, as well as providing a vaccine testing platform in a key risk population. For items 5—6, the EHPC programme could also provide incidental surveillance data on colonisation, herd immunity and serotype replacement in adults the community, and be a valuable source of pneumococcal isolates for susceptibility testing.

2 Methodology

This thesis reports results from two studies. The "Aging and Immunity" study was the expansion of the Experimental Human Pneumococcal Carriage (EHPC) model into a cohort of older adults; in this chapter, we describe how the study was designed and executed, and the clinical and laboratory methods we employed. The "Natural Carriage" study explored the rates and distribution of baseline pneumococcal carriage throughout the history of the EHPC programme; in this chapter, we report how these isolates were serotyped and tested for antimicrobial resistance.

2.1 The NHS approval process

Establishing an EHPC study requires careful coordination between the clinical team and numerous approval bodies, including the local NHS Research Ethics Committee (REC) and the relevant National Health Service (NHS) trust Research Development and Innovation (RD&I) department, as outlined in the flowcharts below (Figure 2.1, Figure 2.2). All previous EHPC studies had undergone a similar approval process, and the flowcharts include specific dates and identifiers for the "Aging and Immunity" study. Following ethical approval, protocol amendments required provisional approval by the sponsor(s) before review by the REC.

REC applications were made using the online Integrated Research Application System (IRAS) form, supported by other documents including a participant information sheet, protocol, questionnaires, provisional intention to sponsor letter, advertisements and General Practitioner (GP) letters (see APPENDIX 5).

The NHS REC defines a sponsor as a*n individual, organisation or group taking on responsibility for securing the arrangements to initiate, manage and finance a study.* The sponsor's role is to ensure that the research safeguards the rights, safety, dignity and

wellbeing of participants. In addition, the sponsor ensures the research is registered on a public database and reviews all REC amendments prior to submission, while confirming that research complies with legislation and Good Clinical Practice (GCP) requirements.

The decision regarding single sponsorship versus co-sponsorship was taken on a study-by-study basis. For the "Aging and Immunity" study, both LSTM and the local NHS trust requested that, as the study would take place on NHS premises (the clinical research unit (CRU) of the Royal Liverpool University Hospital (RLUH)) and involved a degree of risk, the study should be co-sponsored by the Royal Liverpool and Broadgreen University Hospitals Trust (RLBUHT) in addition to LSTM. A contract was drawn up between the two institutions, including designated oversight and liability roles for each party and a material transfer agreement between the two sites. In addition, specific approval was obtained from the CRU.

General outline of ethical approval process	Specific to "Aging and Immunity" study
Develop study design and initial version of protocol	
Develop all documentation for ethics: including patient information leaflet (PIL), consent form, protocol, GP letter, hospital advert.	
Arrange date for Research Ethics Committee (REC)	**Ref** 16/NW/0031
Complete Integrated Research Application System (IRAS) form – complete REC, R&D and Clinical Research Network (CRN) portfolio sections and submit	Submitted 16 December 2015
Attend REC meeting	Attended 21 January 2016
Address all REC amendments and submit	15 February 2016
REC approval	Favourable opinion 4 February 2016, full approval 15 February 2016
ISRCTN (International Standard Randomised Controlled Trials No.) registration	**ISRCTN ID** 10948363 *As part of good research practice, even though this study was not a randomised trial, it was prospectively registered with ISRCTN*
Additional amendments during the study, as required	**Amendment 1** (Technical modifications requested by local RD&I, alterations to sampling schedule) *Submitted 18 March 2016* **Amendment 2** 14 April 2016 (Adding the Royal Liverpool Hospital as co-sponsor) *14 April 2016* **Amendment 3** (Adding text of a study website) *10 May 2016* **Amendment 4** (Expanding inclusion criteria and adding extra samples) *7 February 2017* **Amendment 5** (Specific approval to use the DocMail® service to send letters from primary care practices within the Merseyside Clinical Commissioning Group) *19 July 2017* **Amendment 6** (Amending the protocol to remove certain criteria of restricted medications and the placebo group) *18 October 2017*

Figure 2.1: Overview of ethical approval process

General outline of NHS approval process	Specific to "Aging and Immunity" study
Research Development and Innovation department (RD&I) - Study number and title established	**Number** 5174 **Title** Experimental Human Pneumococcal Carriage Working towards a nasal vaccine for pneumonia: The effect of age on immune function **Short title** EHPC: Aging and Immunity
Clinical Research Unit (CRU) approval: risk management plan, discussion at operational group meeting, testing of emergency phone number	Discussed at operational group meeting on 10 February 2016
Set up Data Monitoring and Safety Committee (DMSC)	Already in place from previous studies
Complete Governance Registration Information Document (GRID) and Declaration of Interest (DoI) form from RD&I; Engage with finance and pharmacy	11 February 2016
JRO (Joint Research Office) application	11 February 2016
Review at local RD&I meeting (with independent peer review) – provisional intent to sponsor letter granted	29 February 2016 *From this point, we were permitted to advertise and hold information sessions, but not to consent participants for the study*
Finance and insurance agreements	31 May 2016
Material Transfer Agreements (MTAs)	
Other approvals – Directorate manager, pharmacy	
Site Specific Information Form (SSIF) submission on IRAS – only to be submitted when ready to start (commences 70-day countdown for recruitment)	24 March 2016
Trust agree to sponsor	31 May 2016
Advertising and recruitment	First consent 1 June 2016

Figure 2.2: Overview of NHS approval process

2.2 Recruitment and advertising

All advertisement materials required approval by the REC before use (See APPENDIX 3 for an example), and only locations listed in the ethically-approved study protocol could be used for advertising. Advertisements inviting volunteers to participate were placed on:

- Physical notice boards in public and private areas, including libraries, bingo halls, gymnasia and GP waiting rooms;
- The intranet/internet of local universities, colleges, LSTM and Royal Liverpool University Hospital (RLUH);
- Social media including Facebook and Twitter;

Participants were also approached based on their prior consent to receive generic research communications (the Consent4Consent database, established by the RLUH). For studies of particular population groups, we conducted targeted outreach and public engagement events at (among others) adult education centres, bowling clubs, food fairs, libraries, retirement associations and Women's Institute meetings. Finally, a collaboration between the Clinical Research Network (CRN) and local primary care centres could request practices to screen their patient lists against the study inclusion/exclusion criteria and send letters of invitation to potentially eligible participants.

Interested persons were asked to contact the research team by phone, text message or email for further information. Potential participants were sent a copy of the participant information sheet and invited to contact a member of the research team if still interested in participating.

Potential participants were invited to discuss the study during a 45-minute appointment (including a 30-minute group presentation); family members were welcome to attend these appointments as well. They were asked to demonstrate that they understand the study's objectives, associated risks and potential benefits. At this point, they had an opportunity to discuss the study with a study clinician and ask questions privately; they

were given an unrestricted amount of time to decide whether to participate or not. If individuals agreed to participate in the study and met the inclusion criteria, they were invited to provide written informed consent. Once consent was obtained, the participant's GP was permitted to share their medical records (either by sending an electronic summary or filling out a questionnaire (see APPENDIX 5)), and this also contributed to the eligibility assessment.

2.3 Safety considerations

The natural history of pneumococcal colonisation suggests that the risk to adults of developing pneumococcal infection during EHPC very low: < 5% of adults are colonised at any time, while the incidence of invasive disease is 20/100 000 patient years. In addition, the following factors further supported our confidence in our ability to safely run the study:

- Specific inclusion/exclusion criteria were in place, to minimise the risk of pneumococcal disease;
- Serotype selection meant that bacteria of lower pathogenicity were used, rather than those typically associated with severe disease;
- Dose ranging studies were performed and published at the start of the EHPC programme, to lay a foundation for future studies (60);
- Specific safety measures were in place, including 24-hour access to the study team and standby antibiotics;
- At the time of ethics application for the "Aging and Immunity" study, the study team had six years of experience in human challenge studies involving over 400 participants, following very similar protocols, and facing the same safety issues. No episodes of pneumococcal infection or severe adverse events (SAEs) had occurred in any of our participants.

The risk for pneumococcal disease increases with both age and comorbidity (182, 183) and so the inclusion/exclusion criteria for the "Aging and Immunity" study were designed to minimise any risk of pneumococcal disease, balanced with a desire to recruit as representative a sample as possible.

2.3.1 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.

2.3.2 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;
- Any current treatment for asthma—to avoid excess risk of infection and minimise confounding effect of medications such as corticosteroids (an EHPC study exclusively enrolling participants with asthma was carried out at the same time as this study);
- 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

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

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;
- 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.

Of note, prior PPV23 receipt was not an exclusion criterion, as it is recommended for all adults aged ≥ 65 years (100) and therefore it was expected that the majority of participants above this age would have received this vaccine. PPV23 is not believed to affect the acquisition of nasopharyngeal colonisation (102), and so a pragmatic decision was made to accept volunteers who had received the vaccine.

Specific "stop criteria" based on findings on pre-screening physical examination and investigations were also defined for this study.

2.3.3 Pre-screening and "stop criteria"

In EHPC studies of younger volunteers, baseline clinical screening (medical history, clinical exam and full blood count) were performed on the same day as their baseline samples (e.g.

pre-inoculation nasal wash, baseline serum etc). For older volunteers, a more comprehensive clinical assessment was required to determine that a participant was safe to enrol, and there was greater potential for participants to be excluded pre-inoculation on clinical grounds. Therefore, all clinical/safety assessments were bundled together as a "pre-screening" visit (which could take place some weeks before inoculation), and scientific baseline samples performed at a screening visit the week before inoculation.

In addition to clinical history, medication review and clinical cardiac and respiratory examination (standard for all EHPC studies), all participants underwent electrocardiography (ECG) and spirometry. Two blood samples were sent at pre-screen: a full blood count (including haemoglobin, platelet count, white blood cell count and differential) and a renal profile (including sodium, potassium, urea and creatinine). If any of these assessments breached the protocol-defined "stop criteria" (Table 2.1), the participant would be excluded.

Table 2.1: Stopping criteria for use during participant assessment prior to commencing the "Aging and Immunity" 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

2.3.4 Risk stratification

Participants were stratified in age-defined risk categories (Table 2.2). The rationale for the age groups related to published pneumococcal disease rates (Centres for Disease Control and Prevention (183)) and clinical guidance for general use of PPV23 (age ≥ 65).

Table 2.2: 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

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.

2.3.5 The Data Monitoring and Safety Committee

A DMSC reviews accumulating data during a clinical trial and advises the sponsor on the future management of the trial. A DMSC for the EHPC studies (consisting of two experienced trialists—one clinician and one statistician) has been in place for 6 years. The DMSC's role was to discuss the safety report containing information on all participants, either at the request of the study's chief investigator, at the end of the study and/or in the event of any serious adverse events (SAEs). The study team would provide the DSMC with general safety reports at key points during the study and update them regarding progression to higher risk groups.

2.3.5.1 Serious Adverse Events

An SAE is an adverse event or adverse reaction that:

- Results in death, *or*
- Is life-threatening, *or*
- Requires hospitalisation or prolongation of existing hospitalisation, *or*
- Results in persistent or significant disability or incapacity, *or*
- Consists of a congenital anomaly or birth defect.

According to the EHPC study protocols, any unexpected (i.e. not listed in the protocol as an expected occurrence) related (i.e. resulting from administration of any of the research procedures) SAEs would be recorded and reported to the sponsors and DMSC (within 24 hours of the principal investigator becoming aware). In the event of any unexpected related SAE, the study would be stopped temporarily for investigation and any further work referred back to the REC for consideration within 7 days.

2.4 Study design

The study schedule and procedures are outlined in Figure 2.3. Following inoculation, participants were seen for a follow-up nasal wash on days 2, 7, 9, 14, 22 and 29. If experimental colonisation was not identified on or by day 14, the participant could omit the day 22 visit, as they were deemed to be at lower risk of pneumococcal diseases and thus required less intensive follow-up. Serum samples were taken at baseline and on day 29. All follow-up visits included a clinical assessment, and participants were asked to record their temperature daily for the first seven days, sending their temperature to the research team via text message.

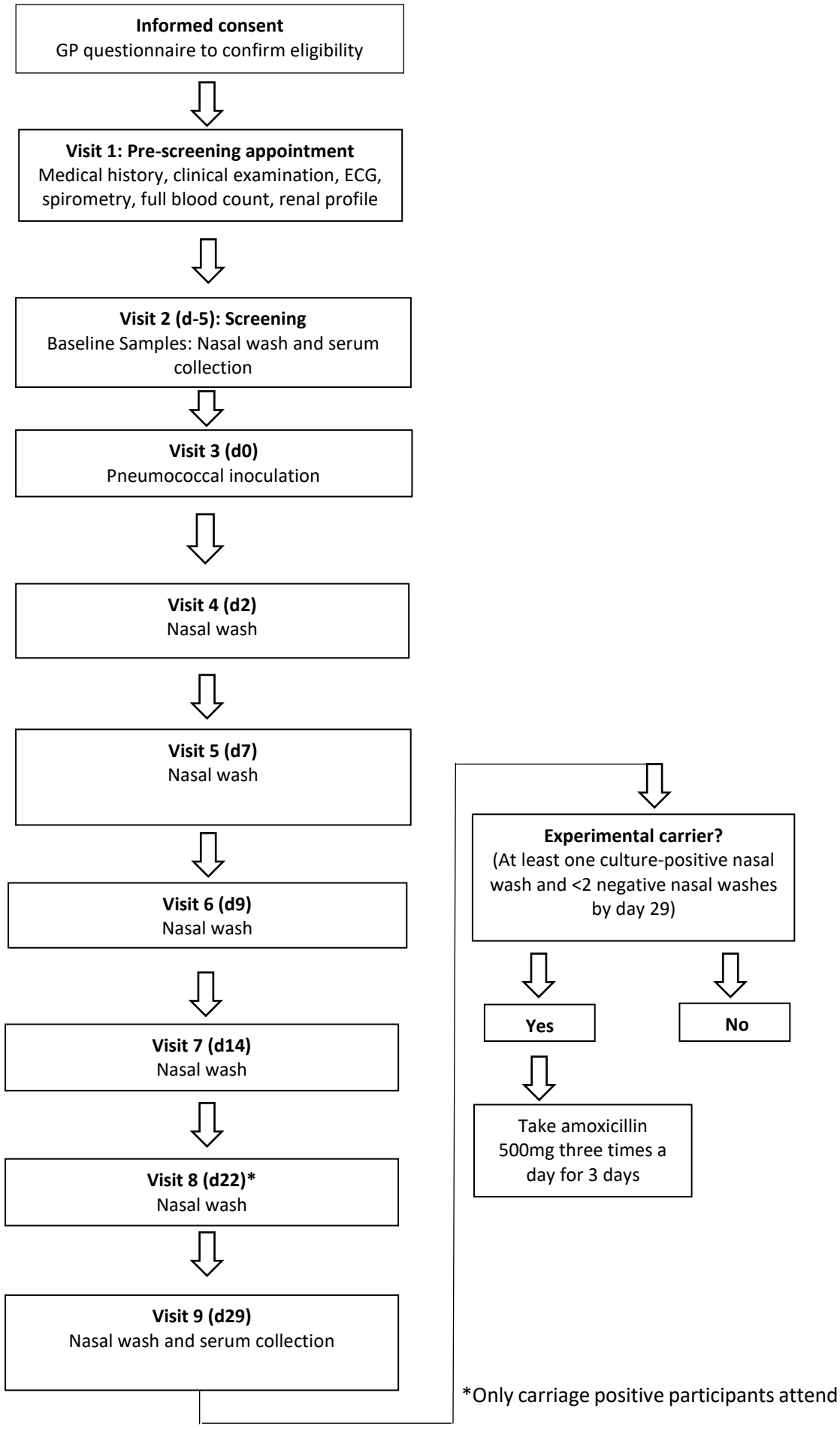

Figure 2.3: Study flowchart

2.5 Sample size calculation

In order to compare this group with previous healthy volunteers, a total sample of 64 inoculated subjects was required. A cohort of this size would permit the detection of a difference of 0.78 at $\alpha = 0.05$ with a power of 0.80, allowing for study non-completion. The underlying assumptions were:

- Experimental colonisation rate is 45% in healthy volunteers (previous published data) (60);
- Experimental colonisation rate falls to 10% in older people (i.e. relative risk is 0.22, expected from published cross-sectional studies of carriage prevalence suggesting very low carriage rates amongst older people) (39);
- Participant drop-out would lead to 10% non-completion rate.

Of note, we did not allow for natural colonisation with pneumococcus at baseline when calculating our sample size, as our literature review suggested that this would occur at negligible rates in an older population. In fact we did identify three "natural carriers" during this study. Therefore we performed post-hoc sensitivity analyses which excluded these participants.

2.6 Clinical procedures

2.6.1 Pneumococcal challenge

On inoculation days, each participant was seen by a study clinician to assess for any acute symptoms, to ensure that inoculation was safe to go ahead. The participant was then placed in a semi-recumbent position on a reclining chair. A P200 micropipette was used to draw up 80,000 colony-forming units (CFU) of pneumococcus suspended in 100µL 0.9% sodium chloride solution (normal saline) The plunger was depressed slowly and the pipette tip arced to and fro along the apex of the nostril. Direct visualisation of the tip was

maintained at all times. The participant was advised to breathe through their mouth, and then requested to remain in the reclining chair for 15 minutes.

2.6.2 Safety briefing

Participants were given a post-inoculation advice sheet, including emergency contact details and "red flag" symptoms (see APPENDIX 6: Post-inoculation safety leaflet). 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. They were also given a three-day course of amoxicillin 500mg tablets. 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.

2.6.3 Nasal wash

Nasal wash was essential to determine colonisation. Nasal washes were performed at baseline screening pre-inoculation and at pre-determined study-specific timepoints thereafter.

A modified Naclerio method was used for nasal wash in all EHPC studies (184). 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 (36, 37).

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.

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

2.6.4 Oropharyngeal swab

The participant's tongue was depressed using a tongue depressor, exposing the palatopharyngeal arch. Flocked swabs (Copan diagnostics, obtained from ThermoFisher, Basingstoke, UK) were used to make five small circular motions in contact with the mucosa of the palatopharyngeal arch in contact whilst avoiding the tongue. Swabs were placed in sterile containers with 1mL skim milk, tryptone, glucose, and glycerin (STGG) media (produced in-house) and transported to the laboratory at ambient temperature. Throat swabs were always taken prior to nasal sampling to ensure that the oropharynx was not inadvertently contaminated with nasal pathogens.

2.6.5 Saliva samples

Saliva collection was performed using the Salivette® system (Sarstedt AG & Co, Nümbrecht, Germany), comprising a small absorbent sponge with a sterile plastic container that can be directly transferred to a centrifuge. The participant was asked to hold the sponge in their mouth until they perceived it as being saturated with saliva. The sponge was transported to the laboratory in the container () on wet ice.

2.6.6 Blood sampling

Up to 50mL of venous blood was collected at protocol-defined timepoints using standard phlebotomy techniques.

2.7 Laboratory procedures

2.7.1 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 thawed isolate 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 (185). 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 (60).

2.7.2 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 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;
5. Gram-positive diplococci on microscopy.

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 (34). 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.

2.7.2.1 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 in CFU/mL of nasal wash. The remaining pellet suspension was stored at -80°C pending molecular testing.

2.7.3 Oropharyngeal swab post-collection processing

Upon receipt in the lab, the STGG samples were stored at -80°C pending molecular testing.

2.7.4 Saliva post-collection processing

Upon receipt in the lab, saliva was extracted from the cotton wool by centrifuging the salivette at 3700g for 3mins at 4°C. The saliva was resuspended in an equal volume of STGG with 50% glycerol and stored at -80°C pending molecular testing.

2.7.5 Pneumococcal detection using qPCR

We compared the performance of culture and qPCR on nasal wash specimens, and also used qPCR to analyse oropharyngeal swabs and saliva samples. The choice of target gene is key in pneumococcal qPCR, given the high number of shared genes between pneumococci and other commensal streptococci (186, 187). The autolysin gene (*lytA*) is generally considered to be the most specific for pneumococcus. Specificity can be further improved by targeting two genes, and only classifying samples that detect both genes as positive (44). Our analysis was simplified because we knew that our participants had been exposed to serotype 6B. Therefore, we defined a positive pneumococcal qPCR as detection of both *lytA* and the 6A/B capsular polysaccharide gene (*cpsA*).

2.7.5.1 Bacterial DNA extraction for qPCR

DNA was extracted using the Agowa Mag Mini DNA isolation kit (LGC Genomics GmbH, Berlin, Germany) from 200µL of oropharyngeal swab and 300µL of nasal wash pellet and saliva. Thawed samples were centrifuged at 20,200g for seven minutes. The pellet was re-suspended in protease mix with lysis buffer (300µL; one part protease to six parts buffer), 100µL of zirconium beads and 300µL of phenol. The suspension was disrupted twice using a tissue homogeniser (Precellys Evolution, Bertin Instruments, Montigny-le-Bretonneux, France) at maximum speed for three minutes, with cooling on ice in between, and then centrifuged at 9,391g for 10 minutes at 20°C for separation of phases. The aqueous phase was mixed with binding buffer (600µL) and magnetic beads (10µL), then vortexed and incubated for 30-120 minutes in an orbital shaker (Fisher Scientific, Loughborough, UK) at room temperature. The sample and magnetic beads were washed twice with 200µL wash buffer, after which the beads were dried at 55°C for 10 minutes. The bacterial DNA was eluted with 63µL of elution buffer, whereupon the beads were removed using a magnet separator and the eluent stored at -20°C.

2.7.5.2 *Multiplex amplification of* lytA *and* cpsA *genes*

The master mix included 9.375µL of diethylpyrocarbonate-treated water and 12.5µL of TaqMan® Universal PCR Master Mix (from ThermoFisher, Basingstoke, UK) and primers and probes for the two genes of interest (all from EuroGenTec, Southampton, UK). For *lytA*, we added 0.15µL of forward primer (5'-ACGCAATCTAGCAGATGAAGCA-3'), 0.15µL of reverse primer (5'-TCGTGCGTTTTAATTCCAGCT-3') and 0.075µL of probe (5'-(FAM)-GCCGAAAACGCTTGATACAGGGAG-(BHQ1)-3') (186). For *cpsA,* we added 0.1µL of forward primer (5'-AAGTTTGCACTAGAGTATGGGAAGGT-3'), 0.1µL of backward primer (5'-ACATTATGTCCATGTCTTCGATACAAG-3') and 0.05µL of probe (5'-(HEX)-TGTTCTGCCCTGAGCAACTGG–(BHQ1)-3') (188). All volumes were multiplied by the planned number of wells when preparing the master mix. We added 2.5µL of extracted DNA to 22.5µL of the master mix in each well. On each plate, two negative controls used 25µL master mix only, and each plate contained a standard curve of 10-fold dilutions of pneumococcal genomic DNA (10^6-10^1 copies/mL). Samples were assayed as duplicates, using thermal cycling conditions: 10 minutes at 95°C for DNA denaturation followed by 40 cycles of 15 seconds at 95°C and one minute at 60°C. All qPCR thermal cycling was performed in the same MX3005P Real-Time qPCR system (Agilent Technologies, Stockport, UK), with the detection threshold set at 3900 for *lytA* and 650 for *cpsA*.

The lower limit of detection was set at 40 cycles (48). The qPCR plate was repeated if:

1. There was DNA detected in either of the negative control wells;
2. Any of the standards between 10^6-10^2 were not detected;
3. Neither of the 10^1 standards was detected;
4. The cycle threshold difference between any duplicates of 10^3—10^6 standards was >1
5. R^2 of the two standard curves <0.98

6. The slope of standard was not between -3.1 and -3.6;
7. Efficiency was not between 90% and 110%.

2.7.6 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 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 CPS (Statens Serum Institut). The samples were serially diluted from 1:400 to 1:3,200 in the microtiter plates, then incubated for two hours at room temperature. Reference serum 98SF (US Food and Drug Administration (FDA)) was pre-absorbed in a similar fashion and serially diluted from 1:500 to 1:32,000, with one row left blank, as a standard curve. The samples were washed three times with PBS containing 0.05% Tween, 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.

2.7.7 Serotyping of natural carriage isolates

All isolates were initially tested using a commercially available latex agglutination kit (PneumoLatex, Statens Serum Institut, Copenhagen, Denmark). This kit comprises a series of reagents and an identification key. When pneumococci were identified on a plate, a single colony would be added to a drop of each reagent. Depending on which reagent or reagents were agglutinated, the identification key would identify which serogroup the isolate belonged to. Some serogroups (e.g. 3 or 8) cannot be subdivided into serotypes, and therefore no further identification procedures were needed for these. The remainder (e.g. serogroup 9 or 19) were sent to the Bacterial Microarray Group at St. George's University of London (BμG@S Biosciences) for molecular serotyping. This methodology (serotyping from genomic DNA on a molecular microarray) has been published previously and has been validated in a multicentre study (189-191).

2.7.7.1 Bacterial DNA extraction for molecular serotyping

The DNA was extracted using the QIAamp minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly: following centrifugation, the bacterial pellet was resuspended in 180 μL of an enzyme lysis solution comprising 20 mg/mL lysozyme, 20 mM Tris-HCl, 2 mM EDTA and 1.2% Triton. After 30 minutes incubation at 37°C, 200 μL Qiagen buffer AL and 20 μL proteinase K were added, and the mixture was incubated for 30 minutes at 56°C followed by 15 minutes at 95°C. The standard manufacturer's extraction protocol was followed, and DNA was eluted into 200 μL Qiagen buffer AE.

2.7.7.2 Molecular serotyping

The BμG@S microarray contains thousands of oligonucleotide probes corresponding to the genes that (in combination) determine the pneumococcal capsular serotype. Fluorescently-labelled genomic DNA is hybridised to these probes, and the fluorescent intensity is used to determine the serotype. Approximately 300 ng of the extracted DNA

(quantified using a NanoDrop spectrophotometer; ThermoFisher, Basingstoke UK) was fluorescently labelled with either ULS-Cy3 (green) or ULS-Cy5 (red) using the Agilent Technologies Genomic DNA ULS Labeling Kit (Agilent Technologies, Stockport, UK). (The red and green channels are analysed independently, meaning that one array can measure two samples.) The fluorescently labelled samples were hybridised overnight to the Senti-SP v1.6.0 microarray, according to the Agilent Array CGH protocol. The microarrays were washed and scanned using an Agilent microarray scanner and feature extraction software. The BµG@S Biosciences methodology applies a Bayesian algorithm to the results to determine which serotype is present in each sample (190).

2.7.8 Antimicrobial susceptibility testing

- Antimicrobial susceptibility testing of natural carriage isolates was performed according to methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (165), screening using disc diffusion and confirming minimum inhibitory concentrations (MICs) using gradient diffusion. The agents chosen, concentrations within each disc and susceptibility cutoff diameters are shown in Table 2.3. The agents were selected because of their prominence in national and local treatment guidelines (see Table 1.2 and Table 1.3 in the INTRODUCTION), with the addition of trimethoprim-sulfamethoxazole given its global importance. Antibiotics with rare/restricted indications that were unlikely to be encountered in a community setting (and therefore unlikely to exert selective pressure for resistance in healthy, community-dwelling adults, e.g. rifampicin, chloramphenicol) were excluded, but vancomycin was included because of its role as “antibiotic of last resort” against pneumococcus.

Table 2.3 Antimicrobial agents selected for susceptibility testing.

