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Declarations

The work presented in this thesis my own. Some of it was performed in collaboration with colleagues and was carried out at the Liverpool School of Tropical Medicine, the Royal Liverpool and Broadgreen University Hospital and Aintree University Hospital.

The results included in this thesis have not been part of a previous degree.

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List of Abbreviations

AE	Adverse Event
AHR	Airway Hyper responsiveness
BDP	Beclomethasone Propionate
BTS	British Thoracic Society
COPD	Chronic Obstructive Pulmonary Disease
CFU	Colony Forming Unit
DMSC	The Data Monitoring and Safety Committee
EHPC	Experimental Human Pneumococcal Challenge
ERV	Expiratory Reserve Volume
FeNO	Fractional Exhaled Nitric Oxide
GP	General Practioner
Ig	Immunoglobulin
ICS	Inhaled Corticosteroid
IRAS	Integrated Research System
ISCRTN	International Standard Randomised Controlled Trial Number
LABA	Long Acting Beta Agonist
LAMA	Long Acting Anti Muscarinic
LTRA	Leukotriene Receptor Antagonist
NIHR	National Institute for Health Research

OCS Oral Corticosteroids

PEFR Peak Expiratory Flow Rate

PCV Pneumococcal Conjugate Vaccine

PPV Pneumococcal Polysaccharide Vaccine

PCR Polymerase Chain Reaction

PI Principal Investigator

RD&I Research Development and Innovation

REC Research Ethics Committee

SABA Short Acting Beta Agonists

SAE Serious Adverse Event

1 Abstract

1.1 Rationale

Pneumococcal pneumonia is a leading cause of death globally, and susceptibility to invasive and pulmonary infection is increased in chronic respiratory conditions. Pneumococcal colonisation of the nasopharynx necessarily precedes disease, but the relationship of colonisation with *Streptococcus pneumoniae* and chronic respiratory disease (such as asthma) is unknown. Asthma is a heterogenous condition representing several phenotypes which have corresponding clinical characteristics and responses to therapy. Immune responses mediated by cytokines from cells such T helper cells 2 (Th2) and Th17 lead to airway inflammation. Both this inflammatory pathology, and the treatment of it (with inhaled corticosteroids) potentially affect nasopharyngeal colonisation with *S.pneumoniae.* Hypothetically, airway inflammation might increase the potential for bacterial attachment, or increase bacterial clearance, or both. As nasopharyngeal colonisation is immunogenic in healthy adults, the balance of these opposing mechanisms may impact the resulting immune response. We have safely used the experimental human pneumococcal challenge (EHPC) model in healthy individuals to study nasopharyngeal colonisation and its associated mucosal and systemic immune responses.

1.2 Objectives

Using experimental pneumococcal challenge in people with asthma, I examined the acquisition of nasopharyngeal colonisation of *Streptococcus pneumoniae,* its association with clinical characteristics, and systemic immune responses. I compared the results with historic data from earlier EHPC studies of healthy controls.

1.3 Methods

I enrolled people with physician-diagnosed, well-controlled asthma on maintenance inhaled corticosteroids. Participants were challenged with pneumococcus serotype 6B, as this is not isolated

from nasal samples within the local community. Blood and mucosal samples were collected at baseline and on days 2,7, 9, 14, 22 and 29 after challenge. The results were compared to healthy controls from 4 experimental human challenge studies, with 2 performed concurrently and 2 in previous years.

1.4 Main Results

Experimental colonisation rates were not significantly increased in people with asthma compared to healthy controls. The number of colonised participants (nasal wash positive for bacterial culture at any time point) was 28 (56%) in asthma vs 68 (45%) in healthy controls (Pearson's chi square p=0.178). The density calculated using the area under time cure (AUC) was similar in people with asthma compared to healthy controls (median [IQR] asthma 63.49 [14.04-116.3] vs healthy controls 81.18 [48.15-104.5] Mann Whitney U test p=0.060). Acquisition of colonisation was independent of baseline characteristics such as blood eosinophils and fractional exhaled nitric oxide levels.

The duration of experimental colonisation was significantly shorter in asthma compared to healthy controls (median [IQR] 14 [7-29] vs 29[14-29]) p=0.034 Mann Whitney U test. Colonisation led to an increase in IgG titres to capsular polysaccharide and pneumococcal proteins in people with asthma as previously described in healthy controls.