Class of antimicrobial agent	Specific agent used for AMR screening	Concentration of agent within disc	EUCAST zone diameter breakpoint (mm) (165)	
			S ≥	R <
β-lactam	Oxacillin	1µg	20	*
Macrolide	Erythromycin	15µg	22	19*
Fluoroquinolone	Norfloxacin	10µg	11	*
Tetracycline	Tetracycline	30µg	25	22*
Folate antagonist	Trimethoprim-sulfamethoxazole	1.25/23.75µg	18	15
Glycopeptide	Vancomycin	5µg	16	16

**Isolates whose zone diameters were less than the EUCAST breakpoint were subjected to additional MIC determination, as outlined in the text.*

2.7.8.1 Inoculum preparation

An inoculum was prepared by growing bacteria overnight on blood agar and suspending a selection of colonies from the plate in saline. The density was confirmed to be equivalent of 0.5 McFarland standard using a multiplate spectrometer (FluoStar Omega, BMG Labtech, Ortenberg, Germany). 100µL of suspension was pipetted into microplate wells, with 100µL saline as a blank, and optical densities (OD) were measured at 625nm. An OD of 0.08—0.13 at 625nm equates to a 0.5 McFarland standard (192). For internal consistency, both between samples and within replicates of samples, we aimed for a blank-corrected OD of 0.1±0.01—inocula were diluted with additional saline if their OD was too high, or supplemented with bacterial colonies if too low. If an acceptable OD was not achieved within 60 minutes of commencing inoculum preparation, a fresh inoculum would be prepared from the original plate.

2.7.8.2 Inoculation

Plates were inoculated within 60 minutes (ideally within 15 minutes) of inoculum preparation, using MH-F media: Mueller-Hinton agar supplemented with 5% defibrinated

horse blood and 20 mg/L β-nicotinamide adenine dinucleotide (ThermoFisher, Basingstoke, UK). A sterile cotton swab was dipped in the inoculum and streaked over the agar plate three times, rotating the plate by 60° between each pass, thus achieving a confluent lawn of growth.

2.7.8.3 Antimicrobial disc application

Within 15 minutes of inoculation, six discs (one containing each antibiotic of interest; ThermoFisher, Basingstoke, UK) were firmly applied to the surface of the agar, evenly-spaced apart, using a disc dispenser. The plates were inverted and incubated face down in stacks of no more than 5 for 16–20 hours at 35°C (± 1°C) with 5% CO_2.

2.7.8.4 Disc diameter assessment

Following incubation, inhibition zones were read from the front with the naked eye, using reflected light, and disc diameters measured using a ruler. The published EUCAST disc diameters (Table 2.3) were used to classify isolates as sensitive or resistant, with additional MIC measurement if indicated. Isolates identified as intermediate susceptibility on disc diffusion were classified as resistant.

2.7.8.5 Minimum inhibitory concentration determination

MICs for isolates identified as potentially resistant on disc diffusion were determined using Etest strips (BioMérieux, Basingstoke, UK), following the manufacturers' instructions. Briefly, an inoculum of 0.5 MacFarland units was prepared—in brain heart infusion broth (ThermoFisher, Basingstoke UK) rather than saline—and plated on MH-F agar following the same procedures used for disc diffusion. An Etest strip was applied using sterile forceps so that the entire strip was in complete contact with the agar surface. Plates were incubated face down in stacks of no more than 5 for 20—24 hours at 35°C with 5% CO_2. MICs were defined as the point showing no growth viewed from the front of the plate, using reflected light with the lid removed. EUCAST breakpoints are given in Table 2.4:

Table 2.4: EUCAST antibiotic breakpoints

Class of antimicrobial agent	Specific agent tested	MIC breakpoint (mg/L) (165)	
		S ≤	R >
β-lactam	Benzylpenicillin (infections other than meningitis)	0.06	2
	Benzylpenicillin (meningitis)	0.06	0.06
	Ampicillin	0.5	2
	Ceftriaxone	0.5	0.5
Fluoroquinolone	Levofloxacin	2	2
Macrolide	Clarithromycin	0.25	0.5
Tetracycline	Doxycycline	1	2

2.7.8.6 Quality control

When preparing the inocula for disc diffusion, colony counts were performed on serial dilutions from each inoculum to retrospectively validate the OD results from the spectrometer. In the case of *Escherichia coli,* 0.5 McFarland is accepted as equating to 1 to 2×10^8 CFU/mL; our pneumococcal colony counts were all between 0.5 and 3×10^8 CFU/mL.

Following EUCAST recommendations, the AST methodology was optimised using a known resistant strain, and one plate of this strain was included with each batch of test samples. *Streptococcus pneumoniae* NCTC 12977 (also known as ATCC 49619 and obtained from Public Health England), is a serotype 19F pneumococcus originally isolated from a sputum sample in Arizona and has low-level, chromosomally-mediated penicillin resistance. EUCAST has published the antimicrobial disc diameter ranges that should be observed when testing this strain (193), and readings by two independent readers were consistently within these limits, with the exception of co-trimoxazole (1—2mm wider than predicted on

two occasions). No cause for this could be identified (other antibiotic discs on the same plate had diameters within the predicted range). This may bias the study towards underestimating the rates of co-trimoxazole resistance.

Two independent readers measured each inhibition zone. For sensitivity, both the individual readings and the average of the two readings were checked against the EUCAST breakpoints. While inter-reader variability of 1—2mm was common, all readings (separate or averaged) were in agreement when classifying isolates as sensitive or resistant. The full procedure was replicated using a freshly-prepared inoculum for resistant isolates and in cases of discrepancies or suspected cross-contamination. Measurement by two independent reviewers was not mandated for MIC measurements, as the Etest results are simpler to interpret.

3 Establishing Experimental Human Pneumococcal Carriage in Older Adults

3.1 Background

As outlined in the INTRODUCTION, anti-pneumococcal polysaccharide immunity is diminished in older adults. This immunodeficiency affects both natural and vaccine-induced antibodies. It remains unclear whether pneumococcal colonisation occurs at high rates in older adults or, if it does, whether it is an immunising event.

If EHPC could be established in older volunteers, it would answer the questions of how susceptible this population is to nasopharyngeal colonisation. It would also open the possibility of vaccine trials (with an endpoint of colonisation acquisition) in an older population, particularly if the rate of experimental colonisation is high. Finally, EHPC in older people would provide a platform for experimental studies of mucosal immunity and the systemic response to colonisation in this population, including serum anti-capsular polysaccharide (CPS) antibody levels.

3.1.1 Aims

1. To establish EHPC in a cohort of adults aged ≥50 years;
2. To determine the rate of experimental colonisation in older adults;
3. To describe the dynamics of experimental colonisation (density and duration) in older adults;
4. To assess demographic and medical factors associated with experimental colonisation in older adults;
5. To assess the safety of EHPC in older adults.

3.2 Methods

3.2.1 Clinical procedures

Ethical approval, inclusion/exclusion criteria, participant recruitment and clinical procedures are summarised in sections 2.1, 2.2, 2.3, 2.4 and 2.6. Pneumococcal vaccination (PPV23) status was determined by review of each participant's primary care medical record.

3.2.2 Laboratory procedures

Nasal washes were tested for the presence and density of *S pneumoniae* as outlined in section 2.7.2.

3.2.3 Study objectives

The primary endpoint was the rate of colonisation, as determined by the detection of *S. pneumoniae* serogroup 6 by classical bacterial culture methods from one or more nasal wash samples in the first 14 days following initial pneumococcal challenge. Secondary endpoints included colonisation density, colonisation rates by age and by pneumococcal vaccination status, and the safety and tolerability of EHPC in older participants.

3.2.4 Statistical analysis

Descriptive statistics were used for reporting participant characteristics and microbiology results, with χ^2 or Fisher's exact test used to compare colonisation rates between different groups, where appropriate. We did not recruit a dedicated cohort of younger controls to directly compare carriage rates. Instead, for comparative purposes, we use aggregate results from colonisation studies of serotype 6B in young volunteers conducted between January 2015 and April 2017 (n = 225).

Participants who became colonised did not necessarily have colonisation detected at every single time point—this is generally assumed to represent low-density colonisation rather

than true clearance and re-acquisition of experimental colonisation. When calculating average colonisation densities at each timepoint, temporarily non-colonised participants (as opposed to "never-colonised" participants) were assigned a colonisation density of 0 CFU/mL. Total bacterial density over the time of the study was defined as the area under the density curve (AUC). The AUC was calculated according to the trapezoid rule using values of [log10 (bacterial density+1)] for each interval, with all participants assigned a density of 0 CFU/mL on inoculation day.

We used binary logistic regression to look for associations between demographic variables and colonisation outcomes. In particular, we hypothesised that older age would be associated with reduced odds of developing experimental colonisation. We also explored whether male sex, PPV23 status, smoking history and/or receipt of statin therapy would be associated with developing experimental colonisation. We used the p value of the Wald statistic to establish whether each predictor was significantly associated with colonisation in univariate analysis, and pre-specified that we would include any variables with a p value ≤ 0.2 in an adjusted multivariate model, eliminating any that became non-significant using backward stepwise regression.

All analyses were performed using SPSS version 24 (IBM, New York).

3.3 Results

3.3.1 Participant characteristics

The first participant was inoculated on 13 June 2016, and the last was inoculated on 19 February 2018. The recruitment process and screening outcomes are outlined in Figure 3.1. The oldest participant was 80 and the median age was 64 years. The baseline characteristics are outlined in Table 3.1, with a more detailed age breakdown in Figure 3.2. Ten participants were excluded on the basis of abnormal clinical findings at their pre-

screening visit, all of whom were referred for appropriate follow-up investigations and care.

Table 3.1: 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%)
Number prescribed statin therapy	2 (5.9%)	5 (20%)	2 (40%)	9 (14.1%)
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 (23, 3, 15)

**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)*

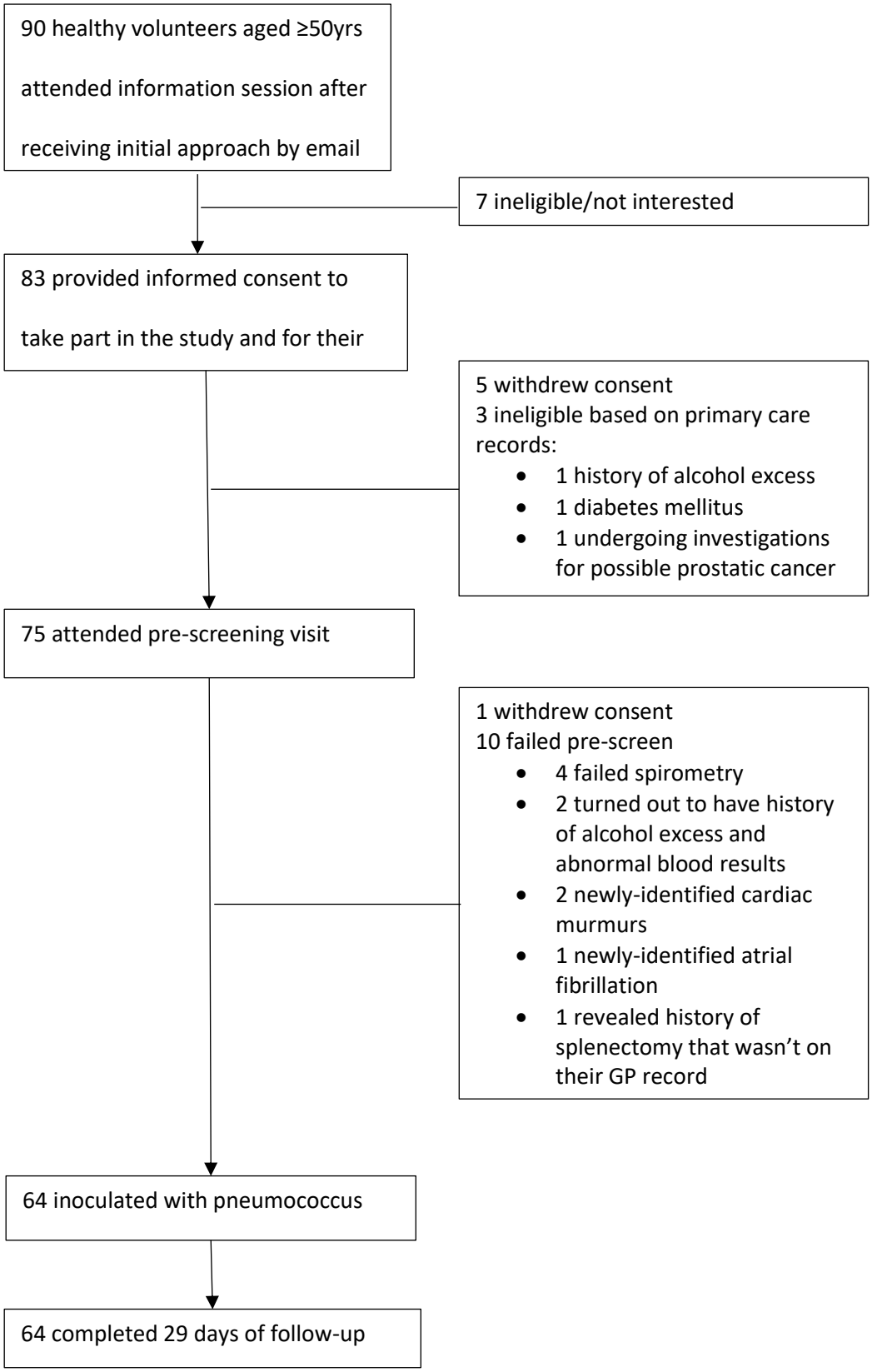

Figure 3.1: Outcomes from recruitment, consent and pre-screening processes

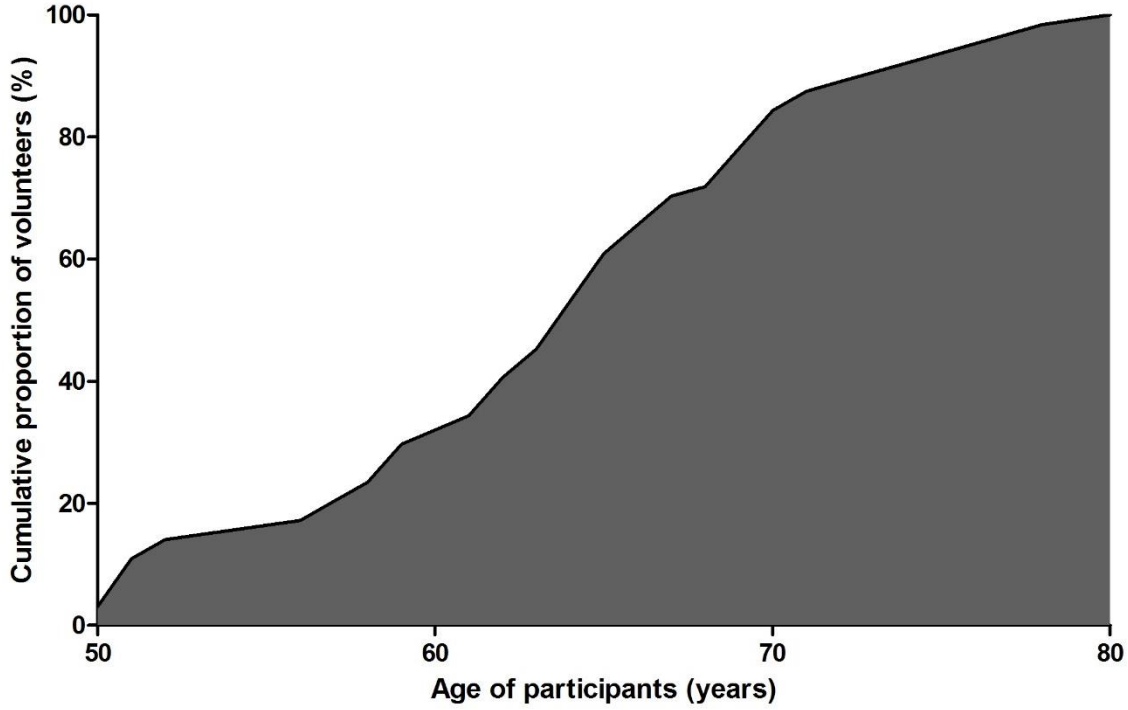

Figure 3.2: Cumulative proportion graph showing the age distribution of inoculated participants

3.3.2 Microbiological results

The median inoculation dose was 84,333 CFU/mL (range 69,333—92,833 CFU/mL). We detected experimental colonisation by day 14 in 25 participants (39.1%) (Table 3.2, Figure 3.3). When baseline natural carriers were excluded, the rate was 37.7% (23/61). Three participants missed visits—two non-carriers missed day 14 (attending day 22 instead) and one carrier attended their day 29 visit on day 22.

Table 3.2: Microbiological results

Total number of participants	64
Developed experimental colonisation	25
Colonised having been negative at baseline	23
Colonised on top of prior natural carriage	2
Maintained natural colonisation without developing experimental colonisation	1
Developed natural colonisation during the study	3
Experimental colonisation density, mean (SD) (CFU/mL of nasal wash)	
Day 2	6,396 (20,544)
Day 7	42,558 (213,895)
Day 9	141,649 (762,178)
Day 14	5,251 (19,187)
Day 22	15,012 (63,566)
Day 29	1,732 (14,699)

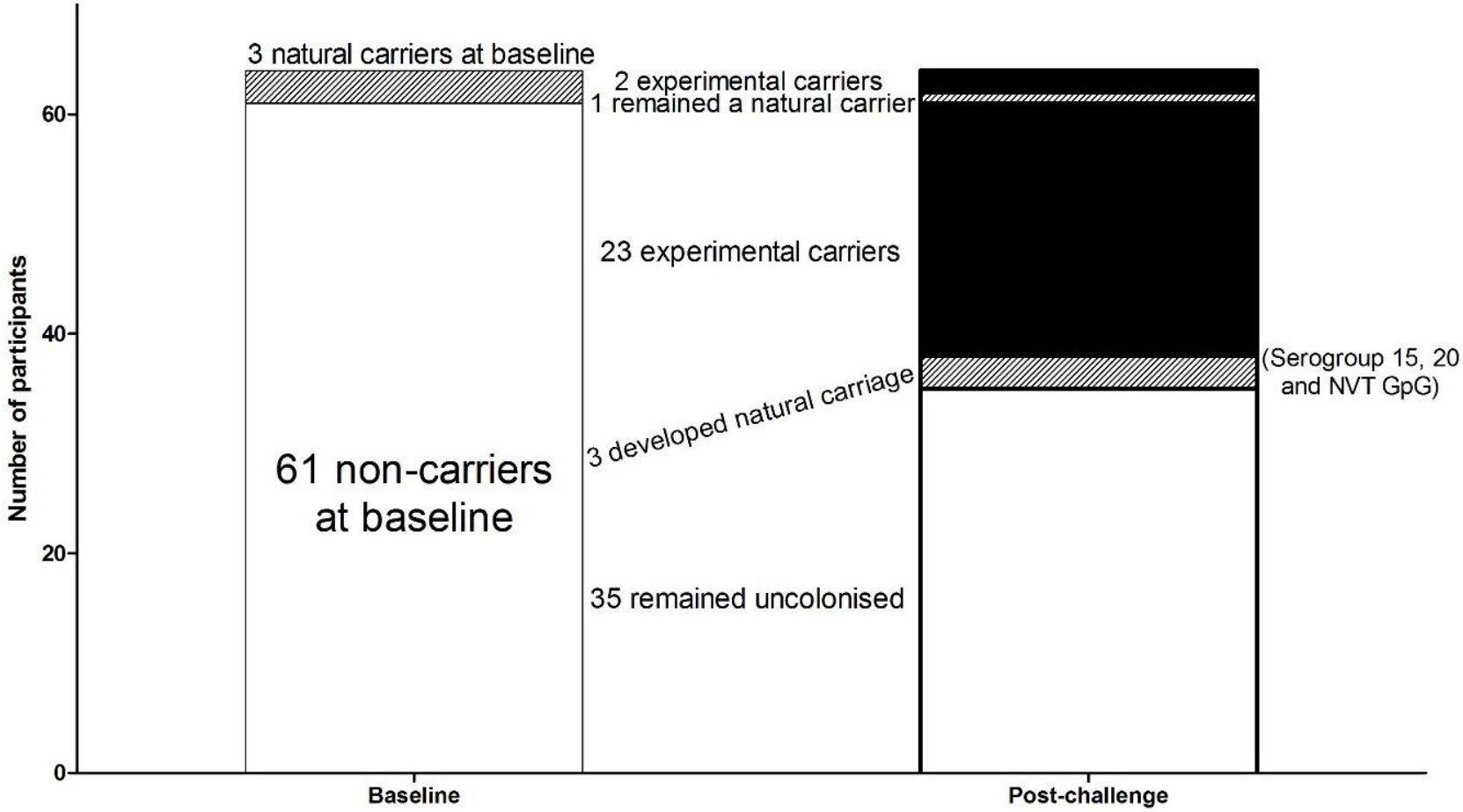

Figure 3.3: Microbiological status of participants pre- and post-inoculation

(NVT GpG: Non-vaccine type, Group G)

As shown in Figure 3.3, three participants developed incident natural carriage during the study. Two carried for only one day, while the third had detectable colonisation over three weeks. Incident natural carriage is relatively infrequent in EHPC studies—as a comparison, in a study of 194 young adults carried out between 2015—2016, it occurred in four participants (study awaiting publication, data from EHPC internal safety records).

3.3.2.1 Colonisation rates in different age groups

The overall colonisation rate of 39.1% did not differ significantly from the 46.7% achieved in 225 young adults inoculated using exactly the same methods and during a similar time period (p = 0.281). A breakdown of carriage rates by age decile is shown in Table 3.3 and Figure 3.4. Carriage rates within the over-50 cohort did not differ by age decile (χ^2 for trend p = 0.146). In a sensitivity analysis excluding those with baseline natural colonisation, the differences still did not achieve statistical significance for the overall comparison (p = 0.212) or the age deciles (bearing in mind that all three natural carriers were aged <60 years).

Table 3.3: 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%	0.281
Age deciles:	50—59	19	9	47.4%	0.953
	60—69	31	13	41.9%	0.620
	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*

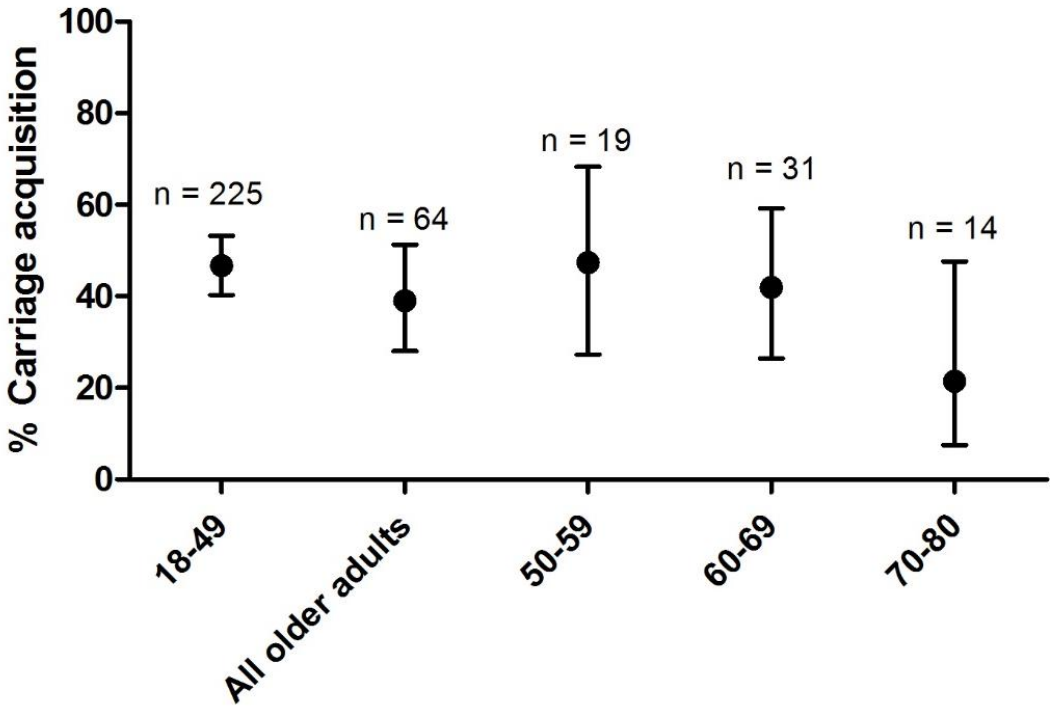

Figure 3.4: Experimental colonisation rates compared with a young adult cohort, and broken down by age decile within the older cohort.
Error bars represent 95% CI

3.3.2.2 Experimental colonisation density and duration

The average colonisation densities at each timepoint are given in Table 3.2. As shown in Figure 3.5, densities varied substantially within and between participants; the average AUC of density was 34.4 CFU.days/mL (95% CI 19.9—48.9). While the majority of carriers were identified as such on their first post-inoculation nasal wash, four were first identified on day 7, two on day 9 and one on day 14. The median duration of colonisation was 22 days, with 8/25 participants still having detectable colonisation at day 29, but four participants never carried beyond day 2. These ranges and fluctuations in density and variable durations of colonisation are similar to those seen in young volunteers following both experimental and natural colonisation (J Rylance et al, manuscript under review, pre-print deposited at https://www.biorxiv.org/content/early/2018/06/14/343319; (48)). The mean AUC of density did not differ between age deciles (p = 0.843 using ANOVA). Using

Pearson's correlation coefficient, we found no significant association between participant age and AUC of colonisation density ($r = -0.099$, $p = 0.636$; Figure 3.6).

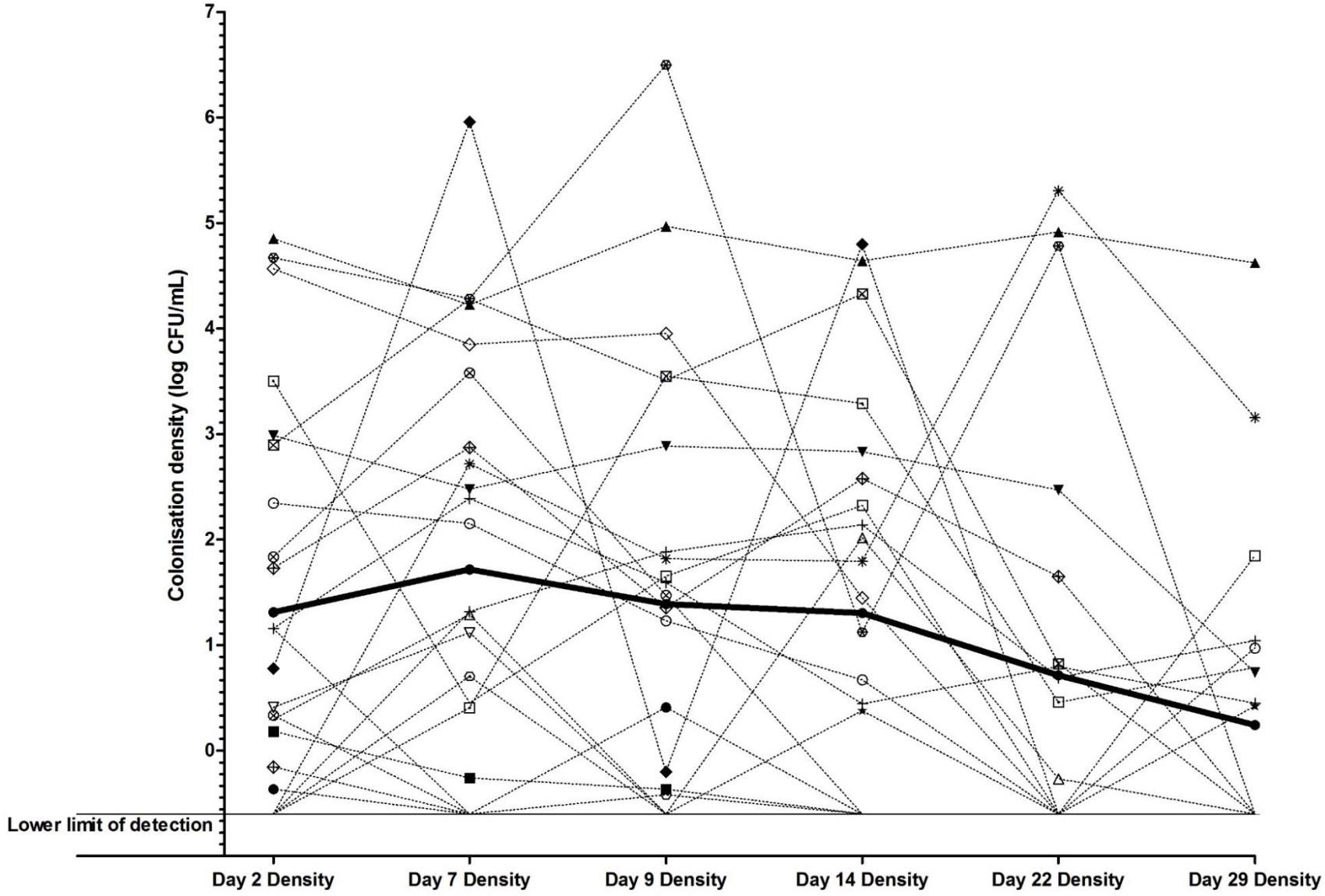

Figure 3.5: Colonisation density over time in experimental carriers
Each dashed line represents an individual participant; the heavy line represents the average density at each timepoint. The lines for participants identified as carriage-negative at certain time points are shown intersecting with the lower limit of detection for colonisation by culture-based methods.

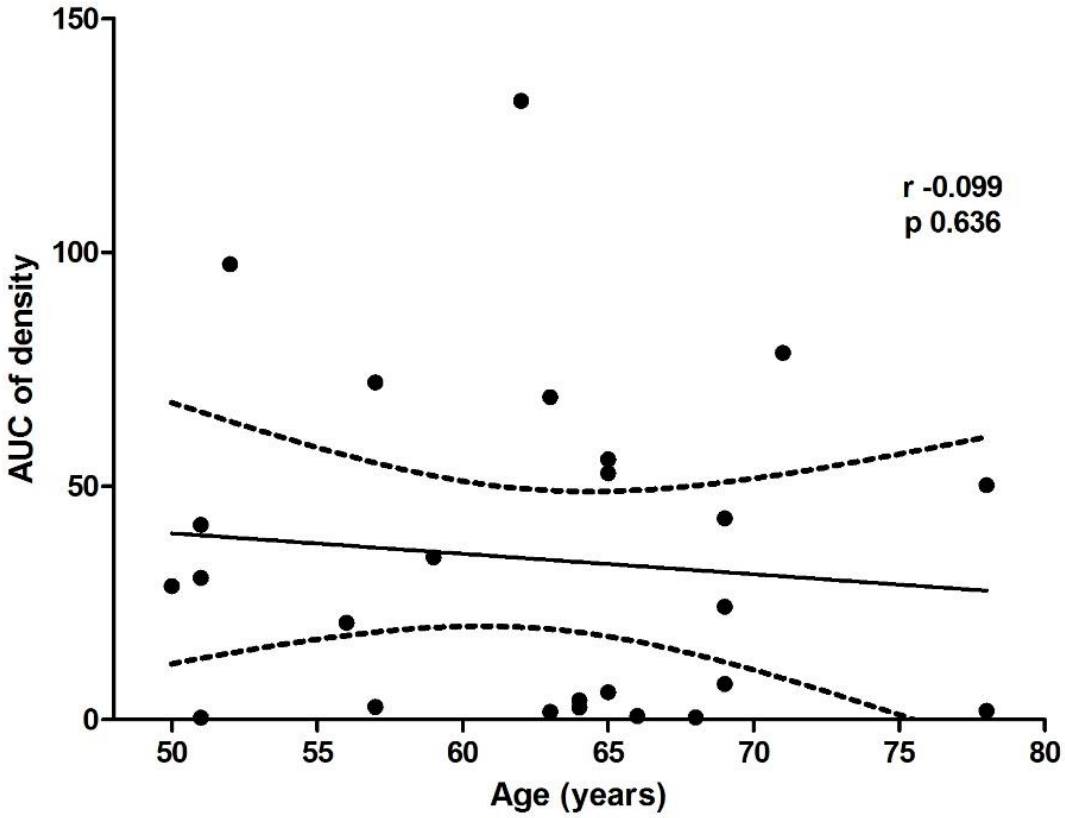

Figure 3.6: Correlation between participant age and colonisation density

(Dashed lines represent 95% CI)

3.3.2.3 Experimental colonisation in PPV23 recipients

Experimental colonisation was not affected by prior receipt of PPV23: 36.4% colonisation (n = 8/22) in PPV23 recipients versus 40.5% (n = 17/42) in 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) in the remainder. These results were similar when excluding baseline natural carriers—the overall comparison was 36.4% colonisation (n = 8/22) in PPV23 recipients versus 38.5% (n = 15/39) in non-recipients.

3.3.2.4 Experimental colonisation and statin therapy

Statins have anti-inflammatory properties, but their utility in infectious disease prevention or mitigation remains unclear (194-196). In our study, the rate of colonisation in participants taking statins was 33.3% (n = 3/9) versus 40% (n = 22/55) in the remainder (p = 0.5 by Fisher's exact test).

3.3.2.5 Logistic regression model

The results of univariate logistic regression exploring predictor variables for experimental colonisation are shown in Table 3.4. Since none of our predictor variables approached a statistically significant association with the outcome, we did not progress to multivariate regression.

Table 3.4: 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	0.313
Male sex	0.59	0.21—1.66	0.319
PPV23 receipt	0.84	0.29—2.43	0.749
Ex-smoker	0.56	0.19—1.65	0.292
Statin therapy	0.75	0.17—3.32	0.705

3.3.3 Safety of EHPC in older adults

There were no incidences of confirmed or suspected pneumococcal disease following inoculation, and no serious adverse events. No temperatures ≥38°C were detected while the participants were monitoring their temperatures daily for the first seven days post-inoculation. Six participants developed symptoms that required clinical review by the research team:

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

- 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.
- 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.
- One colonised participant (age 64) developed an earache on day 14; otoscopy was normal, and the symptoms gradually resolved spontaneously.
- 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.
- 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.

In addition, one participant (non-colonised, age 71) attended his GP with a sore throat 20 days after inoculation, and was given an analgesic spray which improved his symptoms. None of the above symptoms required extra appointments or reporting to the DMSC.

3.4 Discussion

EHPC is safe and feasible in older adults. The rates of colonisation in the oldest participants in this cohort were lower than those seen in younger adults, but these differences are not statistically significant. If there was a true difference in the rates of colonisation seen in the over-50s versus the under-50s (i.e. a relative risk reduction of 0.14) then the sample size required to demonstrate this would have been >300 subjects. The patterns of density and duration of experimental colonisation are similar to those in younger adults. This cohort was notable for a higher-than-expected rate of natural colonisation at baseline, as well as

three incidences of natural colonisation arising during the study. We include those participants who were natural carriers at baseline in our calculations, but sensitivity analyses without these participants produced similar results. We also found that PPV23 had no discernible effect on experimental colonisation, even when restricting the analysis to over-65s (the target population) who had been vaccinated within five years (the putative duration of protection). Furthermore, PPV23 had no effect on colonisation in a model that was adjusted for age, sex, smoking status and statin therapy. This is consistent with a previous meta-analysis of the effects of PPV23 (102). In the next chapter, we will present the results of anti-capsular antibody measurement in all participants, and assess if these titres are a stronger predictor of experimental colonisation than PPV23 status.

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

4 Experimental Human Pneumococcal Carriage in Older Adults—Immunological Findings

4.1 Background

As outlined in section 1.3.1 of the INTRODUCTION, all adults have circulating antibodies against pneumococcal capsular polysaccharide (CPS). While population studies have demonstrated diminished antibody concentrations with increasing age (65), the antibody kinetics following pneumococcal exposure have not been studied in older people. Therefore, we do not know if the fall in antibody levels with age reflects reduced exposure to pneumococcal antigens (i.e. colonisation) in older people or a true decline in immune function with age. In this chapter, we will explore the early antibody response to pneumococcal exposure.

The ideal timepoint to measure post-exposure anti-CPS antibodies has not been defined. One clinical study obtained "convalescent serum" any time between ten days and 12 weeks after pneumococcal infection (88), while a colonisation study had a ten-month interval between serum samples (29). However, vaccine studies have found that anti-CPS IgG levels reach their peak at four weeks post-PPV23 administration in both young and old adults (126, 127), and therefore we chose this timepoint for our study. This timeframe is also similar to that of previous EHPC studies in young adults, facilitating future analyses directly comparing immune responses in different age groups (60).

Pre-existing anti-CPS antibodies are hypothesised to be protective against pneumococcal colonisation. A previous EHPC study of young adults did not confirm this hypothesis (60)—baseline anti-CPS IgG levels were no different in volunteers who did and did not become colonised. We can explore whether this remains the case in older adults by measuring antibody levels prior to pneumococcal challenge in our cohort.

Antibody-mediated protection against colonisation is a key principle underpinning the childhood PCV13 programme. However, PPV23 has not been shown to prevent colonisation (102), and did not protect against EHPC in our cohort aged 50—80 (section 3.3.2.3). Comparing baseline antibody levels and colonisation outcomes in vaccinated and unvaccinated participants will allow us to delve deeper into this conundrum.

In addition, as outlined in section 1.3, anti-CPS antibody boosting has been demonstrated following pneumococcal colonisation (29), including EHPC (60). The role of pneumococcal colonisation in the maintenance of natural immunity has not been explored in older adults. Comparing pre- and post-challenge antibody levels in our cohort will allow us to examine this phenomenon. Multiple doses of pneumococcal polysaccharide do not necessarily result in proportionate cumulative increases in antibodies, particularly in older people (hyporesponsiveness, (117, 197)). The antibody responses to experimental colonisation in vaccinated participants will therefore be of particular interest.

4.1.1 Hypotheses

We test seven hypotheses in this chapter, informed by previous literature on the subject:

1. Higher baseline anti-CPS IgG levels are found in PPV23-vaccinated participants—if confirmed, this would justify subgroup analysis based on vaccine status for other hypotheses;
2. Post-PPV23 antibody levels are not different in participants for whom a long time had passed since vaccination, compared with more recent vaccinees. Opinions differ on the durability of the immune response to PPV23, and some authorities recommend revaccination after five years (99). However, well-powered studies have found that, while antibodies do decline in the years after vaccination, they remain elevated well above pre-vaccination titres at five years (119). Classifying all

participants who had *ever* received PPV23 as vaccine recipients would simplify our analysis.

3. High baseline antibody levels are not associated with colonisation outcomes (i.e. baseline antibody levels would not be different in carriers and non-carriers, similar to young adults in previous EHPC studies (60));
4. Experimental colonisation results in boosting of anti-CPS antibody levels (similar to young adults in EHPC (60));
5. Pneumococcal challenge without subsequent culture-positive colonisation does not have any effect on systemic anti-CPS antibody levels (similar to young adults in EHPC (60, 137));
6. Higher baseline antibody levels are associated with reduced bacterial density (as measured by AUC) in colonised participants (PCV13 vaccination of children and young adults is associated with lower colonisation density, suggesting that control of density is antibody-driven (107, 198));
7. Antibody levels and responses are diminished in the oldest age decile (65).

4.2 Methods

4.2.1 Clinical procedures

Participant enrolment, inoculation and colonisation determination was carried out as outlined in sections 2.2, 2.4 and 2.6. Pneumococcal vaccination (PPV23) status was determined by review of each participant's primary care medical record. Serum samples were taken at baseline and at 29 days (± 7 days) post-inoculation.

4.2.2 Laboratory procedures

Pneumococci were cultured as outlined in section 2.7, and colonisation density was determined as outlined in section 2.7.2.1. Anti-6B CPS IgG levels were measured in baseline and day 29 serum samples using ELISA, as described in section 2.7.6. We defined

experimental carriers as participants who had *S. pneumoniae* serogroup 6 isolated from nasal wash at any timepoint following inoculation.

4.2.3 Statistical analysis

The antibody titres (in ng/mL) were log-transformed to achieve a normal distribution prior to statistical analysis, and the transformed results are reported in this chapter. Fold change in antibodies was defined as the difference between the log-transformed day 29 and baseline levels. Baseline levels were compared between groups (e.g. carriers and non-carriers) using the unpaired t test, with the paired t test used for before-and-after comparisons. Correlations between continuous variables were assessed using Pearson's correlation. We pre-specified that, if we identified higher baseline anti-CPS IgG levels in PPV23 recipients, we would perform analyses on the whole cohort and also on subgroups defined by PPV23 status.

When reporting changes in antibody levels over time, we had a choice between presenting the mean values pre- and post-challenge within each group (e.g. carriers and non-carriers) or comparing the two groups based on the fold change in antibodies. Previous literature suggests that either approach is acceptable (60, 199), and both absolute levels and fold-changes are used in clinical practice (200, 201). As our analysis is largely exploratory, we present many of our between-group comparisons using both approaches where appropriate.

We used binary logistic regression to assess the effect of baseline antibody levels on the development of experimental colonisation and used linear regression to assess the effect of baseline antibodies on colonisation density (as defined by AUC). We pre-specified that we would adjust the antibody levels for age and sex, given their well-documented effects on anti-pneumococcal immunity (65), regardless of findings on univariate analysis (i.e. forced-entry method of multivariate regression). We excluded PPV23 status because its

effects would be largely accounted for by the antibody levels—this was supported by our initial finding that baseline antibody levels were indeed higher in PPV23 recipients. We used the p value of the Wald statistic (in logistic regression) or ANOVA (in linear regression) to establish whether each predictor was significantly associated with colonisation.

All tests were two-tailed, and a p value of < 0.05 was considered significant. All analyses were performed using SPSS version 24 (IBM, New York).

4.3 Results

For each serum sample, we selected the dilution that gave a blank-corrected optical density (OD) closest to 0.1 (or at least between 0.7 and 2.0) in both replicates, as these fell along the linear part of the standard dilution curve. We excluded replicates whose coefficient of variation (i.e. $\frac{\text{Standard deviation}}{\text{Mean}}$) was ≤25%. Fifteen sample pairs required repeat testing, either for poor agreement between replicates or insufficient dilution. Four participants' results were excluded for poor agreement between replicates despite multiple repeated rounds of testing, and an additional participant did not have a post-inoculation serum sample available; therefore results are reported for 59 participants.

4.3.1 Baseline antibody levels and PPV23

History of PPV23 receipt was associated with higher baseline anti-CPS IgG levels (Table 4.1).

Table 4.1: Baseline anti-CPS IgG levels, by vaccine status

	Prior PPV23 (n = 20)	No PPV23 (n = 39)	p value
Anti-6B CPS IgG at baseline, log ng/mL, mean (SD)	3.58 (0.28)	3.33 (0.37)	**0.01**

Anti-6B CPS Ig denotes immunoglobulin directed against serotype-specific capsular polysaccharides, as measured by ELISA.

There was no association between baseline antibody levels and time since PPV receipt, as shown in Figure 4.1.

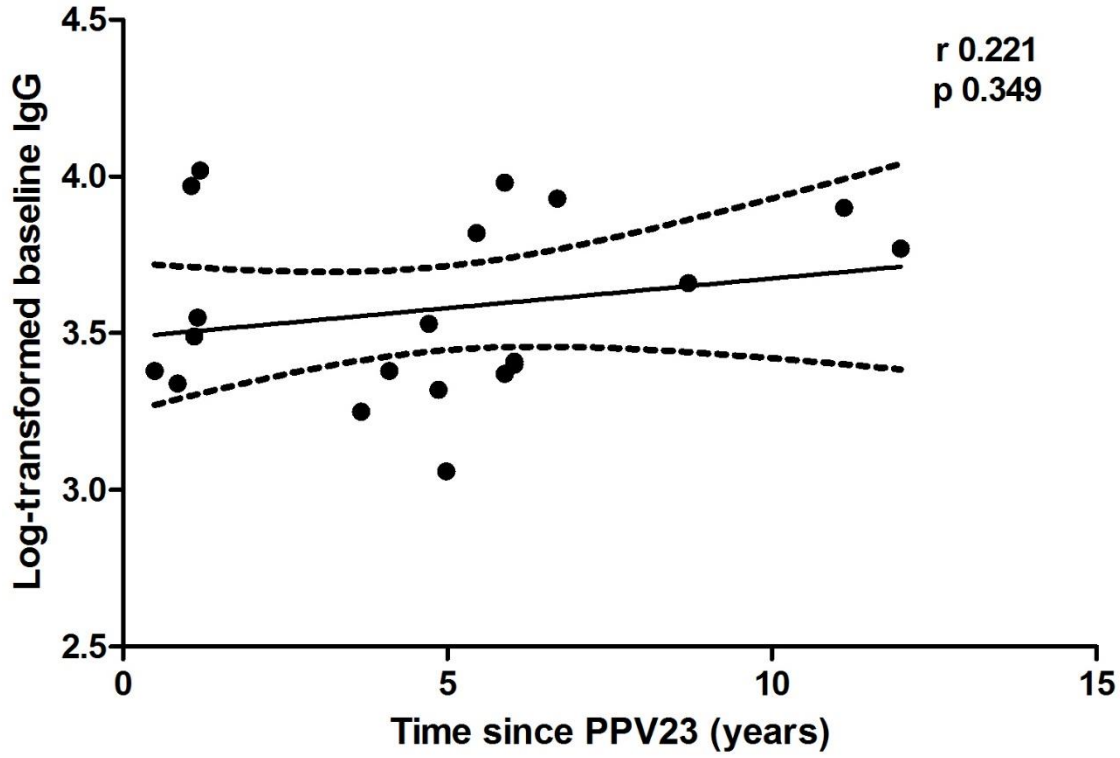

Figure 4.1: Correlation between baseline anti-CPS IgG levels and time since PPV23 receipt (N = 20; dashed lines represent 95% CI)

4.3.2 Antibody responses to experimental colonisation

Baseline and post-challenge antibody titres in colonised and non-colonised subjects are shown in Table 4.2 and Figure 4.2. The baseline levels—3.37 log ng/mL in non-carriers, 3.48 log ng/mL in carriers—did not differ between the two groups (p = 0.25).

We did not identify an increase in antibody levels following colonisation: The mean titre in colonised volunteers was essentially the same at baseline (3.48 log ng/mL) and on day 29 (3.47 log ng/mL). The mean titre in subjects who did not develop experimental colonisation fell to 3.27 log ng/mL at day 29 from 3.37 log ng/mL at baseline (p = 0.039).

Table 4.2: Immunological outcomes following pneumococcal challenge.

	Anti-6B CPS IgG log ng/mL, mean (SD)		p value
	Baseline	**Day 29**	
Colonised (n = 24)	3.48 (0.35)	3.47 (0.43)	0.958
Non-colonised (n = 35)	3.37 (0.36)	3.27 (0.41)	**0.039**

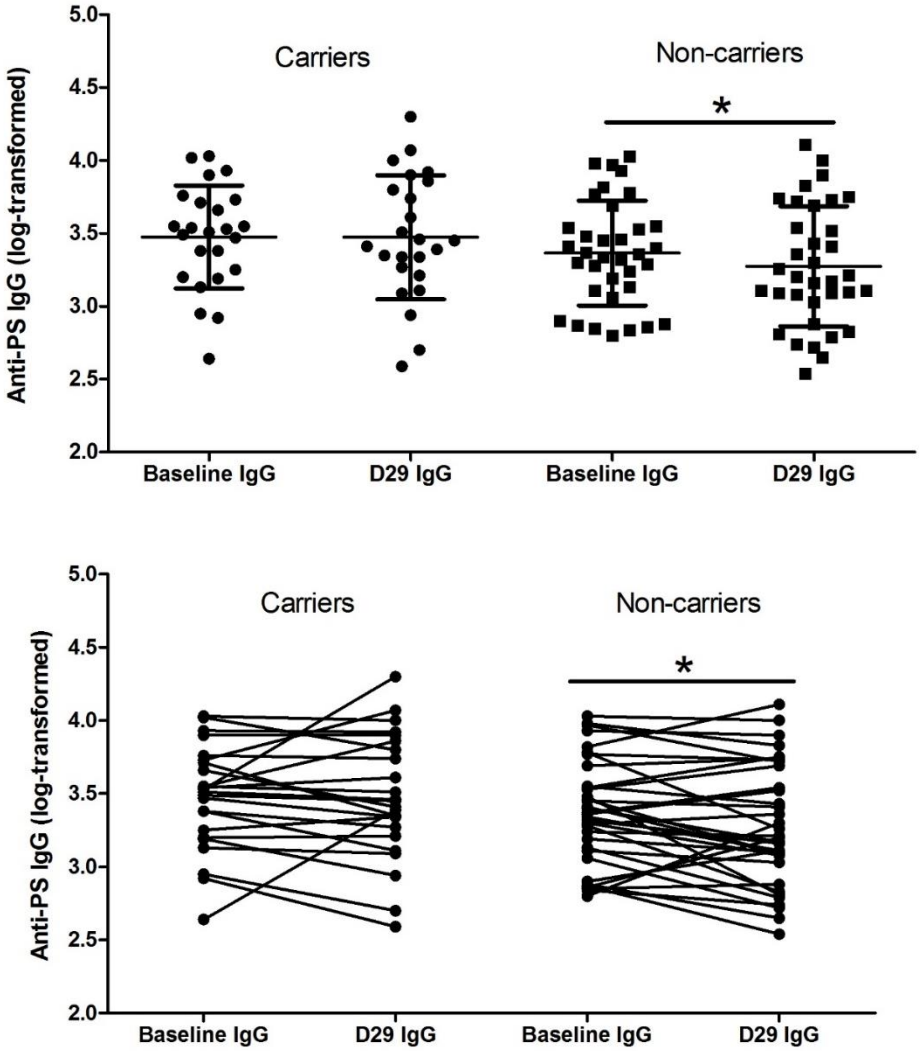

Figure 4.2: Anti-6B CPS IgG levels at baseline and day 29 following inoculation
*Each symbol represents a single participant. The lines and error bars in the first panel represent mean (SD); the lines in the second panel connect the baseline and day 29 values for each participant. N = 24 carriers, 35 non-carriers. * $p < 0.05$*

An alternative approach would be to analyse according to the fold-change in antibody levels following pneumococcal challenge. The differences between log-transformed baseline and day 29 values were normally distributed. The mean fold change in colonised

volunteers was -0.003 (standard deviation 0.29), while in non-colonised volunteers it was -0.09 (SD 0.25). This difference was not statistically significant (p = 0.217 by the unpaired t test).

After finding increased baseline IgG levels in PPV23 recipients, we repeated the analysis separately on PPV23 recipients and non-recipients, for the reasons outlined in the introduction to this chapter. The results excluding all PPV23 recipients are shown in Table 4.3 and Figure 4.3.

Table 4.3: Immunological outcomes following pneumococcal challenge, excluding PPV23 recipients

	Anti-6B CPS IgG log ng/mL, mean (SD)		p value
	Baseline	**Day 29**	
Colonised (n = 16)	3.43 (0.39)	3.47 (0.50)	0.581
Non-colonised (n = 23)	3.26 (0.34)	3.17 (0.37)	0.133

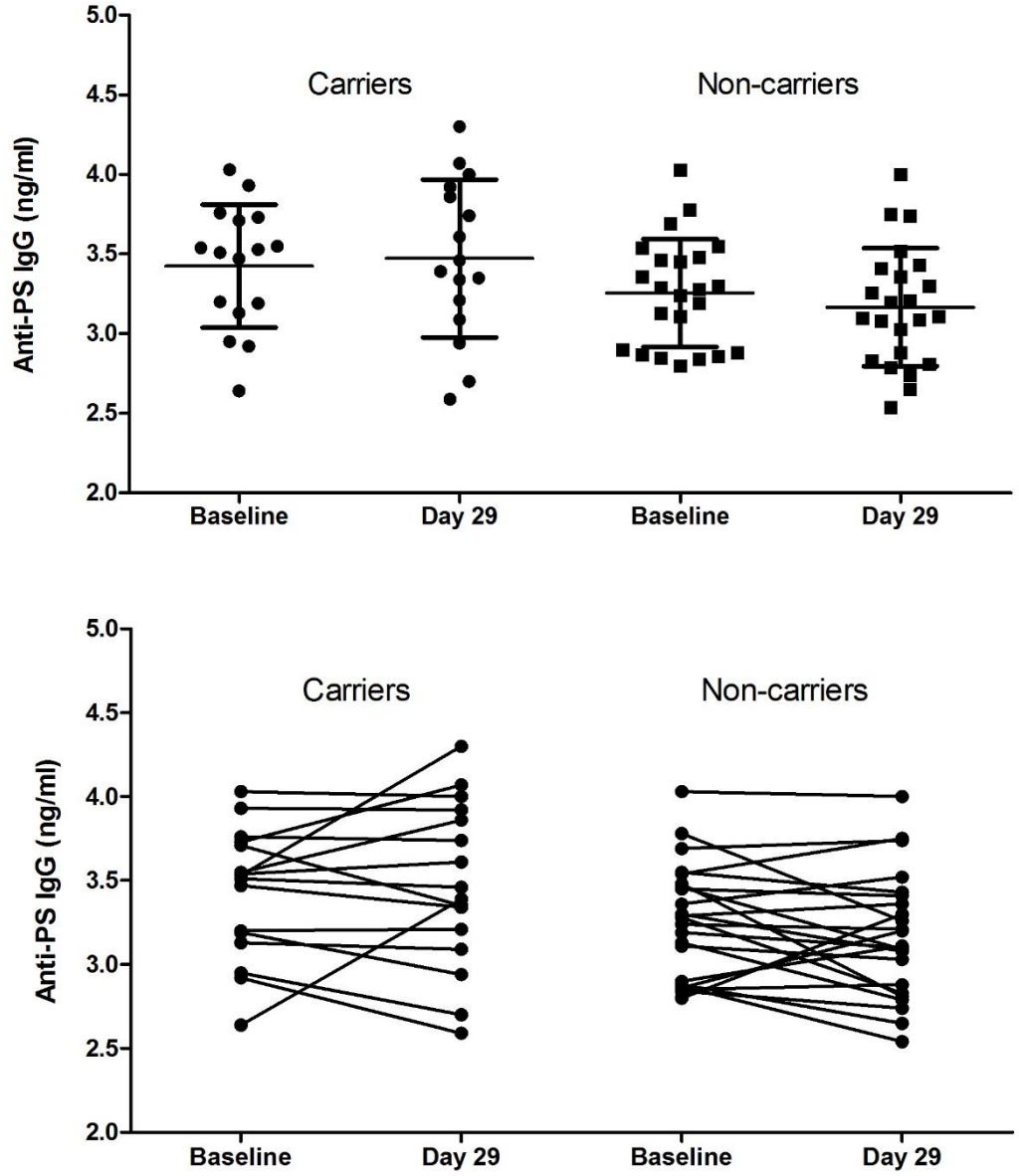

Figure 4.3: Anti-6B CPS IgG levels at baseline and day 29 following inoculation, excluding PPV23 recipients Each symbol represents a single participant. The lines and error bars in the first panel represent mean (SD); the lines in the second panel connect the baseline and day 29 values for each participant. N = 16 carriers, 23 non-carriers. There were no statistically significant differences within or between groups.

When PPV23 recipients were excluded, baseline anti-CPS IgG levels remained similar between carriers and non-carriers (3.43 vs 3.26 log ng/mL, p = 0.16). There were no changes in antibody levels in either colonised or non-colonised participants. The mean fold change in colonised participants was +0.05 (SD 0.34), versus -0.09 (0.28) in non-colonised participants (p = 0.17 using the unpaired t test).