Body mass index correlated positively with likelihood of colonisation in people with asthma. Median BMI for the whole cohort was 24.6 (IQR) (21.6-27.5), with colonised participants (nasal wash positive for bacterial culture at any time point) having a higher BMI median 24.7 (24.1-29.0) vs 23.5 (20.1-26.4) in non-colonised (p=0.019 independent samples t test).

1.5 Conclusions:

Experimental colonisation is not affected by clinical characteristics of asthma, and it is positively correlated with a high BMI. The rate and density of experimental nasopharyngeal colonisation in people with asthma are similar as seen in healthy controls. The duration of colonisation is significantly reduced in people with asthma compared to healthy controls, with a similar immunogenic effect as seen in healthy controls.

Interventions to reduce the likelihood of severe and invasive pneumococcal disease, to which people with asthma are more prone, could be targeted at specific sub-groups. Further investigation could suggest if those with higher BMI should have a lower threshold for vaccination.

2 Introduction

Pneumonia is a common condition and disproportionately affects people at the extremes of age, the immune compromised, and those with chronic respiratory conditions (1, 2). Asthma predisposes to pneumonia, but the magnitude and mechanism of effect are not fully elucidated.

Pneumonia remains a leading cause of morbidity and mortality worldwide, with the burden of disease being greatest in low and middle-income countries (LMIC) (3). It accounts for more deaths in children under the age of five than any other illness. The commonest cause of bacterial pneumonia is *Streptococcus pneumoniae,* resulting in approximately 40,000 hospitalisations and 70,000 primary care consultations annually in the United Kingdom (4). *S. pneumoniae* is commonly found in the nasopharynx, the source of primary spread (5) and initial step towards infection (6). Colonisation is most prevalent in children (7, 8) and declines with increasing age (9), probably due to acquired immunity from exposure. Nasopharyngeal colonisation is a dynamic process with changing prevalence, density and serotype of *S. pneumoniae*. A high colonisation density in the nasopharynx is associated with subsequent development of pneumonia (10), and replacement with more virulent serotypes may lead to pneumonia (11). Perturbation of the airway microbiome may also lead to pneumococcal pneumonia, in some cases without dense nasal colonisation (12, 13).

Systemic immune responses are robustly generated following pneumococcal vaccination, which is now part of the childhood immunisation programme in around half of World Health Organisation member states (14, 15). Vaccination is associated with a reduction in hospitalisations due to pneumonia in children and adults (16), and is effective in reducing the risk of invasive pneumococcal disease (17, 18). However, the incidence of pneumonia is increasing in the elderly (19, 20).

Preventing colonisation is therefore an important alternative target for controlling pneumococcal transmission (6), reducing the incidence of respiratory tract conditions (sinusitis, otitis media and pneumonia) and invasive disease (septicaemia and meningitis) (21) in those most at risk.

Patients with mild, moderate and severe asthma suffer from exacerbations which are mostly due to viral infections (22) but can be secondary to bacteria such as *S. pneumoniae;* the commonest cause of pneumonia. Several risk factors are identified for nasopharyngeal colonisation of *S. pneumoniae* (23) including asthma and an asthma exacerbation in the previous twelve months. Inhaled corticosteroids (ICS) are the mainstay of treatment for asthma, and escalating doses are recommended to achieve adequate control (24). Recent evidence from studies of chronic obstructive pulmonary disease (COPD) suggests ICS therapy increases the risk of pneumonia (25), and has led to a change in guidelines for COPD, which now advise discussion of the increased risk of pneumonia secondary to ICS with patients, and consideration of other therapies (26). There is conflicting evidence in asthma, with review of data from randomised controlled trials showing no increased risk, and observational studies reporting an increased prevalence of pneumonia and invasive pneumococcal disease (27-30).

The focus of this thesis is to determine the rate of experimental nasopharyngeal colonisation of *Streptococcus pneumoniae* in people with asthma, and to understand systemic and mucosal immune responses following colonisation in comparison to healthy controls.

2.1 Asthma

2.1.1 Definition

Asthma is a chronic inflammatory condition, clinically characterised by episodes of recurrent wheeze, shortness of breath, chest tightness and cough secondary to airway hyper responsiveness (AHR). These symptoms often occur at night or early morning, and are associated with airway obstruction which is variable and reversible either spontaneously or with treatment (31).

2.1.2 Aetiology

The exact aetiology of asthma is not clear with several factors contributing to the pathophysiology of this chronic condition. It is increasingly recognised as a complex syndrome with genetic predisposition and more than 100 identifiable gene associations (32, 33). The disease contributing

factors can be broadly grouped into two: i) related to the host such as genetic predisposition and ii) related to symptom triggers or sensitisation such as environmental factors (31). Prenatal factors such as maternal smoking, diet, stress, use of antibiotics and mode of delivery by caesarean section have all been associated with development of atopic disease including wheeze in early childhood (34). In addition, exposure to infections, tobacco smoke, use of antibiotics in early life, having low lung function, being breast fed or not, socio-economic status, allergic sensitisation, and exposure to animals can all modify the risk of developing asthma in genetically predisposed individuals (34, 35). Adults may develop asthma secondary to occupational exposure, from smoking tobacco and other recreational drugs (34). Complex interactions between host and environmental factors are an area of interest for research and further investigation (36).

2.1.3 Asthma Phenotypes

Severe asthma requires treatment with high dose ICS plus a second controller and systemic corticosteroids to achieve symptom control and may remain uncontrolled despite this therapy. 5-10% of total asthma burden is estimated to be that of severe asthma (37). Asthma is well appreciated for its heterogeneity with variable clinical presentation and differing response to therapy. Phenotypes refer to observable clinical and treatable characterstics resulting from hereditary and environmental factors. There is increasing interest in understanding asthma endotypes - the underlying molecular and patholophysiologic mechanisms which determine clinical characterstics and response to treatment. Accessibility to biological therapy for treatment of asthma and approach to personalised medicine has further enhanced research interest in endotypes (38). Personalised medicine has lead to the concept of treatable traits for airways disease such as presence of eosinophilc airway inflammation, increased smooth muscle contractility and reversibility (39). This is an evolving concept with suggestions that these can be used to predict future exacerbations and improve outcomes by providing targeted therapy specially in severe asthma (40). Severe asthma phenotypes are classified either according to clinical characteristics or by pathobiological differences in sputum or bronchoscopy samples (41). Another approach to

classification includes triggers for the disease, such as environmental allergens or occupational exposures (42). Although classifications aim to define and distinguish phenotypes from each other, significant overlap is seen across groups.

2.1.3.1 Eosinophilic Asthma Phenotype

This is characterised by eosinophilic airway inflammation driven by T helper type 2 (Th2) cells and interleukin 5 (IL5) (43). In severe uncontrolled asthma eosinophilia is present in the airway mucosa of both upper and lower respiratory tract, and high counts are seen in sputum and bronchial samples (44, 45). Eosinophils are produced in response to inflammatory mediators released by Th2 cells such as IL5, IL13, and granulocyte colony stimulating factor (GCSF). These are recruited to the airways and act on mast cells to release histamine leading to airway hyper responsiveness, increase mucus production by goblet cells, eosinophil peroxidase causing epithelial cell damage and transforming growth factor β (TGFβ) leading to airway remodelling (46, 47). Eosinophilia is an early sign of an asthma exacerbation and patients with eosinophilic asthma experience recurrent exacerbations (48). Eosinophilic inflammation leads to basement membrane thickening and may result in fixed airflow obstruction.

2.1.3.1.1 Fractional Exhaled Nitric Oxide (FENO) can be used to identify eosinophilic asthma

Nitric oxide (produced by the conversion of L-arginine to L-citrulline, catalysed by NO synthases (NOS)) is an important inflammatory mediator in the lungs and acts as a vasodilator, bronchodilator and non-adrenergic and non-cholinergic neurotransmitter (49, 50). It is produced by epithelial cells, macrophages and T lymphocytes in the lungs (51). High levels of Fractional Exhaled Nitric Oxide (FeNO) noted in eosinophilic phenotype are associated with worse asthma control and peripheral blood eosinophilia. FeNO is reduced by steroid treatment (52). It is a simple non-invasive measurement of airway inflammation widely used in clinical practice (53). The values are recorded in parts per billion (ppb) (54).