Results for the remaining participants (i.e. only those that *had* received PPV23) are shown in Table 4.4 and Figure 4.4. There was a fall in antibody levels post-colonisation (from 3.58 to 3.47 log ng/mL) but this missed the threshold for statistical significance (p = 0.055). Non-colonised participants' antibody levels also dropped. The mean fold change was -0.2 (SD 0.13) in colonised participants versus -0.09 (0.21) in non-colonised participants (p = 0.91 using the unpaired t test).

Table 4.4: Immunological outcomes following pneumococcal challenge, PPV23 recipients only

	Anti-6B CPS IgG log ng/mL, mean (SD)		p value
	Baseline	**Day 29**	
Colonised (n = 8)	3.58 (0.27)	3.47 (0.26)	0.055
Non-colonised (n = 12)	3.58 (0.31)	3.5 (0.4)	0.143

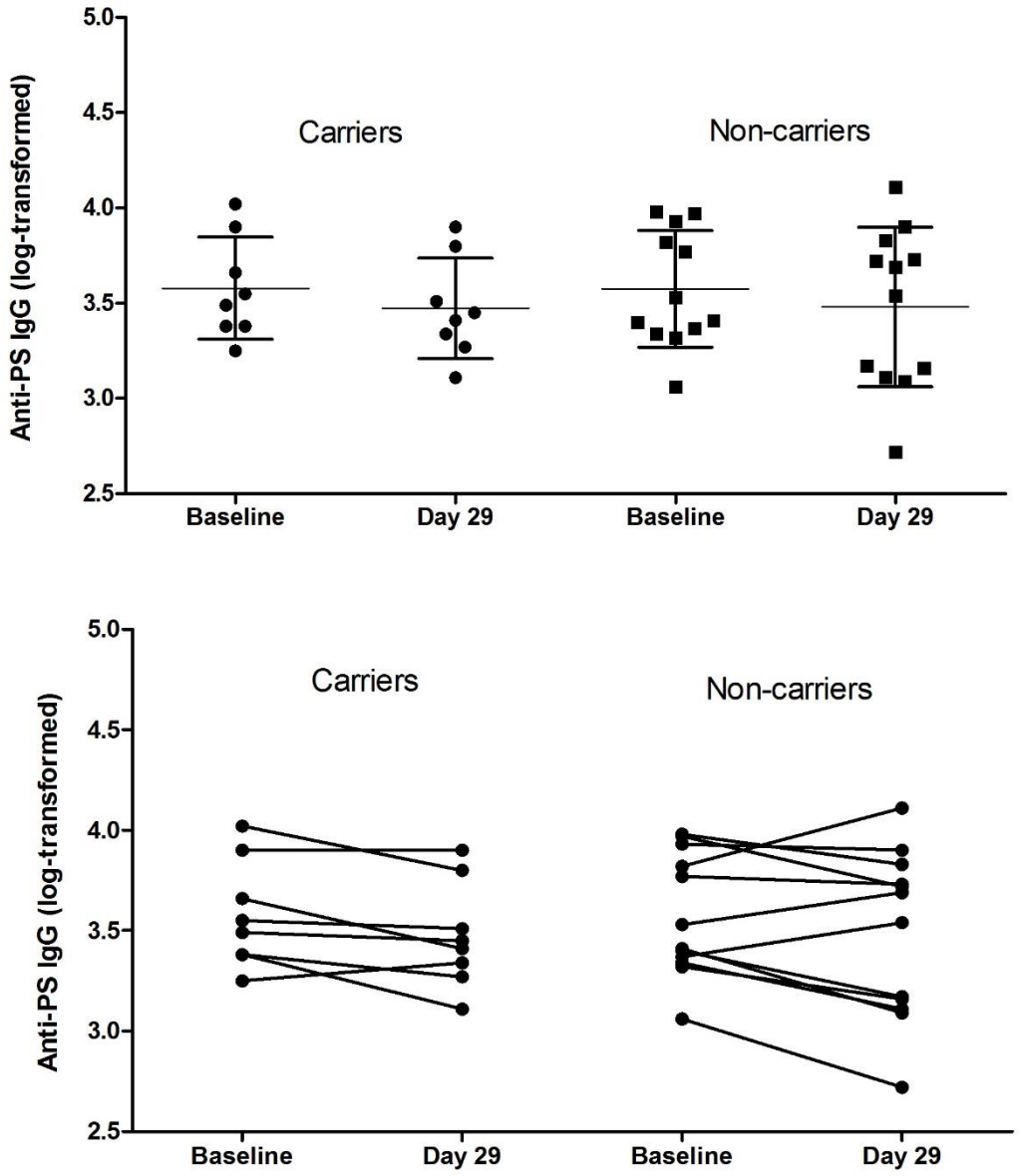

Figure 4.4: Anti-6B CPS IgG levels at baseline and day 29 following inoculation, among PPV23 recipients only

Each symbol represents a single participant. The lines and error bars in the first panel represent mean (SD); the lines in the second panel connect the baseline and day 29 values for each participant. N = 8 carriers, 12 non-carriers. There were no statistically significant differences within or between groups.

The mean fold change in vaccinated colonised participants (-0.1) was in the opposite direction to that of unvaccinated colonised participants (+0.05) but this was not statistically significant (p = 0.235).

4.3.3 Antibody responses and colonisation density

We used Pearson's correlation to assess the relationship between AUC of colonisation density and antibody levels within the colonised cohort. AUC did not correlate significantly

with day 29 antibody levels (r = -0.115, p = 0.59) or with fold change in antibody levels (r = 0.319, p = 0.128). There was a moderate correlation between higher baseline anti-CPS IgG levels and lower colonisation densities, the statistical significance of which was borderline (r = -0.401, p = 0.052; Figure 4.5). The correlations were weaker in participants who had never received PPV23. By contrast, correlation coefficients between antibody levels and reduced colonisation density were stronger in participants who *had* received PPV23, although they did not reach statistical significance. There were non-significant negative correlations between baseline antibodies and colonisation density (r = -0.57, p = 0.204) and between Day 29 antibodies and colonisation density (r = -0.53, p = 0.181), but not between fold change and density (r = 0.122, p =0.773).

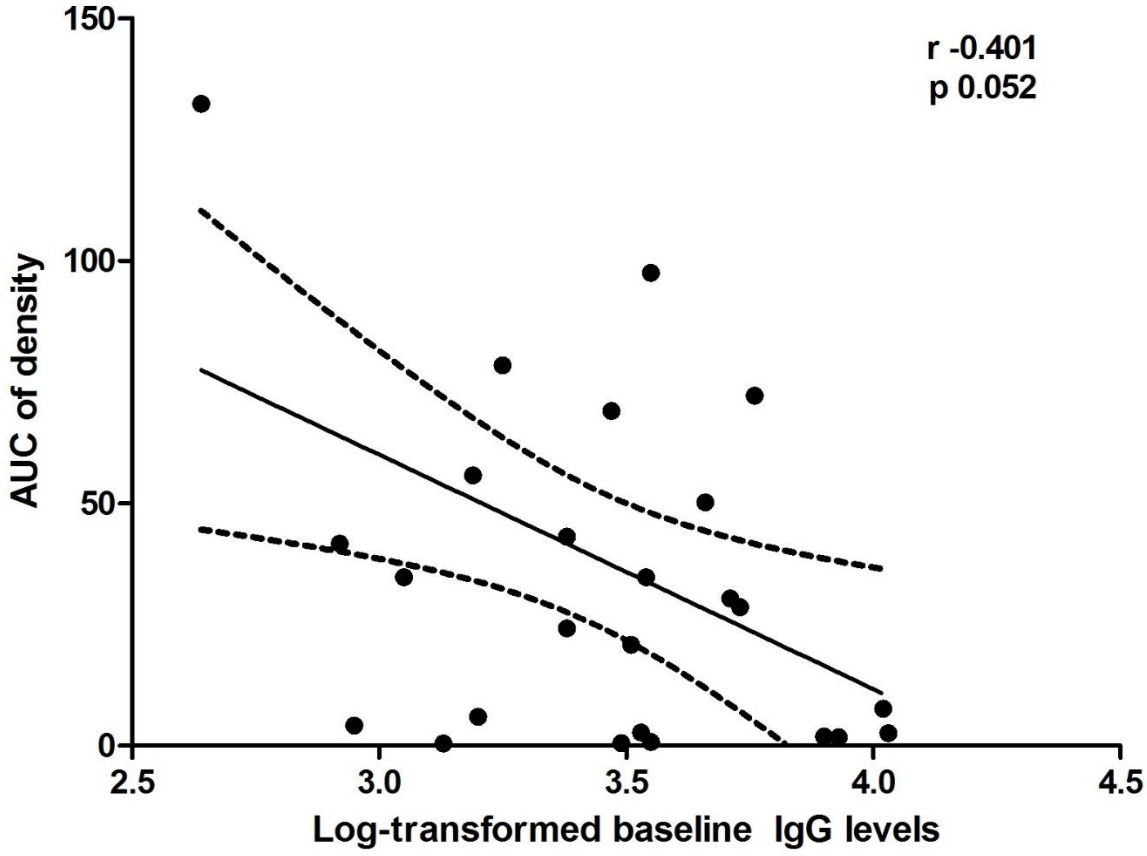

Figure 4.5: Correlation of baseline anti-6B IgG with area-under-the-curve of density in pneumococcal carriers (N = 24; dashed lines represent 95% CI)

4.3.4 Antibody responses and age

The mean baseline antibody concentrations in each age decile are shown in Table 4.5. There was no difference in baseline concentrations between the three groups (p = 0.23 using ANOVA, p = 0.366 when excluding PPV23 recipients). There was also no correlation between baseline antibody levels and age (r = -0.038, p = 0.817; PPV23 recipients excluded).

Table 4.5: Baseline anti-6B IgG levels and responses in different age deciles

		Age deciles			P value
		50—59 (n = 19)	*60—69 (n =26)*	*70—80 (n =14)*	
Baseline anti-CPS IgG titre, log ng/mL, mean (SD)		3.38 (0.37)	3.36 (0.37)	3.55 (0.29)	0.23
Fold change in antibody levels (log-transformed) post-challenge, mean (SD)	*Colonised*	+0.08 (0.35)	-0.05 (0.27)	-0.05 (0.17)	0.606
	Non-colonised	-0.17 (0.31)	-0.05 (0.25)	-0.08 (0.21)	0.510

Using the paired t test, we compared changes in antibody levels between baseline and day 29 in carriers and non-carriers in each decile, and found no significant differences (data not shown). As shown in Table 4.5, there were no differences in the fold change in antibody levels between the different age deciles in either carriers or non-carriers. Of note, the average fold change was positive in carriers aged <60 years while it was negative in older carriers, although this was not statistically significant. Using Pearson's correlation we found that fold change in antibody levels in response to colonisation did not correlate with age in either carriers (r = -0.221, p = 0.229, shown in Figure 4.6) or non-carriers (r = 0.085, p = 0.626). Further subdividing by PPV23 status resulted in no correlations between fold-change of antibodies and age, in either carriers or non-carriers (data not shown).

Of note, reduced antibody levels have been associated with male sex in previous population studies (65). There were no differences in mean baseline antibody titres between males (3.44 log ng/mL, SD 0.42) and females (3.39 log ng/mL, SD 0.30; p = 0.585).

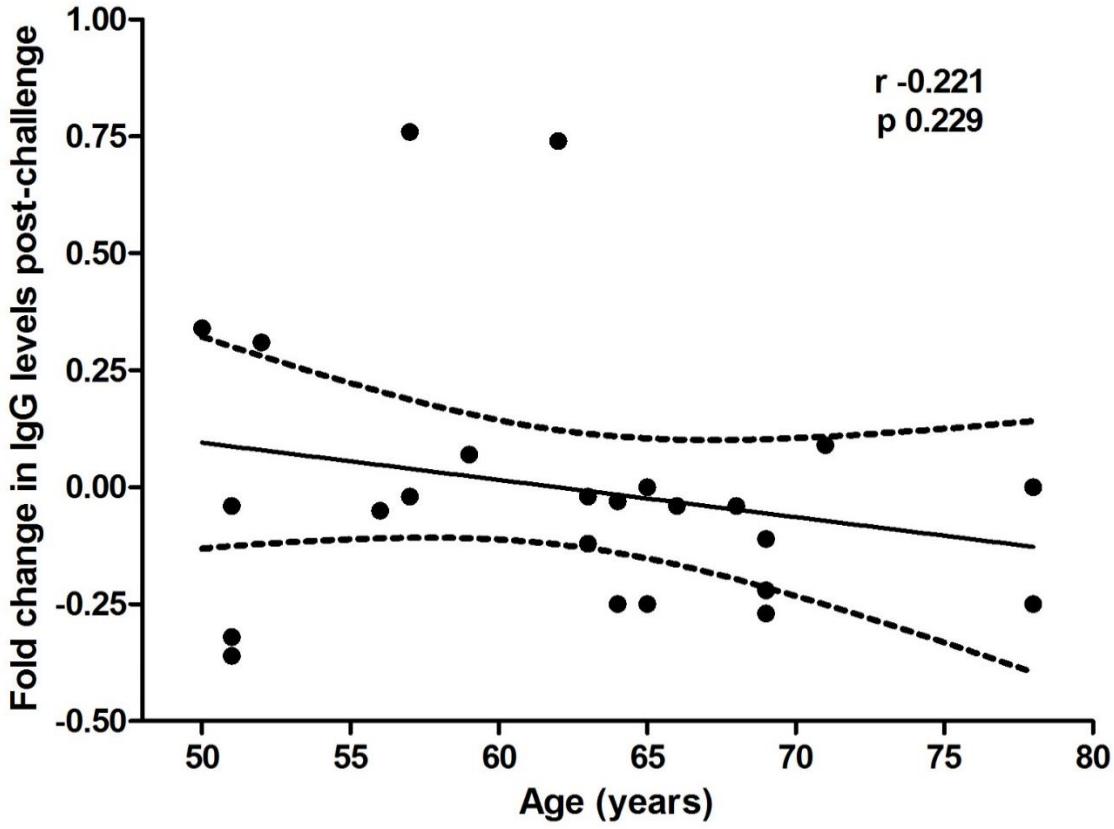

Figure 4.6: Fold change in anti-CPS IgG levels after pneumococcal challenge plotted against age (colonised participants only)

(N = 24; dashed lines represent 95% CI)

4.3.5 Regression analysis—experimental colonisation

The results of univariate logistic regression exploring predictor variables for experimental colonisation are shown in Table 4.6, with the results of multivariate regression in Table 4.7. Baseline antibody levels did not have a significant effect on the odds of developing experimental colonisation, and this was not affected by adjusting for age and sex.

Table 4.6: Predictors of experimental colonisation, using univariate logistic regression

	Odds ratio	**95% CI**	**p value (Wald)**
Age (years)	0.97	0.9—1.03	0.313
Male sex	0.59	0.21—1.66	0.319
Baseline antibody levels (log ng/mL)	2.46	0.54—11.14	0.242

Table 4.7: Predictors of experimental colonisation, using multivariate logistic regression

	Odds ratio	**95% CI**	**p value (Wald)**
Age (years)	0.96	0.89—1.03	0.264
Male sex	0.48	0.16—1.44	0.188
Baseline antibody levels (log ng/mL)	3.54	0.69—18.26	0.132
(Constant)	*0.173*	-	*0.582*

4.3.6 Regression analysis—experimental colonisation density

The results of univariate linear regression exploring predictor variables for experimental colonisation density are shown in Table 4.8. Baseline antibody levels were associated with lower colonisation density, with borderline statistical significance—these results are (by definition) identical to those found when using Pearson's correlation (Section 4.3.3). Age and sex alone were not associated with colonisation density.

Table 4.8: 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
Baseline antibody levels (log ng/mL)	-40.4	19.7	-81.27 — 0.43	-0.4	0.052

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

As pre-specified, we went on to perform multivariate analysis, adjusting antibody levels for age and sex (forced entry method). The results are shown in Table 4.9. Baseline antibody levels, adjusted for age and sex, were significantly associated with reduced colonisation density in experimental pneumococcal carriers.

Table 4.9: Predictors of experimental colonisation density (as defined by AUC), using multivariate linear regression

	Unstandardised			Standardised β	p value
	B	**Standard error**	**95% CI**		
Age (years)	-0.33	0.87	-2.14 — 1.47	-0.08	0.7
Male sex	22.05	14.25	-7.67 — 51.77	0.31	0.14
Baseline antibody levels (log ng/mL)	-44.43	20.0	-86.15 — -2.71	-0.44	**0.038**
(Constant)	*200.72*	*81.1*	*31.56 — 369.88*	-	*0.022*

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

This model shows that, although higher baseline antibody levels do not affect the acquisition of experimental colonisation, a 1 log increase in pre-challenge antibody levels corresponds with a reduction in experimental colonisation AUC density of 44.4 CFU.days/mL for those who do become carriers. The model R^2 was 0.25—i.e. the adjusted baseline antibody levels could only explain 25% of the variation in colonisation density in our sample.

4.4 Discussion

We investigated seven hypotheses in this chapter, and the findings are summarised in Table 4.10.

Table 4.10: Summary of immunological results

Hypothesis		Finding
1	Higher baseline anti-CPS IgG levels are found in PPV23-vaccinated participants	Retained.
2	Post-PPV23 antibody levels are not significantly lower in participants for whom a long time had passed since vaccination	Retained.
3	High baseline antibody levels are not associated with colonisation outcomes	Retained—similar baseline antibody levels in participants who did and didn't develop experimental colonisation.
4	Experimental colonisation results in boosting of anti-CPS antibody levels	Refuted—no change in antibodies following experimental colonisation.
5	Pneumococcal challenge without colonisation does not have any effect on systemic antibody levels	Refuted—fall in antibody levels in the cohort as a whole. However, this was only barely statistically significant.
6	Higher baseline antibody levels are associated with reduced bacterial density in colonised participants	Retained—following adjustment for age and sex, higher baseline antibodies were associated with lower colonisation density, albeit with a p value of only 0.038, i.e. only just clearing the threshold for significance.
7	Antibody levels and responses are diminished in the oldest age decile	Not identified.

The implications of these findings can be explored under two broad headings, as outlined in the introduction to this chapter:

1. Antibody-mediated protection against colonisation
2. Antibody boosting following colonisation

4.4.1 Antibody-mediated protection against colonisation

Baseline anti-CPS antibody levels were not different between participants who did and did not go on to develop experimental colonisation. This remained true when the cohort was subdivided based on PPV23 status. This finding is consistent with EHPC studies in younger adults which drew the same conclusion (60) and our finding that PPV23 was not protective against colonisation in our previous chapter. This does not explain why PCV13—which also induces anti-CPS antibodies—is protective against colonisation in younger participants (107).

Indeed, a study of the effect of PCV13 on colonisation in older adults was published after our study was completed, based on a nested cohort within the CAPiTA study (108, 110). This study found that PCV13 did reduce the rate of vaccine-type colonisation compared with placebo at six months post-vaccine, but not at later timepoints. It may be that PCV13 results in improved antibody delivery to mucosal compartments (73) compared with natural or PPV23-induced immunity. Equally, as outlined in Chapter 1, other facets of immunity must be involved in preventing colonisation.

Higher baseline antibody levels, adjusted for age and sex, were significantly associated with lower colonisation density. Although the model fit was poor, this finding is biologically plausible. It is also consistent with the mechanism of action of vaccine-induced antibodies: in a previous EHPC study in young adults, PCV13 reduced the risk of pneumococcal acquisition (compared with placebo) but was also associated with reduced density in participants who did acquire colonisation (107).

The implications of antibody-mediated control of density will require further study. Higher colonisation density was associated with an increased risk of transmission in a murine model (202). This association is widely held to be true for human children (203), but has yet to be demonstrated. As adults are less likely than children to transmit in any case, the

public health relevance of higher densities in colonised adults is uncertain. However, it is also plausible that higher colonisation density would be associated with higher risk of disease. Adults with pneumococcal pneumonia have been found to have higher colonisation density than controls (24, 204), but in the absence of prospective studies a causal relationship remains to be determined.

Older adults' antibodies have been associated with reduced opsonophagocytic capacity in other studies (80). However, reduction in colonisation density may be mediated by agglutination rather than opsonophagocytic killing (73), and future studies should explore the agglutination capacity of antibodies in older adults.

The reduction in colonisation density by antibodies was small, and the majority of variation in density was unexplained by the multivariable model. Similarly, pneumococcal vaccines are immunogenic but only confer partial protection against vaccine-type pneumococcal colonisation or disease (104, 110). It remains clear that serotype-specific, antibody-mediated immunity alone is not sufficient to control pneumococcal colonisation in older adults.

4.4.2 Antibody boosting following colonisation

In our primary analysis of the entire cohort, we did not identify antibody boosting in colonised recipients. This contrasts markedly with results seen in younger adults (60), and is supportive of a hypothesis that anti-pneumococcal immune responsiveness is diminished in older people.

We demonstrated a small but statistically significant drop in anti-6B IgG levels following pneumococcal challenge without colonisation. This may represent hyporesponsiveness. In previous studies, hyporesponsiveness has not manifested itself as a fall in antibody levels following antigen challenge. Rather, it has manifested as a lower-than-expected rise in antibody titre after a PPV23 booster vaccination compared with the response to the

primary vaccine or to a PCV13 booster (116, 118, 197). Serotype-specific memory B cell levels have been shown to fall following revaccination with PPV23 in older adults (117). These memory B-cells may undergo similar terminal differentiation in response to live pneumococcus. However, these terminally-differentiated B-cells are still capable of secreting antibodies, and therefore a fall in antibody titres after four weeks remains unexpected.

Another possible explanation is that peripheral antibodies were sequestered in the nose following pneumococcal challenge, leading to a drop in circulating levels. This phenomenon has previously been noted for mucosal antibodies in PCV13-vaccinated young adults (preventing colonisation via agglutination), but did not affect peripheral antibody levels (73). Therefore, it may be that some individuals have the capacity to recruit circulating antibodies (or antibody-secreting cells) to the nasopharyngeal mucosal surface and it is this capacity, rather than the absolute antibody concentration in blood, that determines whether the individual is protected against colonisation. Further work specifically exploring the dynamics of mucosal rather than systemic immunity during EHPC in older people could investigate this hypothesis. During the "Aging and Immunity" study we collected a number of mucosal samples, including nasal wash, nasal matrix absorption and nasal microbiopsy (205), and these will allow us to measure mucosal antibody, cytokine levels and immune cell populations over the duration of a challenge/colonisation episode in this older population.

An EHPC study in young adults demonstrated a functional immune boost following colonisation—participants were protected against reacquisition of the same serotype for up to one year (60). Our group plans to re-challenge carriers from the "Aging and Immunity" study with serotype 6B to provide further insight into the true impact of pneumococcal colonisation on immunity.

Recently there has been growing interest in the use of "seroincidence" to estimate pneumococcal colonisation incidence in populations. If our EHPC results are similar to the antibody responses to naturally-acquired colonisation, this suggests that seroincidence would have poor sensitivity for identifying pneumococcal acquisition in older adults. Applying the same definition as a recent paediatric study to our cohort —*any* increase in serotype-specific antibodies between two timepoints (79)— gives seroincidence a sensitivity of 37.5% and specificity of 70.1% for identifying pneumococcal colonisation.

4.4.2.1 Antibody boosting and vaccine status

When we divided the participants by PPV23 status, we did not identify any statistically-significant changes in antibody levels post-challenge. These subgroups were under-powered. In addition, dividing the cohort by PPV23 status results in obvious age imbalances.

4.4.2.2 Age-related trends

While we did not identify an age-related trend within our cohort, clearly the responses to EHPC in this cohort of older adults are different to those previously reported in younger adults (60), and direct comparisons with serum from young controls would be a valuable future project. The antibody response to colonisation may also be serotype dependent. Previous population studies have shown a decline in antibodies against certain serotypes (e.g. serotype 3) with age, but not in other serotypes including 6B (65). By contrast, an observational study of colonisation in young adults found that 6B did not provoke as strong an antibody response as other serotypes (29). It is unlikely that we will be able to study the serological response to experimental challenge with different serotypes in older adults, as EHPC studies with serotypes other than 6B have failed to establish high, reproducible rates of carriage thus far (Victoria Connor et al, unpublished data, and (60)).

While we did not measure functional activity in this study, IgG levels and serum opsonophagocytic activity are reasonably well correlated in older adults (126). As mentioned above, a study of agglutinative as well as opsonophagocytic function in this cohort would provide valuable mechanistic insight.

4.4.3 Conclusions

While this cohort of older adults proved susceptible to experimental colonisation with pneumococcus, their anti-capsular antibody responses to pneumococcal challenge were markedly different to those previously reported in young adults. In particular, pneumococcal challenge with colonisation did not boost antibody levels, and challenge without colonisation was associated with a fall in antibody levels. We found no evidence of superior antibody responses to colonisation in PPV23 recipients. Although pre-existing serotype-specific antibodies were not protective against colonisation acquisition, they may have a role in controlling colonisation density.

5 Defining the Pneumococcal Colonisation Niche using Different Sampling Methods

5.1 Background

As outlined in Table 1.1 and Section 1.2 of the INTRODUCTION, the ideal method to detect pneumococcal colonisation remains a matter of dispute, particularly in older adults. Published rates of colonisation differ substantially, and some of this may depend on the site of sampling—e.g. nasopharynx versus oropharynx—with some authors suggesting that the niche of colonisation changes with age (43). Each site can be sampled using different methodologies—e.g. nasopharyngeal swab versus nasal wash; oropharyngeal swab versus saliva. Finally, the sensitivity and specificity of different laboratory assays—e.g. culture vs qPCR—are quite different, and assay performance can vary between different types of samples from different niches.

Defining both the niche of colonisation and the ideal sampling method is vital to maximise the yield of epidemiological colonisation studies and establish the true underlying rate of colonisation. In addition, colonisation can be an endpoint for vaccine studies, and the definition of colonisation can affect the estimation of vaccine efficacy.

A report from a WHO working group in 2013 recommended that

For adults, both [nasopharyngeal] and [oropharyngeal] samples should be collected. However, if only one sample is possible, collecting from the nasopharynx is more sensitive than from the oropharynx (36).

The report also recommended

The [nasopharyngeal] swab approach for collection of the sample. [Nasopharyngeal] aspirates or washes are also acceptable methods of specimen collection as they have

sensitivity for pneumococcal detection equal to, or greater than, that of [nasopharyngeal] swabs, but may be less tolerated by participants (36).

In our local context, nasal washes have been tolerated better than swabs in adults (37). The WHO working group recommended culture rather than molecular methods for pneumococcal detection, but acknowledged that

Molecular testing of paired [nasopharyngeal and oropharyngeal] samples is needed to establish if the recommendations for anatomic site of sampling apply also to studies using molecular detection of pneumococci (36).

Studies that compared the performance of culture and molecular methods for detecting community-acquired pneumococcal colonisation are summarised in Table 1.1 of the INTRODUCTION. The comparative performances of nasal wash, oropharyngeal swab and saliva have not been assessed in EHPC before. A post-hoc study of EHPC in young adults compared the sensitivity of nasal wash culture and *lytA* qPCR, and found that 42.6% of samples were positive by qPCR versus 27.5% by nasal wash (48). The sensitivity of qPCR was 93.2% while the specificity was 75.9%. The authors concluded that qPCR's advantage was in detecting low-density colonisation. The authors also speculated that *continuous qPCR detection when culture results are negative may represent prolonged low-density carriage, living cells that are in a culture-unfavourable metabolic state, or remaining pneumococcal debris* (48). However, *lytA* qPCR may generate false-positive results by detecting other commensal streptococci; many experts now advocate targeting two genes in pneumococcal qPCR to maximise specificity (47). This approach has not yet been applied to older participants or to oral samples in EHPC subjects.

Along with their respective sensitivities and specificities, numerous other factors affect the choice of test and analysis method, such as patient acceptability, labour intensity, cost,

logistic and infrastructure requirements and ability to harmonise methodology across a variety of settings.

When designing the "Aging and Immunity" study, we pre-specified that colonisation would be defined using nasal wash culture. This facilitated direct comparisons with previous EHPC studies, and was the most logical definition given that participants underwent nasal inoculation. For this reason, nasal wash culture would be the "gold standard" against which other diagnostic methods for experimental pneumococcal colonisation would be compared. Alongside each nasal wash, we also took paired oropharyngeal swabs and saliva samples. In this chapter, we compare the performance of molecular analysis of these samples with nasal wash culture in this cohort.

5.1.1 Hypotheses

We test the following hypotheses in this chapter:

1. Nasal wash qPCR will identify pneumococcus in more samples than nasal wash culture (48);
2. Oropharyngeal swab qPCR will identify pneumococcus in more samples than than nasal wash (see Table 1.1 for literature review);
3. Saliva qPCR will identify the highest number of positive samples (Table 1.1);
4. qPCR targeting *lytA* alone is more sensitive but less specific than qPCR targeting both *lytA* and *cpsA* genes (186);
5. EHPC studies take samples at multiple timepoints, thus maximising the likelihood of detecting colonisation by culture—therefore, at the participant level (rather than the individual sample level) culture and molecular methods have similar sensitivity and specificity for classifying participants as colonised or non-colonised;

5.2 Methods

We obtained nasal wash, oropharyngeal swab (OPS) and saliva samples at each timepoint in the "Aging and Immunity" study (see the timeline in Figure 2.3). We tested the samples from baseline and days 2, 7 and 14 post-inoculation using qPCR. (These timepoints are common to all EHPC studies, and future work will involve molecular tests on archived samples from young volunteers at these same timepoints.)

5.2.1 Clinical procedures

Nasal wash was performed as outlined in Section 2.6.3, OPS as in Section 2.6.4 and saliva collection as in Section 2.6.5.

5.2.2 Laboratory procedures

Nasal wash culture and pneumococcal identification were performed as outlined in Section 2.7.2. OPS and saliva samples were not cultured prior to DNA extraction and qPCR; future work will explore the effect of culture enrichment on test performance.

Nasal wash pellets, OPS and saliva were stored in STGG at -80°C, and for the current analysis they underwent multiplex qPCR targeting both the *lytA* and the 6A/B capsular polysaccharide (*cpsA)* genes, as outlined in Section 2.7.5—two targets were chosen to maximise the specificity, with a positive qPCR requiring detection of both targets within 40 cycles. A schematic outlining the sample timing, samples taken and analytic methods is given in Figure 5.1.

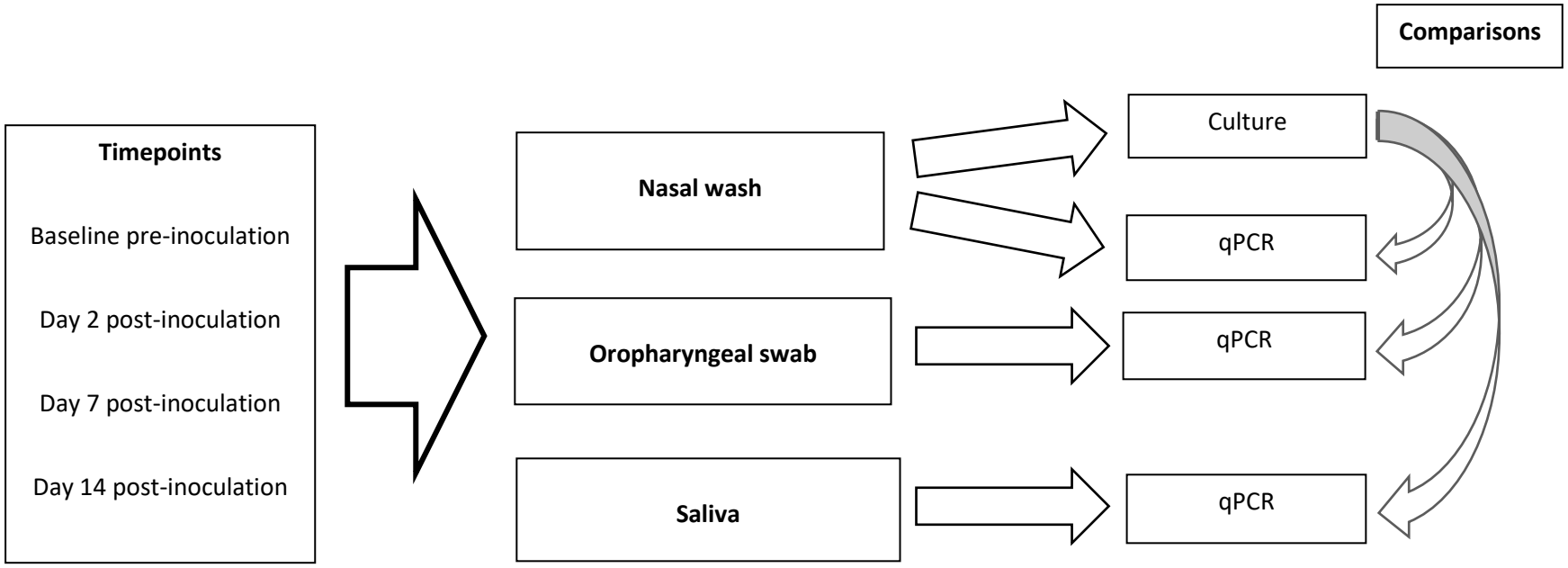

Figure 5.1: Sampling schedule and testing methods employed in this chapter
qPCR: quantitative polymerase chain reaction

5.2.3 Statistical analysis

Nasal wash culture was used as the "reference standard" against which the other tests would be measured. We assessed the relative performance of each sample strategy at each timepoint for our primary analysis. We included participants who were colonised with non-6B serotypes at any timepoint, as our diagnostic methods were intended to be serotype-specific. As a secondary analysis, we re-ran each comparison with qPCR targeting *lytA* alone, to see if qPCR specificity was reduced by only targeting one gene. Another secondary analysis assessed sensitivity and specificity at the participant level, defining a positive result as detection of pneumococci by a given method at *any* post-inoculation timepoint for each participant. We defined sensitivity and specificity as follows:

- Sensitivity = $\frac{\text{Test positives}}{\text{All culture positives}}$
- Specificity = $\frac{\text{Test negatives}}{\text{All culture negatives}}$

Sensitivities and specificities are presented with 95% CIs which were calculated based on Newcombe's efficient score method (206), using an online calculator (http://vassarstats.net/clin1.html, accessed 08 July 2018).

5.3 Results

Sixty-four adults aged 50—84 years provided 254 sample pairs for nasal wash culture/qPCR and nasal wash culture/OPS qPCR (two subjects had missed their day 14 visit), and 251 sample pairs for nasal wash culture/saliva qPCR (two subjects missed their day 14 visit and a further three samples had insufficient saliva volume for testing).

5.3.1 Performance of qPCR versus nasal wash culture

5.3.1.1 Nasal wash qPCR

Using nasal wash culture, 20.1% of samples were positive for *S pneumoniae* serotype 6B, while 18.1% were positive using nasal wash qPCR (Table 5.1).

Table 5.1: Performance of nasal wash lytA/*6AB qPCR versus culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Nasal wash *lytA*/6AB qPCR	Positive	41	5	46
	Negative	10	198	208
	Totals	51	203	254

Sensitivity of qPCR versus the reference standard of culture = $\frac{41}{51}$ = 80.4% (95% CI 66.5—89.7%).

Specificity of qPCR versus culture = $\frac{198}{203}$ = 97.5% (94—99.1%).

At the participant level, two participants were newly identified as carriers using nasal wash qPCR—one was qPCR positive at all three timepoints, while the other was only positive at one timepoint. However, four culture-positive participants were missed by qPCR.

5.3.1.2 Oropharyngeal swab qPCR

As shown in Table 5.2, 7.9% of samples were positive by OPS qPCR.

Table 5.2: Performance of oropharyngeal lytA/*6AB qPCR versus nasal wash culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Oropharyngeal swab *lytA*/6AB qPCR	Positive	16	4	20
	Negative	35	199	234
	Totals	51	203	254

Sensitivity of OPS qPCR versus NW culture = $\frac{16}{51}$ = 31.4% (19.5—46%).

Specificity of OPS qPCR versus NW culture = $\frac{199}{203}$ = 98% (94.7—99.4%).

Two culture-negative participants were newly identified as carriers using OPS qPCR; one of these was also positive by nasal wash qPCR. However, 11 culture-positive participants were missed by OPS qPCR.

5.3.1.3 Saliva qPCR

As shown in Table 5.3, only 1.6% of samples tested positive by saliva qPCR.

Table 5.3: Performance of saliva lytA*/6AB qPCR versus nasal wash culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Saliva *lytA*/6AB qPCR	Positive	3	1	4
	Negative	48	199	247
	Totals	51	200	251

Sensitivity of saliva qPCR versus NW culture = $\frac{3}{51}$ = 5.9% (1.5—17.2%).

Specificity of saliva qPCR versus NW culture = $\frac{199}{200}$ = 99.5% (96.8—99.9%).

One participant was newly identified as colonised using saliva qPCR (the participant was also qPCR positive in both NW and OPS), while 21 carriers were missed entirely.

5.3.2 Performance of *lytA* qPCR alone

We performed two-target qPCR to maximise test specificity. However, *lytA* is generally accepted to be the most specific single gene for pneumococcus (186), and there is a

substantial body of literature in which qPCR for *lytA* alone was used to diagnose pneumococcal colonisation (24, 48). Therefore we did a secondary analysis of the performance of single-target qPCR. Since *lytA* qPCR is serotype-independent, nasal wash culture positivity for any pneumococcal serotype (i.e. including "natural carriers") was considered the reference standard in this analysis.

5.3.2.1 Nasal wash lytA *qPCR*

As shown in Table 5.4, 23.6% of samples were positive for *S pneumoniae* using nasal wash culture, compared with 24.4% using nasal wash *lytA* qPCR.

Table 5.4: Performance of nasal wash lytA *qPCR versus culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Nasal wash *lytA* qPCR	Positive	52	10	62
	Negative	8	184	192
	Totals	60	194	254

Sensitivity of *lytA* qPCR versus culture = $\frac{52}{60}$ = 86.7% (74.9—93.7%).

Specificity of *lytA* qPCR versus culture = $\frac{184}{194}$ = 94.8% (90.5—97.4%).

5.3.2.2 Oropharyngeal lytA *qPCR*

Table 5.5: Performance of oropharyngeal lytA *qPCR versus nasal wash culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Oropharyngeal swab *lytA* qPCR	Positive	22	14	36
	Negative	38	180	218
	Totals	60	194	254

Sensitivity of OPS *lytA* qPCR versus NW culture = $\frac{22}{60}$ = 36.6% (24.9—50.2%).

Specificity of OPS *lytA* qPCR versus NW culture = $\frac{180}{194}$ = 92.8% (87.9—95.8%).

5.3.2.3 Saliva lytA *qPCR*

Table 5.6: Performance of saliva lytA *qPCR versus nasal wash culture*

		Nasal wash culture		*Totals*
		Positive	Negative	
Saliva *lytA* qPCR	Positive	8	6	14
	Negative	52	185	237
	Totals	60	191	251

Sensitivity of saliva *lytA* qPCR versus NW culture = $\frac{8}{60}$ = 13.3% (6.3—25.1%).

Specificity of saliva *lytA* qPCR versus NW culture = $\frac{185}{191}$ = 96.8% (92.9—98.7%).

5.3.3 Molecular methods in natural carriers

Our use of serotype-specific methods (latex agglutination of culture samples and 6A/B qPCR) meant that we did not exclude participants who were "naturally" colonised with pneumococcus from our primary analysis. Colonisation with non-6B serotypes was detected at 11 timepoints by culture, including two episodes of dual colonisation with 6B and a natural strain. NW *lytA* qPCR was positive at 10/11 timepoints. OPS and saliva *lytA* qPCR were jointly positive at one timepoint, and separately positive at one further timepoint each. In the two instances where there was dual colonisation, NW qPCR was positive by both *lytA* and 6AB; in one of these cases saliva *lytA* qPCR was positive but 6A/B was negative.

5.3.4 Classification of volunteers as carriers

When reporting the results of the primary analysis in this chapter, we noted how many participants were "newly identified as colonised" or "missed" by each molecular diagnostic method. In this secondary analysis, we formally assess the performance of molecular methods versus nasal wash culture for classifying volunteers as experimental carriers. If a volunteer tested positive by a given method at *any* of the three post-inoculation timepoints, they were classified as positive. Nasal wash culture for serotype 6B pneumococcus was again considered the reference standard (carriers of non-6B serotypes were classified as culture negative). Missing samples were disregarded, and two volunteers who only tested positive for colonisation by culture on day 9 (i.e. did not have paired samples for molecular diagnostic) are classified as culture negative in this analysis.

5.3.4.1 Nasal wash lytA*/6AB qPCR*

Table 5.7: Performance of nasal wash lytA*/6AB qPCR versus culture at participant level*

		Nasal wash culture		*Totals*
		Positive	Negative	
Nasal wash *lytA*/6AB qPCR	Positive	19	2	21
	Negative	4	39	43
	Totals	23	41	64

Sensitivity of qPCR versus culture = $\frac{19}{23}$ = 82.6% (60.5—94.3%).

Specificity of qPCR versus culture = $\frac{39}{41}$ = 95% (82.2—99.2%).

5.3.4.2 *Oropharyngeal* lytA/*6AB qPCR*

Table 5.8: Performance of oropharyngeal lytA*/6AB qPCR versus nasal wash culture at participant level*

		Nasal wash culture		*Totals*
		Positive	Negative	
Oropharyngeal swab *lytA*/6AB qPCR	Positive	12	2	14
	Negative	11	39	50
	Totals	23	41	64

Sensitivity of OPS qPCR versus NW culture = $\frac{12}{23}$ = 52.2% (31.3—72.6%)

Specificity of OPS qPCR versus NW culture = $\frac{39}{41}$ = 95% (82.2—99.2%)

5.3.4.3 *Saliva* lytA/*6AB qPCR*

Table 5.9: Performance of saliva lytA*/6AB qPCR versus nasal wash culture at participant level*

		Nasal wash culture		*Totals*
		Positive	Negative	
Saliva *lytA*/6AB qPCR	Positive	2	1	3
	Negative	21	40	61
	Totals	23	41	64

Sensitivity of saliva qPCR versus NW culture = $\frac{2}{23}$ = 8.7% (1.5—29.5%)

Specificity of saliva qPCR versus NW culture = $\frac{40}{41}$ = 97.6% (85.6—99.9%)

5.4 Discussion

We investigated five hypotheses in this chapter, and the outcomes are summarised in Table 5.10. A summary of the main results, juxtaposed with the results for *lytA* qPCR alone, is

given in Table 5.11. A heatmap (Figure 5.2) summarises the findings of the primary analysis graphically.

Table 5.10: Outcomes of hypotheses tested in this chapter

	Hypothesis	**Outcome**
1	Nasal wash qPCR will identify pneumococcus in more samples than nasal wash culture	Rejected—similar numbers of samples identified using either method, qPCR 80.4% sensitive versus culture
2	Oropharyngeal swab qPCR will identify pneumococcus in more samples than nasal wash culture	Rejected—oropharyngeal swab 31.4% sensitive
3	Saliva qPCR will identify the highest number of positive samples	Rejected—5.9% sensitivity
4	qPCR targeting *lytA* alone is more sensitive but less specific than qPCR targeting both *lytA* and *cpsA* genes	Retained, but differences in sensitivity and specificity very small between the two methods (See Table 5.11)
5	At the participant level, culture and molecular methods have similar sensitivity and specificity	Rejected—culture was already superior to molecular methods at the individual sample level, and this did not change when a participant-level definition was used; similar sensitivity and specificity within each method at the sample and participant level, although OPS qPCR more sensitive at participant level than at sample level

Table 5.11: Summary of dual-target and single-target qPCR results

	Sensitivity	**Specificity**
(Nasal wash culture)	(100%)	(100%)
Nasal wash *lytA/*6AB qPCR	80.4% (66.5—89.7%)	97.5% (94—99.1%)
lytA *qPCR*	*86.7% (74.9—93.7%)*	*94.8% (90.5—97.4%)*
Oropharyngeal *lytA/*6AB qPCR	31.4% (19.5—46%)	98% (94.7—99.4%)
lytA *qPCR*	*36.6% (24.9—50.2%)*	*92.8% (87.9—95.8%)*
Saliva *lytA/*6AB qPCR	5.9% (1.5—17.2%)	99.5% (96.8—99.9%)
lytA *qPCR*	*13.3% (6.3—25.1%)*	*96.8% (92.9—98.7%)*

95% confidence intervals are given in parentheses.

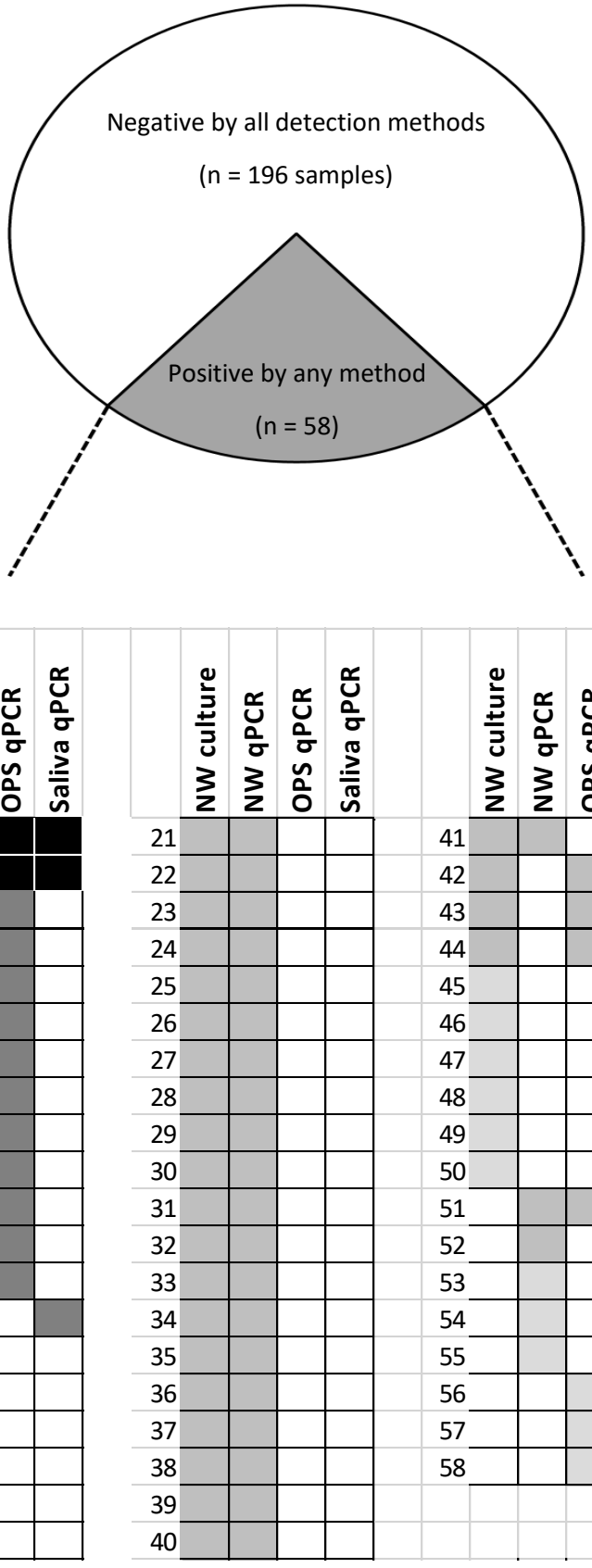

Figure 5.2: Heatmap summarising the results of different pneumococcal tests
Each row represents an individual participant's sampling timepoint, each column a diagnostic method. Shaded squares indicate a positive result, with increasing darkness of shading indicating that a participant was positive by one, two, three or four diagnostic methods at that timepoint. As shown in the pie chart, 77% of samples (n = 196) were negative for pneumococci by all four methods.
NW, nasal wash; OPS, oropharyngeal swab; qPCR, quantitative polymerase chain reaction

None of the molecular sampling techniques demonstrated a sufficiently high sensitivity to replace nasal wash culture as the reference standard for pneumococcal detection in older adults. If we had used only molecular methods, we would have detected fewer experimental carriers than we did using culture, even if we had analysed samples from multiple anatomical sites. Combining culture with molecular methods for routine analysis in the "Aging and Immunity" study would have detected three new experimental carriers if both nasopharyngeal and oropharyngeal samples were tested, while adding a significant workload to the laboratory.

The oral samples showed particularly poor sensitivity for pneumococcal detection by qPCR. This is in marked contrast with the results of community-based colonisation screening studies (44). Unidentified issues with sample collection, processing or storage may have contributed to the high false negative rate. In addition, oropharyngeal pneumococcal detection rates may indeed be low following experimental nasal inoculation, but this may not hold true for community-acquired colonisation.

All molecular methods demonstrated high specificity. Recent studies have reported capsular polysaccharide production by commensal streptococci (such as *Streptococcus mitis*) and the detection of the *cps* gene in these species (207), raising a concern that our *lytA/cpsA* qPCR could be vulnerable to false positives. There were no *lytA*-positive, *cpsA*-positive samples at baseline from any site in any of our participants. Therefore we remain confident that *lytA/cpsA*-positive samples obtained after serotype-6B pneumococcal exposure are more likely to represent genuine detection of experimental colonisation.

5.4.1 Non-serotype-specific molecular testing

Molecular methods are generally considered more sensitive but less specific than culture; hence, refinements to pneumococcal qPCR tend to focus on increasing specificity, e.g. by mandating two gene targets. Our study found that *lytA/*6AB qPCR from any site had low

sensitivity but high specificity. Therefore we looked at the performance of *lytA* qPCR alone. We found that omitting the *cpsA* qPCR came with a modest drop in specificity, and a similarly modest rise in sensitivity (Table 5.11). Nasal wash *lytA* qPCR had a sensitivity of 86.7%, 95% CI 74.9—93.7%; the 93.2% sensitivity of nasal wash *lytA* qPCR in young adults falls (just) within this confidence interval (48).

We cannot state with confidence how many *lytA* qPCR-positive, culture-negative samples represented true pneumococcal colonisation, perhaps in low densities, rather than a false positive assay due to (for example) commensal streptococcal detection. 40% of such samples (12/30) were from participants who were culture-positive at other timepoints, suggesting that the qPCR was detecting either true low-density colonisation or residual pneumococcal debris.

In participants colonised with non-6B serotypes, nasal wash *lytA* qPCR performance did not differ substantially from that of culture. The two "natural carriers" who were positive by nasal wash *lytA*/6AB qPCR also had dual colonisation including serogroup 6 identified on nasal wash culture—this reassures us that no experimental carriers were missed by culture due to overgrowth by non-6B serotypes.

5.4.2 Reliability of saliva sampling

The pneumococcal yield from saliva was surprisingly low—given the high rate of salivary colonisation identified in other studies, it is possible that methodological issues related to saliva collection or processing led to a high false-negative rate in our sample. For example, a Dutch study identified colonisation in 28% of older adults using culture-enriched qPCR of saliva (44). This study used a similar absorbent sponge methodology to ours, but immediately placed the sample in glycerol and transported it to the laboratory on dry ice, whereas we transported our samples without preservative on wet ice, with a 1—2-hour interval between collection and processing. However, other Dutch studies have detected

high salivary colonisation rates using much simpler methods—e.g. a paediatric study (88% colonisation) asked subjects to spit into a sterile container which was transported to the laboratory on wet ice within 4 hours (46). Another study found that pneumococci could be reliably detected in dried saliva that had been stored for up to a month at ambient temperature (208). Of note, these saliva samples were spiked with pneumococci, and thus may have been less representative of epidemiological participant samples. These studies suggest that the salivary pneumococcal yield is not profoundly affected by the collection method. Nonetheless, the discordance between our results and the sizeable body of evidence from the Netherlands remains unexplained. In addition, other studies in EHPC participants have required participants to spit directly into an STGG-filled tube, and these studies detected pneumococcal DNA in saliva up to 48 hours post-inoculation (E Nikolaou, unpublished data). Future EHPC studies in older adults should adopt this methodology. Overall, our findings suggest that, if salivary PCR is employed, it should be used in parallel with other sampling strategies rather than on its own.

Some authors have suggested that culture-enrichment pre-qPCR improves the sample yield (209). Preliminary work with spiked samples in our lab found that qPCR of both culture-enriched and raw samples were complementary, with a combination of the two methods yielding the highest number of positive samples (E Nikolaou, unpublished data). Future work will explore the performance of culture enrichment in samples from the over-50 cohort.

5.4.2.1 Oral fluids other than saliva

A variety of secretions are produced in the oral cavity, including saliva but also gingival-crevicular fluid (210). Gingival crevicular fluid has been found to contain numerous inflammatory mediators and antibodies, which can be reflective of an individual's systemic inflammatory and immune status (210)(211). Did our collection methods truly obtain

samples of participants' saliva, or did we in fact collect an alternative oral secretion, or a mixture of saliva and other secretions?

During saliva collection, we observed that a number of participants found it uncomfortable to hold the sponge in their mouth for the requisite duration; some found it easier to move the sponge around using their tongue, while others preferred to let it rest in a single location. To minimise participant discomfort, we did not mandate a standardised anatomical niche in which to place the sponge. Our collection and analytical methods were based on a modification of a protocol shared by a Dutch team (M-L Chu, personal communication), in which absorbant sponges were placed between gum and cheek. Therefore, we feel that our collection method retains validity, although it would have been ideal to completely standardise the saliva collection manoeuvre between participants. Gingival crevicular fluid is secreted in particularly small volumes, typically being collected using a filter paper strip rather than sponges (211). Therefore, the fact that our participants were able to saturate a sponge suggests that saliva formed a significant fraction of the fluid we obtained.

Gingival crevicular fluid does differ in composition from saliva, although it has been argued that differences in component concentrations are unlikely to translate to different conclusions in research should one fluid be substituted for the other (210). If pneumococcus colonises the oral cavity, there is no reason to suppose that the relative compositions of different oral secretions are more or less inhabitable to this encapsulated pathogen, which has adapted to tolerate inhospitable mucosal surfaces and evade mucosal host defences (212)(72).

5.4.3 Limitations of nasal wash culture as an EHPC reference standard

We chose nasal wash culture as our reference standard based on its use in prior EHPC studies. However, comparison between a culture-based reference standard and a

(potentially more sensitive) molecular diagnostic method poses challenges. Had we been able to culture the samples taken from the oropharyngeal niche, this would have facilitated direct comparisons between culture of oral and nasal samples, and then between qPCR of oral and nasal samples. Overgrowth of oral samples by commensal flora is a major limitation of this approach, but including a culture-enrichment step in future studies could overcome this limitation (209)(47).

Numerous methods exist to establish the performance of a diagnostic test in the setting of an imperfect reference standard, ranging from construction of composite reference standards (sometimes by panel consensus) to correction via statistical modelling (reviewed in (213)). For example, a study assessing a new highly-sensitive PCR methodology (Xpert Ultra, versus the standard Xpert) for the diagnosis of tuberculous meningitis compared each diagnostic method (culture, Xpert and Xpert Ultra) against a reference standard of "positive by any of the three methodologies" (214).

A future EHPC study could adopt a similar methodology—for example, assessing each qPCR from individual niches against a reference standard comprising qPCR positivity from any sample. However, given the low positivity rates of oropharyngeal and salivary qPCR in this chapter, such a study would require methodological refinement—e.g. incorporating a culture-enrichment step and/or a different saliva collection methodology.