2.1.3.2 Obesity related asthma

Obesity is recognised as a risk for asthma and can be secondary to a high body mass index (BMI) or a consequence of therapy for the condition (55). Excessive maternal weight gain during pregnancy may lead to an increased risk of asthma in future for the baby (56, 57). The prevalence of asthma is increased in adults with a high BMI compared to those within normal range (58). Obese patients have more severe asthma, increased hospitalisations, poor symptom control and worse quality of life (59, 60). The response to usual asthma therapy is suboptimal in obese patients due to an altered cytokine profile (61). Obesity alters Th2 driven inflammation by affecting eosinophil recruitment, with an increase in sputum IL5 and submucosal eosinophils, but does not affect sputum eosinophils (62). Obesity alters innate immune responses in the upper (innate lymphoid cells and macrophages) and lower respiratory tract (surfactant protein A) (63, 64). Lung function is altered by obesity itself with a noticeable reduction in expiratory reserve volume (ERV), and an increase in AHR (65, 66). Clinically, weight loss is associated with improvement in not only asthma severity, control, but also quality of life (55).

2.1.4 Asthma Diagnosis

Asthma is a syndrome with variable presentations and symptoms. The diagnosis is often challenging, based on clinical history, examination and diagnostic tests. The tests can measure clinical parameters such as lung function, reversibility and peak flow rates and airway hyper-responsiveness or determine airway inflammation and allergic status (31).

2.1.4.1 Clinical History and Physical Examination

Symptoms of episodic wheeze, cough, shortness of breath suggest a clinical diagnosis of asthma (67). These can show seasonal and diurnal variation, and often occur in response to allergen exposure. In addition, a family history of asthma, and history of atopic disease can be helpful. Presentations and symptoms are variable; patients may present with symptoms related to exercise only, and some with predominant cough often at night only (68). The symptoms can fluctuate over days, months,

seasons or at different times of the day. This is an important part of asthma diagnosis and specifically enquired from the patients (31). Physical examination can be completely normal with polyphonic wheeze being the most common finding (31).

2.1.4.2 Diagnostic Tests

2.1.4.2.1 Lung Function Measurements: Reversibility and Peak Expiratory Flow Variability

These are performed and repeated on different visits, with adequate instructions by trained staff to ensure good quality results (69, 70). A change in forced expiratory volume in one second (FEV_1) of >200mls and >12% after inhaled bronchodilator (salbutamol 200-400mg) is an accepted indication of asthma (71), although lacking sensitivity. An absence of reversibility is seen in patients on optimal treatment with good symptom control, and does not exclude asthma (31).

Peak expiratory flow rate (PEF) is another useful measurement for diagnosis and monitoring of asthma. These are effort dependent and values differ for different peak flow meters and therefore can only be used in addition to spirometry and not in place of it (31, 72). Comparison is made with the patient's best reading, obtained at a time when they are well and symptom free. Airway obstruction may reverse over time after initiation of appropriate treatment such as ICS (31).

2.1.4.2.2 Bronchial Provocation Testing

This test measures bronchial hyper responsiveness (BHR). Inhalation of histamine, methacholine or mannitol is monitored by serial spirometry, starting at a very low concentration and gradually increasing. After each dose FEV_1 is compared to baseline.

This is helpful in assessing individuals with symptoms suggestive of asthma with normal lung function (31). Methacholine challenge test has a better negative predictive value and is therefore useful to exclude a diagnosis of asthma (73). The test should be performed by adequately trained professionals as it can cause bronchoconstriction. It is interpreted according to the value of provocation concentration producing a fall of 20% (PC_{20}) (mg/ml) with >16 accepted as normal, 4-6 borderline, 1-4 mild and <1 moderate to severe BHR (73). This test was considered but not used for

diagnosis in our study, for two reasons: i) It leads to aiway inflammation and may have resulted in an exacerbation. They may have required treatment with OCS and deferred for 4 weeks, ii) it is expensive and the cost of the test was not included in the overall expenses for the study, we did not have funding for this.