5.4.4 The role of nasal wash culture outside the context of EHPC

Nasal wash culture has a number of disadvantages. It can be uncomfortable and technically challenging for participants, although better tolerated than nasopharyngeal swab (37). In addition, saline impairs pneumococcal viability, meaning that the sample must reach the laboratory within an hour of collection. If nasal wash were used for field studies without near-patient laboratory facilities, then the sample would require freezing upon collection

or a preservative like glycerol or STGG to be added immediately afterwards. The reasonable sensitivity of nasal wash qPCR could help overcome this limitation.

In epidemiologic studies, simply detecting pneumococcal colonisation is not always enough—ideally, serotypes should also be identified. Currently, serotype identification can only be performed with confidence on culture-based samples. When performing qPCR in EHPC, we could be confident that a positive 6AB qPCR coupled with a positive *lytA* qPCR, in a participant recently inoculated with serotype 6B, represented serotype 6B colonisation. Serotyping of qPCR-positive samples from a general population is more logistically challenging, as potentially every *lytA*-positive sample must undergo repeat qPCR with separate probes for every serotype. The majority of currently available probes only identify pneumococci at the serogroup level (47). Current vaccines may not target every member of a serogroup: for example, of serotypes 9N, 9L and 9V, only 9V is in PCV13, and thus the detection of serogroup 9 by qPCR is of limited epidemiologic utility. Novel methods, such as microarray analysis of genomic DNA, can classify pneumococci at serotype level (191) and could potentially be adapted for use with qPCR-positive samples.

Culture also allows the phenotypic measurement of antimicrobial susceptibility, another important metric in epidemiologic studies. Potentially, PCR probes directed against specific genes known to encode resistance could be employed—however, the issue of potential contamination with commensal flora cannot be overcome. In addition, there have been no prospective studies correlating the detection of antimicrobial resistance genes with phenotypic susceptibility in pneumococci (215).

5.4.5 Conclusions

Nasal wash culture was superior to qPCR of nasal wash, oropharyngeal swab or saliva for detecting experimental pneumococcal colonisation in older adults. We did not identify any potential benefit to integrating molecular diagnostics and/or alternative niches of

colonisation into routine EHPC practice. We cannot rule out methodological limitations or qualitative differences between natural and experimental colonisation, and our results from qPCR of oral samples are discordant with the majority of community-acquired colonisation studies in older adults (Table 1.1). Hence we cannot state with certainty that nasal wash culture is the optimum pneumococcal detection methodology in all circumstances. Nonetheless, our findings underscore the validity of nasal wash culture in the EHPC model and reinforce the advantages of this strategy. In the next chapter, we will employ culture, serotyping and antimicrobial resistance testing on nasal wash samples collected since the inception of the EHPC programme, demonstrating the utility of this approach in a "real world" context.

6 Community-Acquired Pneumococcal Colonisation in Healthy, Low-Risk Adults in Liverpool

6.1 Background

Previous chapters have discussed how older adults are highly susceptible to colonisation with serotype 6B pneumococcus, and that this appears to be independent of natural or PPV-induced anti-CPS immunity. Serotype 6B is included in the 13-valent pneumococcal conjugate vaccine (PCV13), which is routinely administered to children in the UK. Childhood pneumococcal vaccination has been shown to induce "herd immunity", reducing vaccine-type colonisation in vaccinated children but also in unvaccinated adults (33) and reducing rates of invasive pneumococcal disease at regional and national population levels (216).

A study published after ours was completed suggested that PCV13 could reduce rates of vaccine-type colonisation in older adults, but that the effect did not persist beyond six months (110). PPV23 (which also covers serotype 6B) has not been shown to protect against colonisation, including in our study of EHPC in older adults. There are no plans to introduce PCV13 for older adults in the UK (100), and therefore childhood vaccination programmes will remain the cornerstone of community protection against pneumococcal colonisation, transmission and disease.

6.1.1 The outlook for PCV13 in the UK

When PCV13 was introduced into the UK childhood vaccine programme, it was administered at two and four months of age, with a booster dose at 13 months—this is denoted as a "2 + 1" schedule. Recently the UK Joint Committee on Vaccines and Immunisation announced that the schedule would be changed to "1 + 1" (priming at 3 months, boosting at 13 months), based on new immunological data (217, 218). This was

predicated on the demonstration that vaccine-type pneumococci were circulating at negligible levels, and therefore that vaccine effectiveness on carriage rates could be maintained despite the new schedule's more limited immunogenicity. However, the assertion that carriage was "controlled in all age groups" was based on studies of families rather than the general adult population (34). Adults without regular contact with children may be less susceptible to the ecological effects of childhood PCV13 programmes, and could represent a "reservoir" of vaccine-type pneumococci with important implications for public health (35). This reservoir could reintroduce vaccine-type pneumococci into the paediatric population, reversing the gains made by the PCV13 programme.

Since the most virulent serotypes tend to be included in vaccine formulations, these will have higher disease-causing potential if they continue to circulate. Following the rollout of PCV7 in 2006, serotype 19A (a non-PCV7 serotype) emerged as one of the leading causes of invasive pneumococcal disease (219, 220). This supported a switch to PCV13, which covers serotype 19A. However, despite substantial declines in invasive serotype 19A disease in children since the introduction of PCV13 in the UK, it continues to cause disease in over-65s (5). Rates of disease due to serotype 3 have not fallen in the UK adult or paediatric population since PCV13 was introduced (5), consistent with laboratory studies showing that vaccination against this serotype is relatively less effective (221).

6.1.2 Serotype replacement

Even if vaccine-type pneumococci are eliminated in the young adult population through herd immunity, overall rates of pneumococcal colonisation may still not decrease if non-vaccine serotype (NVT) pneumococci move into the newly-vacant niche. Serotype replacement has been demonstrated for pneumococcal disease of all age-groups in the UK (5). This is highly likely to reflect serotype replacement in colonisation as well, which has been demonstrated in UK children and families (77, 222). Serotype replacement in

colonisation has not been explored in the general UK adult population with the same rigour that has been applied to household and family surveys.

6.1.3 Related research on this topic

A study in the Netherlands specifically compared a cohort of parents to a cohort of adults who had no regular contact with children aged <6 years (47). Nasopharyngeal swab culture detected colonisation in 9% (n = 26/298) of parents compared with 1% (n = 4/323) in "childless" adults (p<0.001). The yield was substantially improved by culture-enriched qPCR targeting two pneumococcal surface proteins in saliva samples, resulting in 30% (n = 89/298) colonisation in parents versus 6% (n = 18/323) in "childless" adults (p<0.001). The reliance on molecular methods meant that the investigators could not precisely assign serotypes to all isolates: 43% of isolates were unserotyped, and many others could only be identified at the serogroup level. The low rates of colonisation in "childless" adults limited the investigators' ability to discern trends in serotype replacement compared with parents, although they still concluded that there was no evidence of a reservoir of NVTs in adults *vis-à-vis* children. However, a study with a more precise method of serotyping would have greater potential to unmask any such trends.

The most recent UK colonisation survey was carried out in 2015/2016 and enrolled children and their parents from two regions in southern England (223). Colonisation was detected by nasopharyngeal swab culture in only 2.8% of parents (n = 8/284)—all were NVTs. The proportion of colonised parents fell between the first survey in 2001/2002 (7.6%) and the most recent survey, while the proportion of under-5s colonised remained stable at around 50%.

6.1.4 Antimicrobial resistance and colonisation

Since neither population immunity, natural immunity nor vaccine-induced immunity provide complete protection against pneumococcus in older adults, pneumococcal disease

will inevitably continue occurring in this population. Antimicrobial therapy will remain necessary to reduce mortality in these cases. Childhood PCV13 programmes can have complex effects on antimicrobial resistance rates (33, 181, 224). Surveillance of invasive pneumococcal isolates in the UK has found rates of penicillin non-susceptibility as high as 10% and macrolide resistance in 5—7% (225). A recent colonisation study in UK adults attending primary care found penicillin and macrolide resistance in 13.9% (each) of isolates (based on 36 carriers out of 3,211 adults screened), but did not have matching serotype data (30). The Dutch study comparing parents with "childless" adults relied on molecular detection of pneumococci and did not assess antimicrobial susceptibility (47). There are no longitudinal data of colonisation with resistant pneumococci in healthy UK adults, but this would provide further insight into the intersection of serotype replacement and antimicrobial resistance.

Healthy volunteers participating in the EHPC programme at LSTM undergo screening for pneumococcal colonisation prior to experimental inoculation. In this chapter we use these volunteers as a surrogate for the general healthy adult population to conduct a pilot study of the rates of colonisation, serotype distributions and antimicrobial susceptibility profiles over time.

6.1.5 Hypotheses

We test four hypotheses in this chapter, informed by previous literature on the subject:

1. The prevalence of pneumococcal colonisation is <3% (recent studies in UK adults have found rates of colonisation between 1.2—3.4% (30, 34, 223));
2. The serotype composition changes over time in favour of non-vaccine-type colonisation (i.e. serotype replacement (222));

3. Pneumococcal colonisation rates in young adults remain stable over time, with increases in non-vaccine-type colonisation balancing out any reduction in vaccine-type colonisation (222);
4. Antimicrobial resistance rates are low (<10% of isolates) and remain unchanged during the study period, reflecting the low exposure to healthcare settings/antibiotics anticipated among our cohort.

6.2 Methods

We reviewed all nasal washes from screening visits of EHPC volunteers between October 2010 and March 2017 to identify natural carriers at baseline. These isolates were subjected to serotyping and antimicrobial susceptibility testing.

6.2.1 Inclusion and exclusion criteria

No nasal washes were excluded from this analysis. While the inclusion/exclusion criteria for EHPC varied to a small degree between studies, in general they remained similar to the criteria outlined in section 1.8.1. In particular, smoking, recent antibiotics and close contact with the chronically ill or children under 5 were always exclusion criteria. (However, contact with these "at-risk" groups *prior* to participation was not recorded, because researchers were only concerned about the risks of post-inoculation contact.) Age >50 years was an exclusion criterion until the "Aging and Immunity" study commenced in 2016, therefore the age distribution of participants is heavily weighted towards younger adults.

Two trials of pneumococcal vaccines were performed during the period of analysis—one of the licensed PCV13 (n = 100 (107)), one of the experimental serotype-independent GEN004 vaccine (n = 96; NCT02116998). Pre-vaccine nasal washes were available for the PCV13 study, but the baseline nasal washes in the GEN004 study were taken 64 days post-vaccine. Interim results suggest that this vaccine does not have a statistically significant effect on

colonisation (147). It may still cause a small reduction in rates of colonisation, in which case our results would be biased towards underestimating true natural carriage rates. The vaccine was designed with the aim of affecting all serotypes equally, therefore we worked under the assumption that serotype distributions were not biased by including participants from this trial (226). It is unknown (but unlikely) whether rates of antimicrobial resistance were biased by including these participants.

For studies with multiple inoculations ("re-challenges"), only the first screening nasal wash was included for each participant. Similarly, participants who were screened twice within the same study (e.g. due to rescheduling their inoculation) were only counted once (their first screen). However, volunteers who participated in more than one EPHC study were included on each occasion. If a participant was recorded on our internal database as having been colonised at baseline but did not have a stored sample of their isolate, they were classified as "carriage negative" for this analysis.

6.2.2 Clinical procedures

Nasal washes were performed as outlined in section 2.6.3.

6.2.3 Laboratory procedures

Pneumococcal identification was performed as outlined in section 2.7.2, molecular serotyping as in section 2.7.7 and antimicrobial susceptibility testing as in section 2.7.8. Pneumococci were classified as vaccine or non-vaccine serotypes based on their presence or absence in PCV13, whose serotypes are shown in Table 6.1 alongside the additional serotypes included in PPV23.

Table 6.1: Serotypes in the two licensed pneumococcal vaccines

Serotype	PCV13	PPV23	Serotype	PCV13	PPV23
1	■	■	11A		■
2		■	12F		■
3	■	■	14	■	■
4	■	■	15B		■
5	■	■	17F		■
6A	■		18C	■	■
6B	■	■	19F	■	■
7F	■	■	19A	■	■
8		■	20		■
9N		■	22F		■
9V	■	■	23F	■	■
10A		■	33F		■

Shaded boxes indicate that the serotype is included in the indicated vaccine

We determined antimicrobial susceptibility using EUCAST clinical breakpoints (Table 2.3, Table 2.4). Meningitis breakpoints were used when determining penicillin susceptibility, and intermediately-susceptible isolates were classified as resistant. We defined multiple drug-resistant (MDR) pneumococci as those that were resistant to at least three classes of antibiotic (224, 227).

6.2.4 Statistical analysis

For purposes of comparison, we made the assumption that maximal vaccine coverage of under-5s with the primary series of PCV13 was first achieved on 1 April 2015 (i.e. five years after its introduction) and divided our period of analysis into before and after this timepoint. The timing and magnitude of serotype replacement following vaccination have varied substantially between different studies, regions and age groups (97); our assumption is supported by UK IPD data, showing an inflection in the rate of NVT IPD in young adults around 2014-2015 (5). We compared the proportion colonised, the proportion colonised with PCV13 serotypes and the proportion of isolates displaying antibiotic resistant phenotypes before and after 1 April 2015 using χ^2 or Fisher's exact test where appropriate.

We compared serotype diversity during the two time periods using the Simpson index. The Simpson concentration, λ, sums the squares of the proportion of each serotype relative to the total number of isolates, and its inverse (1/ λ) is commonly used as a measure of species diversity (228, 229). The closer the value of 1/ λ is to the total number of serotypes, the more evenly dispersed the serotypes are in the population. All analyses were performed using SPSS version 24 (IBM, New York).

6.3 Results

Between September 2011 and March 2017, 795 healthy volunteers met the inclusion criteria for EHPC studies, signed informed consent and underwent at least one nasal wash. A demographic breakdown is given in Table 6.2. The median age was 21 years (IQR 20—23 years) and 452 (56.9%) were female. We detected natural colonisation in 52 subjects (6.5%, 95% CI 5.0—8.5%). Another three participants were recorded as colonised but had no samples for analysis—we classified these three as non-colonised. Two of the natural carriers reported in the "Aging and Immunity" study (Section 3.3.2) are included in this analysis, with the third being detected after the dataset for this study was finalised.

We identified PCV13 serotypes in 17/52 (32.7%); the numbers of each serotype are given in Table 6.3 with numbers of vaccine versus non-vaccine serotypes over time shown in Figure 6.1. The microarray detected dual colonisation in one subject—99% of the signal was attributed to serotype 37 and 1% to 19F; we classified the participant as colonised with serotype 37.

Table 6.2: Demographic characteristics of participants

		Non-carrier (n = 743)	**Carrier (n = 52)**	**Total (n = 795)**
Age, median (IQR)		21 (20—23)	21 (19—23)	21 (20—23)
Female sex, n (%)		428 (57.6%)	24 (46.2%)	452 (56.9%)
Year of screen, n (% of annual total):	2010	8 (100%)	0	8
	2011	65 (97%)	2 (3%)	67
	2012	129 (89.6%)	15 (10.4%)	144
	2013	31 (96.9%)	1 (3.1%)	32
	2014	66 (93%)	5 (7%)	71
	2015	154 (94.5%)	9 (5.5%)	163
	2016	178 (93.2%)	13 (6.8%)	191
	2017	112 (94.1%)	7 (5.9%)	119

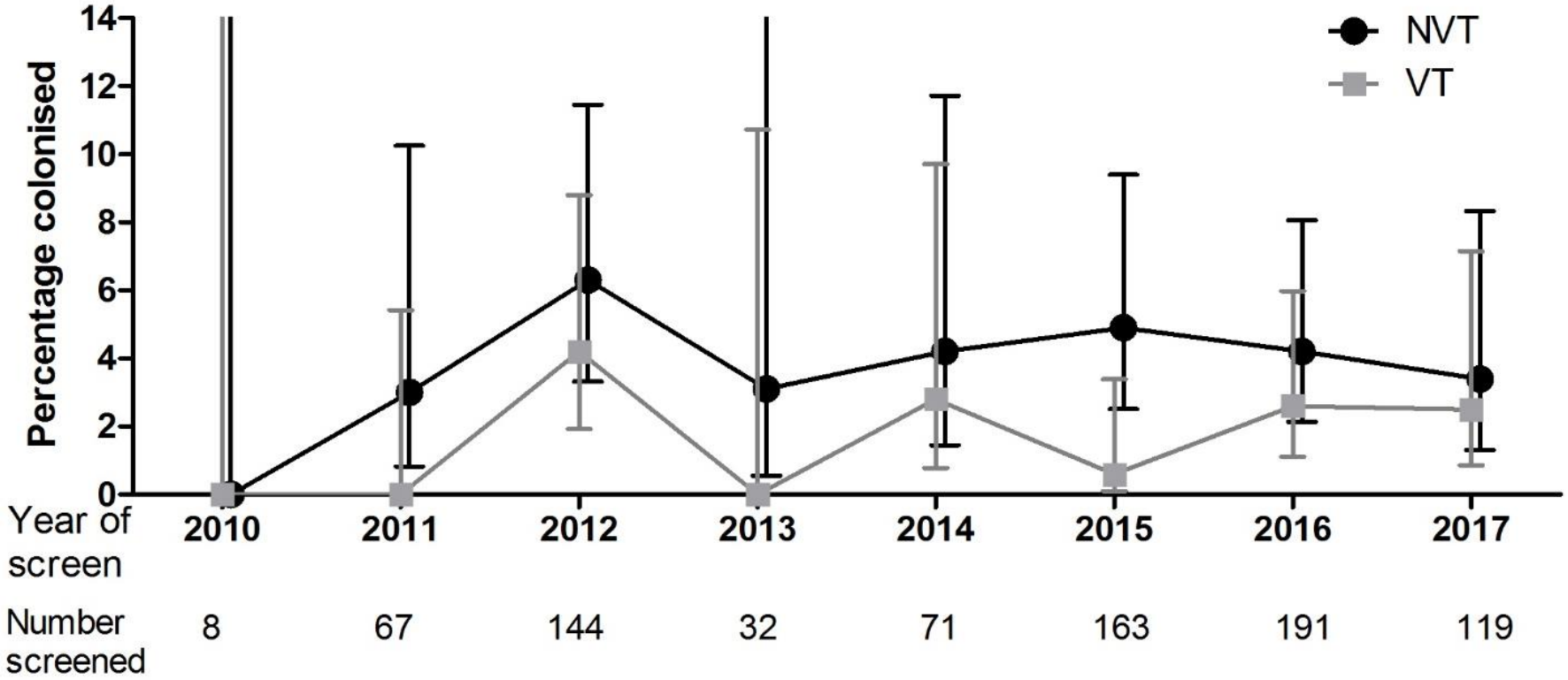

Figure 6.1: Percentage of volunteers colonised with pneumococcus each year
NVT, non-vaccine type; VT, vaccine-type. Error bars represent 95% CI. Note the scale on the y axis.

Prior to 01 April 2015, 24/368 subjects (6.5%) were colonised, with 28/427 colonised after 01 April 2015 (6.6%; p = 0.98 by χ^2). Among colonised participants, 8/24 (33.3%) were carrying PCV13 serotypes before 01 April 2015 versus 9/28 (32.1%, p = 0.93) afterwards (Table 6.3, Figure 6.2). The Simpson diversity index of the pre-01/04/2015 sample was 10.3 while post-01/04/2015 it was 11.2.

Table 6.3: Number of volunteers colonised with each serotype

Serotype	Pre 01/04/2015	Post 01/04/2015	Total
3[*†]	5	5	10
23B	2	3	5
8[†]	2	2	4
11A[†]	2	2	4
35F	2	2	4
37	2	2	4
15A	1	2	3
19F[*†]	1	2	3
19A[*†]	1	2	3
9N[†]	1	1	2
31	0	2	2
33F[†]	2	0	2
35B	1	1	2
6A[*]	1	0	1
10A[†]	0	1	1
15C	1	0	1
24F	0	1	1
Total	**24**	**28**	**52**

Total numbers are provided in addition to numbers before and after the five-year anniversary of PCV13 introduction
** included in PVC13; † included in PPV23*

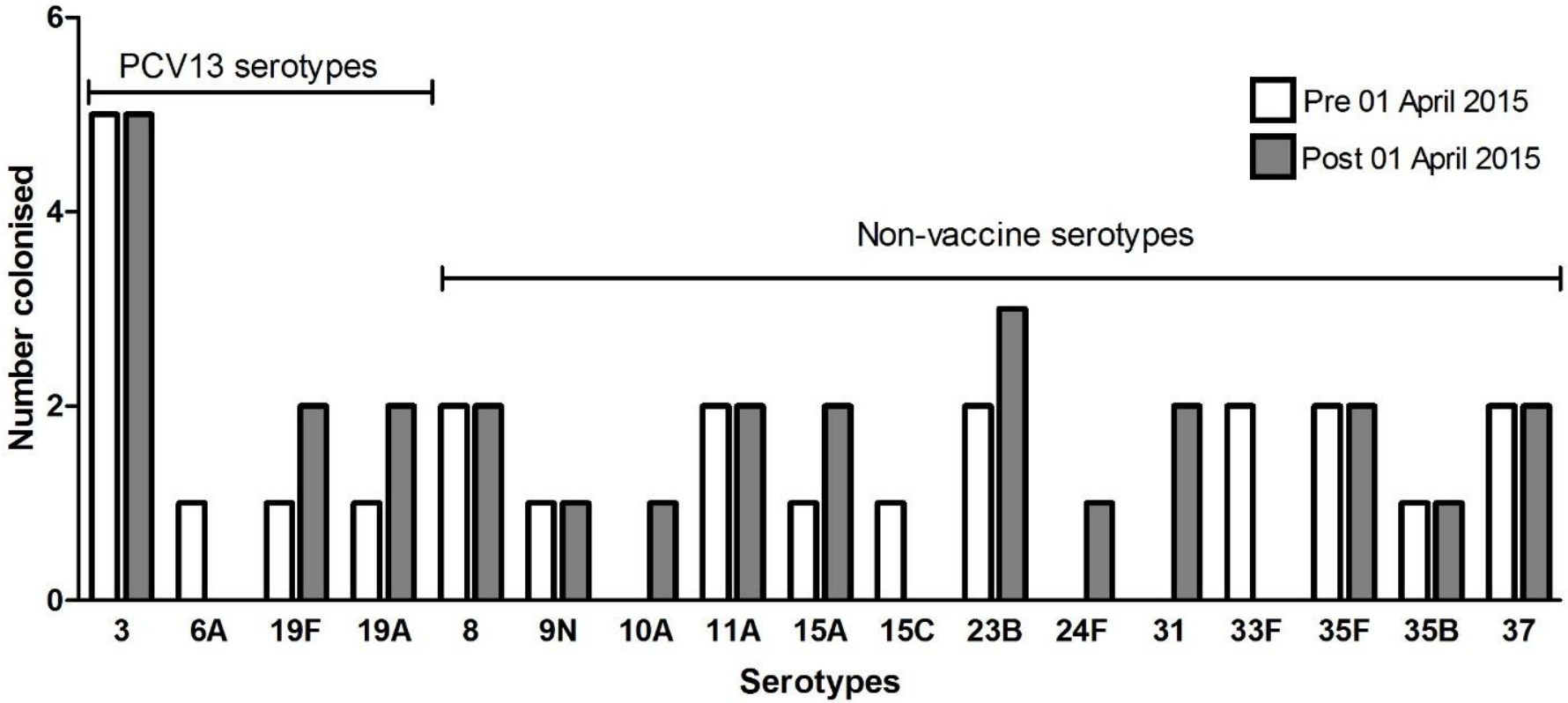

Figure 6.2: Serotype distribution during the two time periods
This bar chart shows the numbers of volunteers colonised with vaccine (left) and non-vaccine (right) serotypes, before (white bars) and after (grey bars) the five-year anniversary of PCV13 introduction. (This figure has been included in a published paper (230), included in this thesis as Appendix 11)

6.3.1 Antimicrobial resistance

We identified resistance to at least one antibiotic in 8/52 isolates (15.4%), and the susceptibility profiles are summarised in Table 6.4. Two of the eight resistant isolates were PCV13 serotypes. Therefore, 2/17 (11.8%) PCV13 serotypes harboured antimicrobial resistance versus 6/35 (17.1%) of NVTs (p = 0.99 by Fisher's exact test; Figure 6.3).

There was one case of discordance between disc diffusion screening and Etest MIC measurements. Norfloxacin disc screening suggested that an isolate was quinolone resistant, but it was determined to be sensitive when the levofloxacin MIC was measured (MIC 1.5 mg/mL, with 2 mg/mL being the cutoff for resistance).

The highest rate of resistance was against penicillin (5/8 isolates), followed by clarithromycin and doxycycline (4/8 each). We did not detect any levofloxacin or vancomycin resistance. Two isolates displayed MDR phenotypes. Using EUCAST non-meningitis breakpoints, all penicillin non-susceptible isolates would have been deemed "intermediate susceptibility", and all were fully-susceptible to amoxicillin and ceftriaxone.

All antimicrobial resistant isolates were detected after 01 April 2015—i.e. 0/24 natural carriers harboured resistance before 01 April 2015, versus 8/28 (28.6%) after. This difference was statistically significant, with a p value of 0.005 by Fisher's exact test.

Table 6.4: Antimicrobial susceptibility profiles among the eight isolates with any resistance detected

Subject ID	**Date isolated**	**Age (yrs)**	**Sex**	**Serotype**	**Susceptibility to antimicrobial agents**			
					Penicillin G	*Clarithromycin*	*Doxycycline*	*TMP-SMX*
402/109	07/04/2015	27	F	24F	S	S	S	**R**
402/057	21/04/2015	23	M	15A	**R**	**R**	**R**	S
016/040	29/10/2015	18	M	15A	**R**	**R**	S	S
016/174	18/02/2016	21	F	23B	**R**	S	S	**R**
018/010	06/07/2016	57	M	23B	**R**	S	S	**R**
019/020	13/10/2016	20	M	3*	S	S	**R**	S
019/099	16/11/2016	19	F	8	S	**R**	**R**	S
019/165	08/02/2017	20	M	19F*	**R**	**R**	**R**	S

**Included in PCV13*
F: Female; M: Male; R: Resistant; S: Sensitive; TMP-SMX: Trimethoprim-sulfamethoxazole

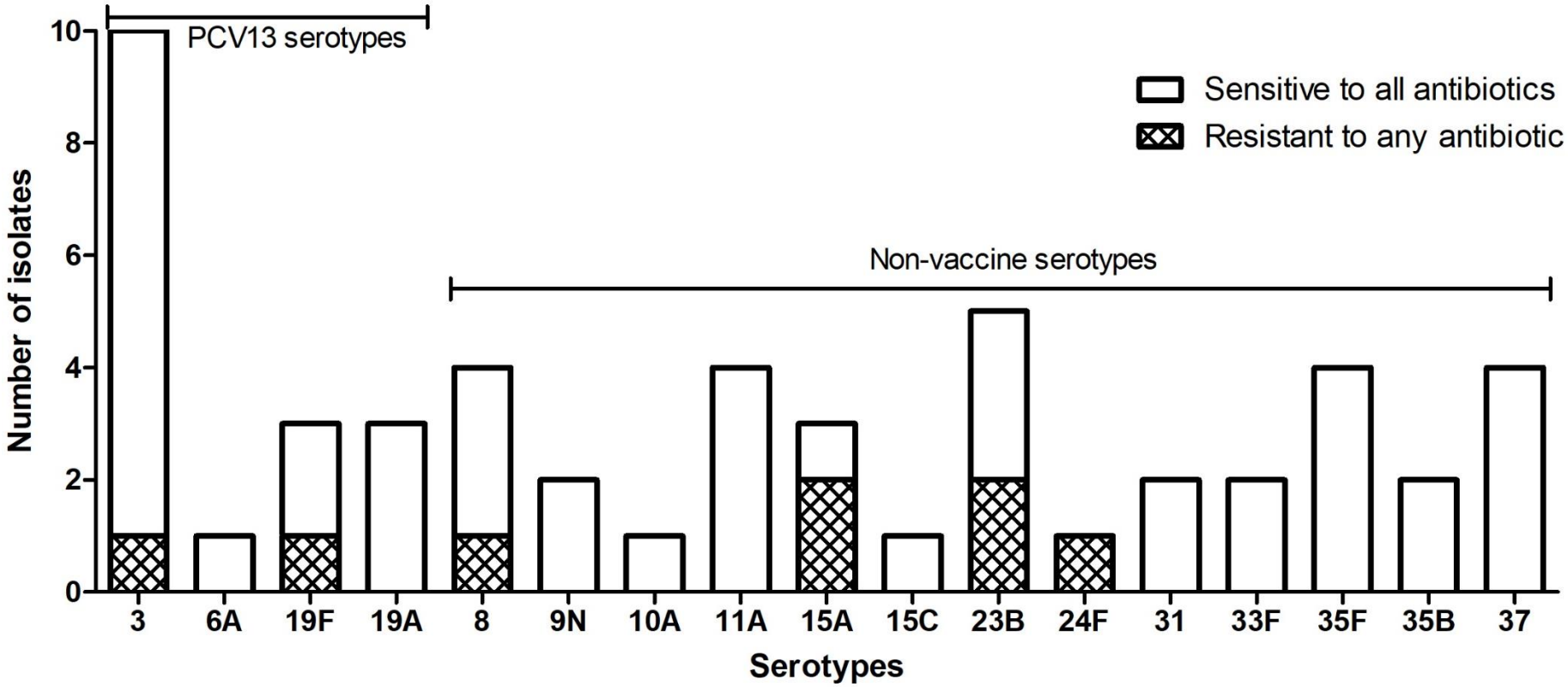

Figure 6.3: Numbers of isolates from each serotype in whom resistance was identified to any antimicrobials
Solid components represent pan-sensitive isolates, while cross-hatched components denote numbers resistant to any antimicrobial.
(This figure has been included in the supplementary appendix of a published paper (230), included in this thesis as Appendix 11)

6.4 Discussion

In this pilot study, conducted over 7.5 years of screening healthy adult volunteers for participation in clinical research, we incidentally detected pneumococcal colonisation in 6.4%. The prevalence of PCV13 serotypes was low, dominated by serotype 3 and did not change over time. Antimicrobial resistance was identified in 15.4% of isolates, all of whom were screened in 2015 or later. The outcomes for each of our hypotheses are outlined in Table 6.5.

Table 6.5: Outcomes of each hypothesis investigated in this chapter

	Hypothesis	**Finding**
1	The prevalence of pneumococcal colonisation is <3%	Refuted—colonisation identified in 6.4% (95% CI 5—8.5%).
2	The serotype composition changes over time in favour of non-vaccine-type colonisation	Refuted—low prevalence of PCV13 serotypes throughout, and grossly similar serotype distributions/diversity over time.
3	Pneumococcal colonisation rates in young adults do not decline during the study period	Retained.
4	Antimicrobial resistance rates are low (<10% of isolates) and remain unchanged during the study period	Refuted—antimicrobial resistance rates low but >10%, and all detected after 01 April 2015. However, resistance rates against individual agents all <10%.

6.4.1 Serotype findings

We found low rates of PCV13-serotype compared with NVT colonisation in young adults, with the exception of serotype 3, and did not identify any change in the proportion of PCV13 versus non-vaccine type colonisation over time.

6.4.1.1 Vaccine type colonisation

Three PCV13 serotypes continued to circulate during our period of analysis—3, 19F and 19A. One explanation is that these serotypes are less susceptible than others to herd protection from childhood PCV programmes.

PCV7 (covering serotypes 4, 6B, 9V, 14, 19F and 23F) had been included in the childhood vaccine programme since 2006, while PCV13 was introduced in 2010, i.e. the same year that screening for EHPC commenced. Therefore, it appears that circulation of PCV7 serotypes was already controlled within the adult population, except serotype 19F. A similar persistence of serotype 19F in IPD has recently been reported in the Alaskan native population (231).

It is concerning that carriage of serotypes 3 and 19A has continued in adults throughout the PCV13 era. Both serotypes are generally associated with above-average virulence and case fatality rates (220, 232). PCV13 is known to have poor efficacy against serotype 3 (221), which continues to cause invasive disease among all ages in the UK (5). Invasive disease due to serotype 19A is declining in children in the UK, but it remains an important cause of pneumococcal disease in over-65s (5).

A reservoir of colonisation among adults could explain these findings. The immune threshold for protection against pneumococcal colonisation is higher than that for disease (79), and therefore these serotypes may also continue to circulate in children. However, the most recent UK childhood colonisation survey found only one serotype 3 and two 19A isolates in 150 colonised children aged <5 years (223).

6.4.1.2 Non-vaccine type colonisation

We did not identify any major patterns, dominant serotypes or changes over time among the NVTs isolated from healthy adults. Serotype 23B was the most common NVT (five isolates), followed by 8, 11A, 35F and 37 (four each). Serotype 8 has emerged as the

commonest cause of invasive disease in the UK population (particularly over-65s) in recent years, responsible for one in five of all cases (5).

Of the ten commonest serotypes identified in colonisation in our study, half (serotypes 3, 8, 15A, 19A and 23B) were also among the top ten serotypes causing invasive disease in England and Wales in 2016—2017 (5). Thus, our pilot data are somewhat reflective of current national trends in IPD. The most recent UK childhood colonisation survey did not identify any children colonised with serotype 8, and serotypes 3 and 19A were among the least frequently isolated (223). However, this survey did identify high rates of childhood colonisation with (for example) serotypes 10A and 22F, which are important in disease and were under-represented in our adult cohort. A colonisation study including all age groups and covering a wider geographical area would be the ideal. If such a survey could successfully identify a range and ranking of serotypes that reflect disease incidence, then colonisation surveys could have the potential to predict serotype replacement in disease following the introduction of higher-valency conjugate vaccines.

6.4.1.3 Serotype diversity

We used the Simpson index to quantify serotype diversity. The closer the Simpson index is to the total number of serotypes in the sample, the more diverse the sample is. We identified 14 distinct serotypes both before and after 01/04/2015, as shown in Table 6.3. With respective Simpson indices of 10.3 and 11.2, it appears that both time periods were similar in terms of serotype richness. Visual inspection of Figure 6.2 gives a similar impression.

6.4.2 Antimicrobial resistance

Overall 15.4% of isolates were resistant to at least one antibiotic. We identified penicillin non-susceptibility in 10% of young adult pneumococcal carriers, and macrolide resistance in 8%, both of which mirror the resistance rates in invasive isolates (225). Among those

colonised with resistant pneumococci, co-resistance to two or three agents was common, again reflecting emerging patterns in invasive disease (224). In the penicillin non-susceptible subjects, the penicillin MICs were generally low, meaning that all would be classified as "intermediate susceptibility" using non-meningitis breakpoints. However, one subject (#019/165) had a penicillin MIC of 0.75 mg/L, which would be less likely to respond to intravenous benzylpenicillin at routine UK doses (1.2g QDS) even in non-meningeal infections (165).

Most of the resistant isolates were from non-vaccine serotypes. We identified dual resistance to penicillin and clarithromycin in two out of three carriers of serotype 15A. Serotype 15A has been among the top ten serotypes causing IPD in the UK since 2011, with a recent study finding that over 30% of these invasive isolates were resistant to three antibiotic classes (224). Further analysis found that the majority of these multi-resistant 15A isolates belonged to multilocus sequence type 63, suggestive of clonal spread within the population. In our results, clonal expansion of resistance is also likely for serotype 23B, where we identified two carriers with resistance to both penicillin and TMP-SMX. Multilocus sequence typing or indeed whole genome sequencing would be required to confirm this hypothesis.

However, not all resistance is due to clonal expansion—we also found co-existent clarithromycin and doxycycline resistance in 3/8 resistant isolates, all with different serotypes. This suggests the spread of AMR via mobile genetic elements. The Tn916 family of conjugative transposons commonly carries genes for both macrolide (e.g. *Mef(E)* or *Erm(B)*) and tetracycline (e.g. *Tet(M)*) resistance in pneumococci (233, 234). Mobile genetic elements allow the spread of AMR between populations of pneumococci but also to other unrelated bacteria in the nasopharyngeal niche. Again, genetic analysis would be needed to investigate this hypothesis further.

6.4.3 Limitations

Our study has some important limitations. We lack detailed medical history for the participants, as we did not have consent to retain these after the participants' original trial participation had concluded. However, the strict inclusion criteria mandated by EHPC studies mean that the study population is quite homogeneous, defined as it is by the absence of significant risk factors for pneumococcal colonisation. Most recruitment events for EHPC studies were carried out in local universities, and it is reasonable to assume that the majority of recruits were themselves students or university employees, but we lack precise data on the source of each individual participant. Prior antimicrobial exposure is unknown, beyond a requirement that all participants must not have received antibiotics in the month prior to enrolment. Contact with children or healthcare settings pre-enrolment was not an exclusion criterion; it is unlikely that people would give up childcare roles simply to participate in a clinical trial. However, some volunteers may have attended hospital for non-infectious indications, visited a relative in hospital or undergone university placement in a hospital before screening. All of our screenings took place well after PCV7 had been introduced and during the period when PCV13 was being introduced, limiting our ability to identify vaccine-induced trends over time.

Molecular testing may be more sensitive than culture for detection of community-acquired colonisation, particularly if the oropharyngeal niche is sampled in addition to the nasopharynx (47). However, this would not allow confirmation of antimicrobial resistance. Phenotypic resistance testing is an important strength of our study, as the evidence base for genotypic-phenotypic correlation of AMR in pneumococci is poor (215).

Nasal wash is not commonly performed in epidemiological colonisation studies. However, it has been recognised as being better tolerated than nasopharyngeal swab while maintaining equal sensitivity for pathogen detection (36, 37). Nasal wash may in fact be a strength of

our study if it explains why we found higher-than-expected rates of colonisation in our population—nasopharyngeal swabs of UK adults who shared a household with children only identified colonisation in 2.8% (223). Nasal wash is technically challenging for participants, but all participants in this study had (by definition) performed a successful nasal wash on their first attempt. Child-friendly nasal-wash methods have also been reported (235). It should be noted that nasal wash requires near-patient laboratory facilities, as the pneumococcal yield falls if kept in saline for >1 hour; this limits the potential for widespread nasal wash use in community colonisation studies. To overcome this limitation, basic near-patient facilities to centrifuge samples and re-suspend them in STGG media would be needed, or else investigators would need to rely on molecular testing methods.

6.4.4 Implications of this study

Using nasal wash culture in healthy adults, our study—admittedly a pilot—found a similar rate of colonisation to a Dutch study of a similar population that relied on combined oral and nasal sampling, analysed by both culture and qPCR (47). Our methodology had the advantage of allowing full serotyping and antimicrobial susceptibility testing on every isolate, maximising the utility of our samples.

Our results suggest that serotype replacement is well-established among young adults and that vaccine-type circulation is mostly controlled with the important exceptions of serotypes 3, 19F and 19A. The relatively high rates of colonisation and of AMR were surprising, given that our study population was specifically selected to lack important risk factors for pneumococcal colonisation (smoking, contact with children < 5) and exposure to healthcare (caring roles, recent antibiotics).

Surveillance of invasive pneumococcal disease by Public Health England has found an overall reduction in IPD in the UK population since the introduction of PCV7, but also that

serotype replacement has cancelled out any additional benefit from PCV13 (5). The failure of herd protection is most apparent in the over-65 population. We have identified ongoing carriage of epidemiologically important serotypes—e.g. 3, 8, 15A, 19F and 19A—in young adults, which might be contributing to ongoing disease in older adults independent of paediatric colonisation dynamics. In addition, the adult reservoir of serotypes 19A/F could re-colonise the paediatric population after the abridged 1 + 1 PCV13 schedule is implemented.

The emergence of AMR in the last three years of our study is concerning, representing a reservoir of antimicrobial resistance genes in the community. As discussed above, AMR in young colonised adults could be driven by both clonal expansion and mobile genetic elements (although whole-genome sequencing would be required to confirm this). Our study population was generally healthy and subjects were excluded if they had recently received antibiotics—i.e. there was low selective pressure for AMR. The AMR we identified probably represents transmitted resistance, reflecting the background population rate of pneumococcal AMR; it is therefore likely that rates of AMR are higher in populations with greater antibiotic exposure. The rates of resistance to individual drug classes are not high enough to warrant changes to treatment guidelines for pneumococcal disease. However, ongoing vigilance for AMR in colonisation at the population level is warranted, to detect trends and increases in rates of AMR as early as possible. Resistant isolates were mostly from non-vaccine serotypes, suggesting that the childhood PCV13 programme is unlikely to further reduce rates of AMR.

The serotype distributions and rates of antimicrobial resistance in young adults were broadly similar to those seen in studies of invasive disease (5, 225). The yield from screening for colonisation in adults is lower than in children, and studies of invasive disease can avail of routinely-collected clinical samples rather than the dedicated research

sampling programme needed for a colonisation study. However, nasopharyngeal sampling of adults is quick and well-tolerated. The BμG@S molecular serotyping methods used on our samples could potentially be adapted for saliva samples, increasing the yield further (albeit without concomitant phenotypic AMR measurement). Colonisation studies of young adults could augment the ongoing surveillance for trends in pneumococcal serotype distributions and susceptibility profiles in communities.

7 Conclusions

In this thesis, we have established that adults aged 50—80 years develop experimental colonisation at similar rates to those seen in young adults. We have also demonstrated that the immune response to colonisation in older adults is different to that of young adults, with no serotype-specific immune boosting following colonisation, and a drop in serotype-specific antibody levels following pneumococcal challenge. This observation, coupled with the finding that prior PPV23 did not protect against experimental colonisation, adds to the existing body of evidence that anti-CPS immunity is deficient in older adults.

Our comparison of different diagnostic strategies within the EHPC model confirmed that nasal wash was the most sensitive method for detecting pneumococcal colonisation within our cohort of older adults. We did not find any evidence for a change of "niche" with aging, or to support the addition of oropharyngeal or saliva samples when assessing pneumococcal colonisation in older adults. These findings come with the caveat that they may not be generalisable from experimental to community colonisation. However, our study of community-acquired pneumococcal colonisation in young adults supported the high diagnostic yield of nasal wash, as well as reaffirming the advantages of culture-based pneumococcal detection methods for serotyping and antimicrobial susceptibility testing.

Our findings present 3 avenues for future research:

1. The use of the EHPC model in older adults
2. Antibody-based anti-capsular immunity in older adults
3. The role of colonisation surveys to inform vaccination policy

7.1 Future EHPC studies in older adults

Our EHPC study established that this model has excellent safety, tolerability and rates of colonisation in older adults. This study generated a wealth of samples and data, including nasal lining fluid samples, nasal microbiopsies and peripheral blood mononuclear cells, many of which have yet to be analysed. Future studies may pursue in-depth analysis of putative biomarkers or correlates of protection identified by these secondary analyses. Candidate vaccines—designed by our team based on these findings, or from external partners—could be trialled in older adults using the EHPC model. A negative outcome of such a trial would efficiently down-select candidates at Phase II level, avoiding the logistical and financial challenges of a Phase III efficacy trial. A positive outcome at Phase II level, preventing experimental colonisation in an at-risk population (rather than a pure immunogenicity study or an EHPC study of young adults) would give vaccine developers and funding agencies confidence in progressing to Phase III.

EHPC could also be used to explore ongoing unanswered questions regarding pneumococcal biology and ecology in older adults. We found that saliva qPCR had poor sensitivity for detecting colonisation in older adults, in marked contrast to community-based studies. A future EHPC study employing alternative saliva collection methods would help explore this conundrum further. In addition, alternative inoculation methods could explore whether the route of exposure influences the pneumococcal niche.

Our cohort was not representative of the general older population: the exclusion of participants with chronic cardiac, respiratory or immunosuppressive conditions, while necessary for safety, is the main limitation of our study. Under-representation of the "oldest old" (over-70s) within the study population is a further limitation. These limitations may explain some of the discrepancies between our findings and other published studies—for example, we did not demonstrate falling anti-CPS antibody titres with age, while others

have done so (65). However, even in a particularly healthy cohort of older adults, we identified antibody responses to pneumococcal challenge that are different to those of younger adults. EHPC was safe and well-tolerated in our cohort, meaning that future studies could consider adopting less restrictive inclusion criteria for older adults; indeed, an EHPC study recruiting volunteers with chronic obstructive pulmonary disease is due to commence in 2019. Future studies could also focus specifically on enrolling over-70s, and using biomarkers of biological rather than chronological age (236, 237) either as inclusion criteria or to guide sub-group analyses.

7.2 Anti-capsular immunity in older adults

Anti-CPS immunity is the mechanism underpinning current pneumococcal vaccines. We did not find evidence that pre-existing anti-CPS antibodies confer protection against colonisation. Other researchers have found that PCV13 confers partial protection against vaccine-serotype CAP, IPD and colonisation in older adults. We found that anti-CPS antibodies may contribute to control of pneumococcal density in older adults; however, evidence linking lower pneumococcal density with reductions in pneumococcal disease incidence, morbidity or mortality is lacking. Higher pneumococcal nasopharyngeal density has been found in HIV-infected patients with pneumococcal pneumonia compared with controls with either non-pneumococcal pneumonia or asymptomatic HIV infection (24), while another study found higher nasopharyngeal pneumococcal density was measured in patients with more severe pneumococcal pneumonia (204). Neither of these studies was sufficient to establish a prospective causal relationship between colonisation density and subsequent clinical outcomes.

We did not find any indication that nasopharyngeal pneumococcal exposure improved anti-capsular immunity in older adults, with static anti-CPS IgG levels in colonised participants and decreased levels in non-colonised participants. Therefore, if a mucosal vaccination

strategy is pursued in older adults, an alternative antigen would have a greater likelihood of success.

However, until an appropriate antigen, adjuvant, delivery system and correlate of protection are identified, polysaccharide vaccination—and particularly protein-conjugated polysaccharide vaccination—remains the only proven method for improving anti-pneumococcal immunity and preventing pneumococcal disease in older adults. The limitations of this strategy lie partially with its imperfect efficacy, but more with its suboptimal effectiveness in "real-world" settings. Although PCV13 confers a degree of protection against vaccine-type CAP and IPD in older adults, there are no plans to add it to the adult vaccination schedule in the UK. Given the high rates of serotype replacement in pneumococcal disease in the UK, there is no reason to revisit this decision, unless a vaccine with greater (or more relevant) serotype coverage is introduced.

7.3 Colonisation surveys to inform vaccine policy

Herd immunity, conferred via the childhood PCV13 programme, has become a cornerstone of protection against pneumococcal disease in older adults. However, our study of community-acquired colonisation in young adults in Liverpool found ongoing colonisation of the vaccine serotypes 3 and 19A. This raises the possibility of an adult colonisation reservoir of these serotypes, since they continue to cause IPD in the general adult population. Nonetheless, this incomplete herd protection is restricted to a small number of serotypes, and would not be sufficient in itself to justify expanding PCV13 coverage to older adults.

In any case, herd protection is currently being diminished by serotype replacement. This was borne out by our community colonisation study, with the majority of isolates being NVTs. We also identified the emergence of antimicrobial resistance in recent years, again clustered in NVTs, representing a further pneumococcal threat to the population.

Our study showed that nasal wash has a reasonable yield in young adults—higher than achieved by nasopharyngeal swab in comparable populations—and our findings complement existing surveillance data on circulating pneumococcal dynamics. Colonisation surveys are a valuable measure of vaccine impact. However, they could have even greater public health benefit if they could inform future vaccine serotype composition.

7.4 A roadmap towards colonisation survey-driven vaccine policy

For colonisation surveys to truly inform vaccine policy, an adaptive pneumococcal vaccine design for older adults would be required. With such a strategy, a system of pneumococcal serotype surveillance would identify trends in serotype replacement and inform the serotype composition of the adult vaccine in as close to real time as possible. The vaccine itself could maintain the current protein-conjugated polysaccharide backbone but would target serotypes more relevant in adult disease. Others have proposed such a strategy before (238), and adaptive vaccine strategies driven by circulating pathogen strains already exist for influenza, with data provided by the Global Influenza Surveillance and Response System (239).

Pneumococcal serotype replacement does not occur at the same rate as influenza virus genetic drift, and therefore annual vaccine updates would not be required. The key unanswered question remains: to what degree does serotype replacement in carriage precede and predict serotype replacement in disease? Mathematical modelling could combine our longitudinal colonisation data, historic childhood colonisation surveys and disease surveillance to address this question, although larger-scale adult colonisation surveys might be needed for statistical power. If models establish the relationship between colonisation serotypes in different age groups and subsequent serotype-specific invasive disease rates, then colonisation surveys at regular intervals could be combined with disease surveillance to determine the serotype composition of the next vaccine.

Superior in-hospital diagnostics (for example, techniques to identify the serotypes in non-bacteraemic pneumonia (240)) would enhance disease surveillance.

Rapid reformulation of vaccines in response to colonisation and disease surveys would potentially require vaccine manufacturers to license their methodologies to other producers, in order to meet global demand for vaccines with different serotype combinations. In addition, it would require pre-licensure studies demonstrating that introducing new serotypes into a standardised manufacturing process would consistently result in vaccines that are immunogenic in the target population. EHPC studies in older adults could play a role in such studies, especially if new serotypes were successfully introduced into the EHPC model. In the meantime, the impetus for novel, serotype-independent, "universal" pneumococcal vaccines would remain, and EHPC studies, feasible in adults of all ages, could continue contributing to the pursuit of this goal.

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Appendix 1: Approval from NHS Research Ethics Committee

NHS
Health Research Authority

North West - Liverpool East Research Ethics Committee
Barlow House
3rd Floor
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7109
Fax: 0161 625 7919

04 February 2016

Dr Jamie Rylance
Clinical Lecturer
Liverpool School of Tropical Medicine
Pembroke Place
Liverpool
L3 5QA

Dear Dr Rylance

Study title:	**Experimental Human Pneumococcal Carriage Model (Programme Grant) Research: working towards a nasal vaccine for pneumonia The effect of age on immune function**
REC reference:	**16/NW/0031**
Protocol number:	**15-053**
IRAS project ID:	**196461**

The Research Ethics Committee reviewed the above application at the meeting held on 21 January 2016. Thank you for attending to discuss the application along with Dr Hugh Adler.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Manager Mrs Margaret Hutchinson, nrescommittee.northwest-liverpooleast@nhs.net. Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below. .

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

1) Please amend the Participant Information Sheet as follows:

a) Please reword the heading 'What are the benefits of taking part?' to 'Will there be benefits to taking part in the study?
b) Under the heading 'How much will I get paid?' please change the word 'risks' to 'discomfort'.

You should notify the REC once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. Revised documents should be submitted to the REC electronically from IRAS. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which you can make available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for HRA Approval (England)/ NHS permission for research is available in the Integrated Research Application System, at www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from the HRA. Guidance on where to register is provided on the HRA website.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Summary of discussion at the meeting

Social or scientific value; scientific design and conduct of the study

The Committee commended the researchers on a well presented proposal for research of significant potential future benefit.
The Committee also noted that the research team had conducted similar research in younger age populations, and were now looking to include an older population. As ageing affects the immune system this was an important target group.

The researchers explained as participants would be older it would be a more challenging group. Therefore they would be incrementally more cautious as the age of the groups increased due to the increased risk of complication they may experience from increased discomfort related to inoculation. However the research team had received requests from older groups to be involved in this research and were confident all the additional risks had been considered.

The Committee noted that page 8 of the IRAS Form included an unfinished sentence which included 'by appropriate' and asked the researchers to confirm what the complete sentence should read.

The researchers confirmed the sentence should read 'by appropriate screening of patients with appropriate inclusion/exclusion criteria' and advised the Committee this had been a copy error.

The Committee were satisfied with the responses given.

Informed consent process and the adequacy and completeness of participant information

The Committee agreed the Information and Consent Form were very well presented and only requested minor corrections as detailed above.

Other general comments

The Committee asked for confirmation if the Grant awarded for the study would be sufficient to cover the costs involved.

The researchers confirmed the grant would be sufficient to cover the costs for the main study. The Sub-Studies would be funded by external collaborators.

The Committee were satisfied with the response.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Copies of advertisement materials for research participants [Poster advert]	1	24 November 2015
Covering letter on headed paper [Cover letter]		
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [LSTM insurance policy]		30 November 2015
GP/consultant information sheets or letters [GP Questionnaire]	1	10 December 2015
IRAS Checklist XML [Checklist_04012016]		04 January 2016
Letter from funder [Funding confirmation]		03 March 2015
Letter from sponsor [LSTM sponsorship]	1	30 November 2015
Non-validated questionnaire [Daily Symptom Log]	1	14 December 2015
Participant consent form [Consent]	1	24 November 2015
Participant information sheet (PIS) [Participant information sheet]	1	25 November 2015
REC Application Form [REC_Form_16122015]		16 December 2015
Referee's report or other scientific critique report [Referee report]		08 September 2014

Research protocol or project proposal [Protocol]	1	25 November 2015
Summary CV for Chief Investigator (CI) [CI CV]		24 November 2015

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/NW/0031	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp
Mrs Glenys J Hunt
Chair

E-mail: nrescommittee.northwest-liverpooleast@nhs.net

Participant information sheet

Research: working towards a nasal vaccine for pneumonia

Exploring the effect of age on immunity against pneumonia

Would you like to take part in our research? This information leaflet tells you how you could take part. A member of our team will also discuss it with you: please ask us if you have questions. You may want to talk to other people about the study: please do so. Take your time to decide if you want to be involved.

What is the purpose of the study?

We are developing a new vaccine to protect against bacteria called **Pneumococcus.**

Small numbers of these bacteria are often found in the nose. Usually, the carrier does not know the bacteria are there. In most adults this is present at least once per year and more often in children. We think that small numbers of bacteria present in the nose ("nasal carriage") can help to protect people against disease.

Mild infections with pneumococcus are very common, such as ear infections in children. But pneumococcus can also infect the lung (causing pneumonia) or the brain (causing meningitis) or the blood (causing sepsis). These severe infections are very uncommon in healthy adults: about 50 cases in Liverpool per year. Very young children and adults who are elderly (mainly those who have other illnesses) are more likely to become ill.

We may be able to protect people against severe disease from pneumococcus using a vaccine which could be sprayed into the nose. We don't yet know if this will work.

To test the idea, our research team want to study what happens when small numbers of the bacteria are put up the nose of healthy volunteers. We have already studied this using more than 400 volunteers, and have found this type of study to be safe.

All of the volunteers we have studied so far have been less than fifty years of age. In order to develop a vaccine that will protect older people, we need to understand the immune responses to bacteria in adults aged over fifty.

Do I have to take part?

No. Taking part in this study is voluntary.

Why have I been asked to take part?

We are looking for volunteers who are fit and healthy. We check for reasons which may put you at higher risk from the study. We also make sure that your participation will provide helpful information to us. If we find any reason you may be at higher risk of infection, then we will not invite you to take part.

You will not be eligible if:

- You are aged less than 50 years
- You are a regular smoker or have a significant history of daily smoking
- You are in close contact with those who have lower immune levels (such as young children and people with chronic ill health)
- You have taken part in similar research before
- You are allergic to penicillin
- You have heart or lung disease
- The study doctor thinks that a health condition, or medication means that you are at increased risk of infection

What happens if I choose to take part?

1. **Health check** – for safety, we check that you are healthy. This includes a clinical assessment and checklist (as above).
2. **Consent** –We ask you to sign a consent form when you are sure you want to take part.
3. **Taking samples** – We take samples from the nose, throat and blood *(see below*). We also do a heart tracing (ECG) and breathing test (spirometry)—again this is to check that you are healthy
4. **Being given drops of pneumococcus in the nose** - We put a few drops with a small numbers of bacteria up your nose
5. **Monitoring**– we will ask you to contact us daily (by phone or text) to make sure you are well
6. **Monitoring visits** – We take samples from your nose to see whether the bacteria is present.

Part one takes less than four weeks then after three to six months we will invite some participants to repeat this for part two.

What kind of samples do you take?

Samples from the nose: To collect cells from your nose we place a small piece of blotting paper inside your nostril for a few minutes ("nasosorption"), and also run a small plastic rod along the inside of each nostril ("nasal probe"). We also perform a "nasal wash", where we squirt a little salty water into your nose. After a few seconds the water runs out into a sample bowl. This will tell us about the bacteria in your nose and your immunity.

Throat swab: we wipe the back of your mouth with a sterile swab (like a cotton bud). The laboratory can use this to find out if there are any bacteria or viruses.

Saliva samples: we will ask you spit into a small tube or to chew on a small sponge for two minutes. We can then test the saliva in the sponge for bacteria.

Blood samples: We take blood samples from a vein in your arm (using a needle). We will never take more than 50 mL (about the same as 10 teaspoons).

You may choose to allow the researchers to study the DNA from your blood sample. If you choose not to donate your DNA you may still take part in the study.

Some participants will also be asked to collect four nasosorption papers and eight saliva samples over 48 hours at home (or workplace etc). This is an additional option which would be discussed with you in greater detail at the start of the study. If you choose to take home samples they must be collected

within 15 minutes of a set time. Therefore we will ask you to send a photograph of the sample to confirm the time taken.

What will happen to my samples?

We will process your samples in laboratories at the Liverpool School of Tropical Medicine (LSTM) and at the Royal Liverpool University Hospital. We will measure the levels of bacteria and viruses in your nose, and we will look in detail at how your immune system responds to the pneumococcus bacteria.

To make full use of your samples, we will store the remainder. In the future, we can then go back to them with new tests to answer new questions. For some specialist tests, we may send samples to laboratories in the UK and abroad.

What will happen at each visit of Stage 1?

Visits 1 - 3:

Consent and screening check (spread over about two weeks)

First we will explain the study in detail, obtain your signed consent if you are happy to take part in the study, and ask some basic questions to ensure that you are eligible. We will also write to your GP to confirm some aspects of your medical history (e.g. what medications you are taking, and which vaccinations you have had before).

At the next visit, we make sure you are fit to take part in the study. We ask routine questions about your medical health, check your blood pressure, temperature and listen to your heart and lungs, perform a heart tracing (ECG), breathing test (spirometry) and blood test.

If you are well enough to take part in the study, we do the throat swab, saliva collection, nasal swab, the nasal wash, nasal probe and another blood test.

We then book your next appointments. If you have problems and can't come at a specific time, we can be flexible to accommodate you.

⇩ *Between one to seven days after Visit 3:*

Visit 4: *Being given pneumococcus up the nose*	We collect a sample from your nose using blotting paper. We then use a dropper to put a small amount of water containing a small number of bacteria into each nostril. Usually, volunteers have no symptoms afterwards. There will be a doctor or nurse available by telephone 24 hours a day to answer questions. We will give you a course of antibiotics to keep with you, in case you are unwell, as well as a thermometer to check your temperature at home. ***Every day for the next week, we will need to be in contact with you by phone or text to check that all is well.***
	⇩ *Up to six visits over the next five weeks*
Visits 5 - 10: *Monitoring*	At each visit, a number of samples will be taken, which may include throat swab, saliva collection, nasal swab, nasal wash, nasal probe and blood tests
	⇩
End of Part 1 *of the study*	If our laboratory test finds that the pneumococcus bacteria stays in your nose, at this stage we will ask you to take a course of antibiotics to clear it, and we may ask you to be in part 2 of our study.

What about Part 2?

We think that having small number of bacteria in your nose—even for a short time—might protect you against illness from this bacteria, possibly for a long time. But we cannot be certain. To test this, we may ask you to have the pneumococcus put into your nose a second time, after 3 to 6 months. Y*ou do not have to take part in Part 2 if you do not want to*. In total, Part 2 visits will take about 2 to 3 weeks.

What will happen at each visit of Part 2?

Visit 1: *Screening check, consent, and taking samples*	We make sure you are still fit to take part in the study, by repeating the questions and examination done at Part 1. We do the throat swab, saliva collection, nasal wash, nasal probe and blood test.
	⇩ *1-7 days later*

Visit 2: *Being given pneumococcus up the nose*	We use a dropper to put a small amount of water containing a small number of bacteria into each nostril, just like before. Each day for the next week we will ask you to contact the research team by phone or text for seven days to ensure that all is well and to check your temperature reading (again, antibiotics and a thermometer are provided in the study).
	⇩ *Daily phone call or text message. 2 days later:*
Visit 3: Monitoring	Throat swab, saliva collection, nasal wash, nasal probe and blood test
	⇩ *Daily phone call or text message. 5 days later:*
Visit 4: Monitoring	Throat swab, saliva collection, nasal wash and nasal probe
	⇩ *Up to 7 days later*
Visit 5: End of the study	At the end of Part 2, after a final throat swab, saliva collection, nasal wash, nasal probe and blood test, if our laboratory confirm that you have had pneumococcus in your nose, we will ask you to take the antibiotic course to clear it.

What are the risks of being in the study?

Risks of being given live bacteria

Because the bacteria are alive, there is a very small risk of infection to you or your close contacts. We do not expect anyone to develop an infection but this is why we choose participants carefully, and why we monitor them closely. We provide a thermometer and antibiotics that treat these bacteria. We give you a separate leaflet which explains the safety precautions, and what to do if you feel unwell. If you carry the pneumococcus bacteria in your nose at the end of the study, we will ask you to take the antibiotics to kill the bacteria.

Risks of medical tests during the study

The only side effect of nasal sampling is a little discomfort. Some people experience a runny nose. Some people can feel light-headed after blood tests, and sometimes may have a bruise.

What if there is a problem?

You can contact the research team 24 hours-a-day by phone. They will answer any questions, and an emergency service will be available day and night, including visits to your home if you are unwell and unable to come in to see us. Any medical care you need will be provided by the NHS.

What if I wish to complain?

If you wish to complain about any aspect of the study, you can contact the study doctor. The NHS complaints procedures are also available to you. Complaining will not affect the medical care you receive now or in the future.

What if I change my mind, or want to stop?

Even if you do start in the study, you are free to stop at any time and without giving a reason. If you decide not to take part, or to withdraw from the study, this will have no effect on your future health care.

If you decide to stop, we will continue to use the samples and information that we have already collected unless you tell us not to. You will be paid for the visits completed up to that point.

Will my details be kept confidential?

Yes. For safety, we collect information about your medical history and contact details before you take part. The clinical research team use this information to check you are healthy, and to contact you when needed. We will ask your permission to ask your GP to share some of your medical history with us.

We will also collect information which allows us to understand more about the samples, for example, you age or sex. However, those outside of the clinical team are never given information that can identify you. Your samples are given a unique number, and your name is not used.

We do not expect to find anything which would affect your health care. If we do, we will let you and your GP know about it.

All data will be collected and stored at the Royal Liverpool University Hospital and the Liverpool School of Tropical Medicine. It will be stored for a minimum period of 10 years. Your medical notes and research data are may be looked at by those who monitor the research.

What are the benefits of taking part?

There are no direct benefits to you. You will be a part of what we believe is a valuable research study that may help us to improve medical care for others.

How much will I get paid?

The money you are paid is compensation for inconvenience, loss of income, and possible discomfort of taking part. The first payment will be made at the end of part one. If you are eligible and choose to take part in the second study you will receive a second payment at the end of part two. Our payments are listed below:

Part 1	**Visit length**	
Visit 1: Study information, signing consent	45 min	-
Visit 2: Health check, screening and samples	60 min	£40
Visit 3: Screening and samples	30 min	£30
Visit 4: Having pneumococcus put up your nose. This includes you making daily telephone/text message contact for the first 7 days. (We will withhold £5 per day if you do not contact us)	30 min	£50
Visits 5 and 6: Nasal samples, throat swab and blood samples	30 min	£20
Visit 7: Nasal samples and throat swab	20 min	£15
Visits 8 and 9: Nasal wash and throat swab (*not all participants will be called for visit 9)*	15 min	£10
Visit 10: Nasal samples, throat swab and blood samples	25 min	£20
Optional home samples (for a subset of participants)	8 timepoints over 48 hours	£48
Part 2		
Visit 1: Screening and samples from the nose and blood	45 min	£30
Visit 2: Having pneumococcus put up your nose, and follow-up, as above	60 min	£50
Visits 3, 4 and 5: Nasal samples, saliva collection, blood tests and throat swab	15 min	£15

Contact details

General questions: please contact the research team on 0151 706 3381 during normal working hours. Web site: www.lstmed.ac.uk/pneumoniavaccine

The Chief Investigator for this study is Dr Jamie Rylance. You may contact him at the Liverpool School of Tropical Medicine, Pembroke Place, Liverpool, L3 5QA, UK. Telephone: 0151 705 3775. This research is sponsored by the Liverpool School of Tropical Medicine and the Royal Liverpool and Broadgreen University Hospitals. It is funded by the Medical Research Council. The research has been reviewed for scientific content by an external panel.
The National Research Ethics Service Committee (16/NW/0031) has reviewed the study and given approval for it to take place.

Appendix 3: Advertising material

LSTM

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

Would you like to take part in our research?

Working towards a nasal vaccine for pneumonia

Exploring the effect of age on immunity

We are developing vaccines to protect against a bacteria called Pneumococcus. Vaccine are needed as this bacteria can cause infection particularly in young children, frail elderly people and those with other illnesses.

Researchers in Liverpool have developed a way to test vaccines against these bacteria.
We invite healthy volunteers to have a few drops of the bacteria in the nose then monitor the response carefully.

Please contact the respiratory research team for more information if you are a

- **Healthy**
- **Non-smoker**
- **Aged 50 to 84 years**

(you will be paid for your time and inconvenience)

Please contact Respiratory Research for further information on:

Tel: 0151 706 4856 or 0151 705 2500
Email: 2volresearch@lstmed.ac.uk
Text 2VOL to 88802

Experimental Human Pneumococcal Carriage (EHPC) Model Effect of age on immunity
Poster Advertisement Version 1: 24th November 2015
REC Ref 16/NW/0031

Appendix 4: Letter of invitation from primary care

<To be printed on GP Letter Headed Paper>

<Insert Patient Title and Name>
<Insert Patient Address1>
<Insert Patient Address2>
<Insert Patient Address3>
<Insert Patient Address4> *<Insert Date>*

Dear *<Insert Patient Title and Name>*

We'd like to introduce you to ground-breaking research looking at ways to prevent pneumonia. The researchers are based in the Liverpool School of Tropical Medicine and Royal Liverpool University Hospital, and they study the immune response to a bacteria placed in the nose. A better understanding of these immune responses could help them develop a new vaccine which could be used to protect children and other vulnerable populations all over the world.

The team have been working on this for five years and have extensive experience in carrying out similar studies in over 500 volunteers.

As part of our commitment to medical research, we have agreed to assist the research team at the hospital in identifying potential people registered at our practice who may be interested in taking part in their studies. Our records indicate that you may be eligible to take part in a study for non-smoking, healthy people who are between 50 and 84 years of age. The study will involve a full medical assessment followed by the placement of bacteria in the nose and regular follow-up with doctors and nurses. All volunteers are paid for their time and inconvenience.

This study has been reviewed and approved by the National Research Ethics Service Committee to ensure the safety of the people taking part.

Taking part in a study can be an enjoyable experience where you get to meet like-minded people in a relaxing environment with a host of facilities and an opportunity to learn more about research—and about your own health—from medical experts.

To find out more about the study please:

- **Call 0151 706 4856**
- **Text 2VOL to 88802**
- **Email:** 2volresearch@lstmed.ac.uk
- Website: **********

Yours Sincerely,
<Insert GP Signature>

Dr *<Insert GP Title and Name>*
< Insert Name of Practice >

Appendix 5: General practitioner questionnaire

GP questionnaire

The Royal Liverpool and Broadgreen University Hospitals NHS Trust

Dear Dr _____

Your patient _____,

DOB _____, address _____ has agreed to participate in the following study: **Experimental Human Pneumococcal Carriage: Effect of age on immune function.** This study involves inoculation with pneumococcal bacteria and monitoring over 4 weeks for experimental carriage of the bacteria.

To ensure that your patient is eligible and safe to participate, we require some background information. We appreciate that your practice is extremely busy, and therefore a printed patient summary, including **current medication**, **past medical history** and previous **immunisation** and **allergies** would suffice. Alternatively, please would you **complete, sign, date and stamp** the attached questionnaire at your earliest convenience. Please return the completed forms by fax to Helen Hill on **(0151) 706 4856**. Thank you very much for your kind assistance in our research project. If you have any concerns or questions regarding this study please feel free to contact us.

Sister Helen Hill

helen.hill@lstmed.ac.uk

Tel (0151) 706 4856

Fax (0151) 706 4856

Please tick one box

1. *Printed patient summary attached* ☐
2. *Questionnaire completed* ☐
3. *This patient is not registered at my practice* ☐

Medical Report to be completed by the General Practitioner

General Medical History	Please Circle	Please Provide Details
Does the patient have cardiovascular and/or respiratory disease?	YES NO	Details _____
Does the patient have an immunosuppressive condition? (Including diabetes, active malignancy, immunosuppression secondary to medications etc)	YES NO	Details _____
Is the patient allergic to penicillin/ amoxicillin?	YES NO	
Has the patient been prescribed any regular medications?	YES NO	Details (or please attach copy of latest prescription) _____
Has the patient ever been microbiologically diagnosed with pneumococcal disease? (Culture-proven or molecular diagnosis)	YES NO	Details _____
Has the patient received a pneumococcal vaccine at any point? (Prevenar/ pneumovax)	YES NO	Vaccine and date _____

Please provide any additional relevant information:	
Date	**Practice stamp**
GP signature	
Print name	

Please fax these forms back to the Research nurses at the Royal Liverpool University Hospital on 0151 706 4856 (safe haven fax).

For office use only

Review of GPQ by study physician (please sign box below)

Eligible to proceed with study based on GPQ	YES NO (please circle)	
Name	Signature	Date

Appendix 6: Post-inoculation safety leaflet

EXPERIMENTAL HUMAN PNEUMOCCAL CARRIAGE MODEL

Working towards a nasal vaccine for pneumonia:

The effect of age on immune function

Information Sheet

EMERGENCY RESEARCH TEAM

XXXXX XXX XXX

7 days a week

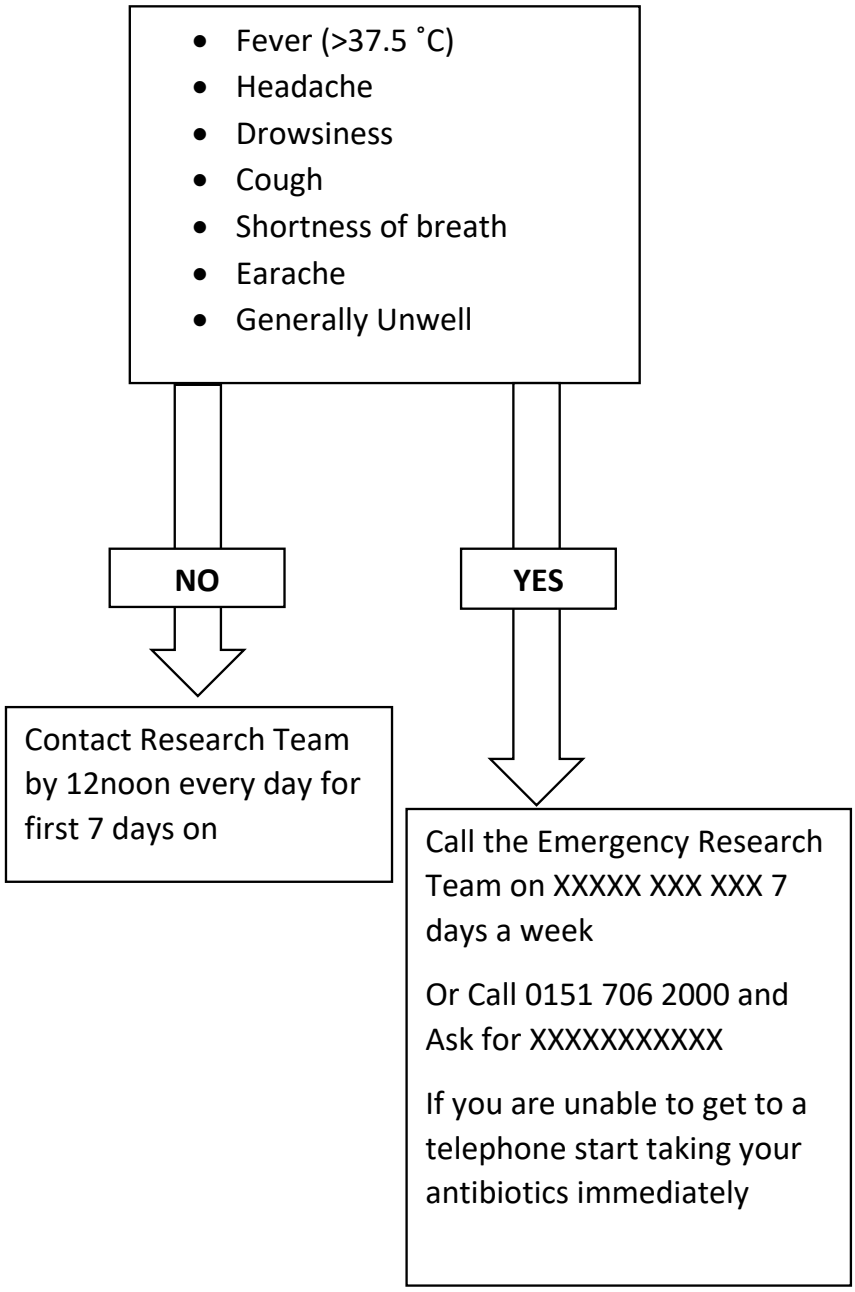
OR CALL

0151 706 2000

Hospital Switchboard

Ask for XXXXXXXXXXXXX

Do you have any of the following?



What should I do?

If you have any of the above symptoms we would ask that you should contact the research team on the following numbers without delay

Xxxxx xxx xxx 7 days a week

0151 706 2000 Hospital switchboard - ask for xxxxxxxxxxxxx who will be available by telephone 7 days a week for advice.

What if I feel very unwell?

In the unlikely event you feel very unwell, the research team emergency number (xxxxx xxx xxx) is available seven days a week. If for any reason you are unable to make contact with the team (or are not able to access a phone) we recommend that you start taking the antibiotics immediately (one tablet (500mg) of AMOXICILLIN to be taken three times per day) and attend your nearest Emergency department.

What do I tell the doctor?

If, for any reason you have to attend your doctor or the hospital you need to inform them that: *You have had live Streptococcus pneumoniae inoculated into your nose on ___/___/_____ as part of a clinical study. The bacteria you carry are fully sensitive to amoxicillin and you have no history of allergy to this antibiotic.*

Do I need to do anything if I feel well?

We ask that for the **first 7 days** you text or phone **the research nurse** by 12noon every day on the following number: **xxxxx xxx xxx.** .

This is to ensure that you are not experiencing any problems. If we do not hear from you by 12noon we will contact you to make sure you are not experiencing any problems. In the event that we cannot contact you, your next of kin will be contacted.

Things you should know.....

Following inoculation with pneumococcus

After the pneumococcus is put into your nose it is possible that it may cause an infection. Although this is very unlikely it is sensible that you familiarise yourself with symptoms or signs that may indicate infection to make sure they are recognised and treated early.

Keep your thermometer, antibiotics and contact numbers with you at all times during the study.

WHAT SHOULD I LOOK OUT FOR?

If you feel generally unwell or have any of the following:

- **Fever (temp>37.5 ˚C)**
- **Shivering**
- **Headache**
- **Drowsiness**
- **Shortness of breath**
- **Cough**
- **Earache**

If you have any of the symptoms or signs marked in bold please call the emergency number immediately.

Xxxxx xxx xxx

7 days a week

OR Phone 0151 706 2000 and ask for xxxxxxxxxxxxx

Appendix 7: Poster presented at the International Symposium on Pneumococci and Pneumococcal Diseases, 2016

Developing Experimental Human Pneumococcal Carriage (EHPC) in elderly subjects

Hugh Adler[1,2], Helen Hill[1,2], Angela Wright[1,2,3], Seher Zaidi[1,2], Katherine Piddock[1], Stephen B Gordon[4], Daniela M Ferreira[1], Jamie Rylance[1,2,5]
1. Liverpool School of Tropical Medicine, Liverpool, UK; 2. Royal Liverpool and Broadgreen University Hospitals NHS Trust, Liverpool, UK; 3. NIHR Comprehensive Research Network Northwest, UK; 4. Malawi-Liverpool-Wellcome Trust, Blantyre, Malawi; 5. Aintree University Hospital, Liverpool, UK
Email: Hugh.Adler@lstmed.ac.uk

Background

People aged >65 years are particularly vulnerable to pneumococcal disease,[1] and respond suboptimally to currently-available vaccines.[2]

Nasopharyngeal pneumococcal carriage is common in young people and has an immunizing effect,[3] but is rare in the elderly, in whom its immunologic effects are unknown.[4]

We have previously shown that experimental human pneumococcal carriage (EHPC) is feasible and safe in healthy young adults,[3] and is an invaluable source of immunologic data as well as showing promise as a vaccine testing platform.[5]

We have launched a new study to establish and assess EHPC in older adults. This poster outlines our study protocol.

Objective

Inoculate 64 healthy community-dwelling volunteers aged ≥50yrs with 80,000 CFU pneumococcal serotype 6B per naris.

Inclusion Criteria

- Adults aged 50-84 years with good performance status (0 or 1 on WHO scale) who are willing to attend study visits and maintain daily contact with study team.

Exclusion criteria

- Current smoker or ex-smoker with >10 pack years history
- Increased risk of pneumococcal disease (alcohol abuse, immunosuppression, cardiac/respiratory/renal disease).
- Abnormal findings on pre-screening physical examination, spirometry, electrocardiogram or routine blood tests.
- Penicillin allergy.
- Close physical contact with at-risk individuals (children under 5yrs, immunosuppressed adults).

Questions to be answered:

What is the rate of EHPC in older adults?

- *30—50% of younger adults can be experimentally colonised[4,5]; we hypothesise a rate of 10% in an older cohort.*

What is the functional significance of any antibody response to EHPC?

- *In younger subjects, pneumococcal challenge induces mucosal antibodies, but carriage is required for a systemic antibody response.[3]*
- *Anti-pneumococcal antibodies (both "natural" and vaccine-induced) have reduced opsonic function in elderly people[6]—will this be the same in experimentally-colonised subjects?*
- *There is a dearth of knowledge about nasal mucosal immunity in the elderly, the role of mucosal antibodies, and whether a mucosal vaccine would be efficacious[7]—all of these avenues can be studied using the EHPC model.*

Is the local inflammatory response different to that of young subjects?

- *Flow cytometric analysis of nasal cell samples could assess the degree of "inflammaging" of the nasal mucosa.*

Is carriage protective against subsequent re-challenge with the same serotype?

Where is the niche of pneumococcal carriage in the elderly?

- *Oropharyngeal sampling may have a higher yield for detecting pneumococcus than nasal sampling[8]—paired oral and nasal samples and salivary PCR will be performed on all subjects in this study.*

Latest results
2 subjects inoculated
Ages 59 and 62
Carriage rate: 0/2

Nasal cell scraping, used for:
- Nasal mucosal cell culture
- Ciliary beat analysis
- RNA expression analysis
- Characterisation of local inflammatory responses

Nasal wash, used for:
- Pneumococcal culture (defines carriage)
- *lytA* and capsular PCR (to compare culture with molecular methods)
- Mucosal immunoglobulin and cytokine assays

1.Navarro-Torne A et al. *EID* (2015).
2.van Werkhoven CH et al. *CID* (2015).
3.Ferreira DM et al. *AJRCCM* (2013).
4.Flamaing J et al. *J Am Geriatr Soc* (2010).
5.Collins AM et al. *AJRCCM* (2015).
6.Park S, Nahm MH. *Infect Immun* (2011).
7.Fujihashi K, Sato S, Kiyono H. *Exp Gerontol* (2014).
8.van Deursen AM et al. *Vaccine* (2016).

Bill & Melinda Gates foundation
The Royal Liverpool and Broadgreen University Hospitals NHS Trust
MRC Medical Research Council

Appendix 8: Abstract presented at the British Infection Association Spring Meeting, 2017

BIA 20th Annual Spring Meeting

ABSTRACT SUBMISSION

Title: Experimental human pneumococcal colonisation in older people is feasible and safe

Abstract No. 0010

Title Experimental human pneumococcal colonisation in older people is feasible and safe

Abstract

Background: Nasopharyngeal colonisation with *Streptococcus pneumoniae* can lead to invasive disease, but is usually asymptomatic, and generates beneficial adaptive immune responses. Colonisation may be safely replicated experimentally in young adults, with 45% of volunteers typically developing colonisation after experimental inoculation. We report the initial results from the use of this experimental model in older adults, where natural colonisation is less frequent and the immunological effects are uncertain.

Methods: Healthy volunteers between 50 and 75 years old were inoculated with 80,000 CFU of *S pneumoniae* (serotype 6B) in each nostril. Colonisation was determined by nasal lavage (at baseline and days 2, 7, 9, 14, 22 and 29 post-inoculation). Anti-6B capsular polysaccharide IgG antibodies were quantified by ELISA from serum samples at baseline and 29 days post-inoculation.

Results: Sixteen volunteers have been inoculated to date; no adverse events were observed. The median age was 61 years (range 50-73). Two were naturally colonised with pneumococcus at baseline (one serotype 3, one 23F). Experimental colonisation was established in three volunteers. The mean baseline antibody titre in those volunteers who did not become colonised was 1285ng/mL versus 1197ng/mL at day 29 (p=0.8), while in those volunteers who become colonised it was 1422ng/mL at baseline versus 4227ng/mL at day 29 (p=0.2).

Conclusions: Pneumococcal colonisation can safely be experimentally established in older people, who are typically under-represented in controlled human infection studies. Early results suggest that experimental colonisation occurs at a lower frequency in older volunteers than in prior studies of young adults.

Permission Yes

Approval Confirm

Affiliations
(1) Liverpool School of Tropical Medicine, Liverpool, UK
(2) Royal Liverpool University Hospital, Liverpool, UK
(3) Malawi/Liverpool Wellcome Trust, Blantyre, Malawi

Authors				
	Hugh	Adler	(1)	Presenting
	Esther	German	(1)	
	Elena	Mitsi	(1)	
	Victoria	Connor	(1) (2)	
	Caroline	Hales	(1) (2)	
	Helen	Hill	(1) (2)	
	Lepa	Lazarova	(1) (2)	
	Catherine	Lowe	(1) (2)	
	Rachel	Robinson	(1) (2)	
	Seher	Zaidi	(1) (2)	
	Simon	Jochems	(1)	
	Elissavet	Nikolaou	(1)	
	Sherin	Pojar	(1)	
	Jesús	Reiné	(1)	
	Carla	Solorzano-Gonzalez	(1)	
	Emma	Smith	(1) (2)	
	India	Wheeler	(1) (2)	
	Stephen	Gordon	(3)	
	Daniela	Ferreira	(1)	
	Jamie	Rylance	(1) (2)	

Registration Confirm

Appendix 9: Poster presented at the LSTM Postgraduate Researcher Conference, 2018

Appendix 10: Review article published in *Clinical and Vaccine Immunology*, 2017

Appendix 11: Research paper published in the *Journal of Infectious Diseases*, 2019