2.1.4.2.3 Fractional Exhaled Nitric Oxide (FeNO)

See section 2.1.3.1.1.

2.1.5 Airway microbiome in asthma

Advances in novel culture-independent techniques to identify bacteria, have improved our understanding of the different human microbiota (74). Pyro sequencing of 16S rRNA gene amplicons from bacteria helps in genus and species identification (75, 76).

The upper and lower airway microbiome is different, with the latter studied using bronchoalveolar lavage (BAL) specimens, and/or in spontaneously expectorated sputum (74) (77, 78).

Bacteria from the phylum proteobacteria (*H.influenzae, M.catarhalis, Neisseria spp*) are significantly increased in asthma compared to healthy controls and COPD in the oropharynx (79). Genus Haemophilus is present in both COPD and asthma in samples from the bronchial tree (BAL and bronchial brushings) (78).

Airway microbial composition and diversity (variation in species in mild, moderate and severe asthma) is correlated to bronchial hyper responsiveness in sub-optimally controlled asthma (80). Specific bacterial groups such as Nitrosomonas species with the enzyme nitric oxide reductase (81) may alter nitric oxide concentration and FeNO levels (82, 83), and the microbiome may influence corticosteroid sensitivity (84).

2.2 Asthma Exacerbations

A severe asthma exacerbation is an acute episode of progressive worsening of symptoms, including shortness of breath, wheezing, cough, and chest tightness, requiring treatment with oral

corticosteroids or an increase from a stable maintenance dose for a period of 3 days or more (85). Moderate asthma exacerbations do not require treatment with systemic corticosteroids, are associated with a deterioration in symptoms and treated with a temporary increase in inhaled therapy such as increased use of salbutamol (85).

2.2.1 Viral causes of asthma exacerbation

Most acute asthma exacerbations are caused by viruses (86, 87), and human rhinovirus is the commonest implicated organism (88). Antibiotics are not recommended routinely for the treatment of acute asthma exacerbation (24) and are usually prescribed to treat exacerbations for patients with a diagnosis of COPD or both COPD and asthma, as opposed to those with a diagnosis of asthma alone (89).

2.2.2 Bacterial causes of asthma exacerbation

Bacteria are recognised as a source of infection, exacerbation (90) and colonisation of airways in asthma (91), although viral infections are accepted as the primary cause (92). Bacteria are also found in sputum of patients with asthma outside of an exacerbation (91). This is further discussed in section 2.5

2.3 Managing Asthma

2.3.1 Inhaled Corticosteroids (ICS) are the mainstay of preventative treatment of asthma

ICS have anti-inflammatory properties improve symptoms, quality of life and lung function (31) (93). They help control airway inflammation, reduce airway hyper-responsiveness, frequency and severity of exacerbations along with mortality (93-96). The available ICS for treatment of asthma are shown in Table 1. These are usually started in low doses and increased as required to achieve optimal control (24); high doses are associated with side effects (97, 98). The dose delivered varies for different formulations and devices. ICS therapy does not cure asthma and symptoms may deteriorate when patients stop taking these (99).

Table 1 Commonly used ICS

	ICS	Brand names available
1	Beclomethasone dipropionate	Clenil Modulite®, Qvar®, Fostair®
2	Budesonide	Pulmicort®, Symbicort®
3	Ciclosenide	Alvesco®
4	Fluticasone propionate	Seretide®, Flixotide
5	Mometasone acetonide	Asmanex®
6	Fluticasone furoate	Relvar®

Fostair, Symbicort, Seretide and Relvar are combination inahlers with long acting beta agonist.

2.3.1.1 Common Side Effects of ICS

High doses of ICS can be associated with local (oral thrush) and systemic side effects (85) such as adrenal suppression, bruising, reduced bone mineral density, cataracts and glaucoma (100-107)

2.3.2 Long Acting Beta agonist (LABA)

These are used as add on therapy to ICS for better symptom control (85). Combination of ICS and LABA is initiated when treatment with low to medium dose of inhaled corticosteroids is not sufficient to control symptoms (108). This is an effective combination and leads to improvement in symptoms, lung function, reduction in exacerbations and use of short acting beta agonist (SABA) (95, 109-113), and is the preferred option when a medium dose of inhaled corticosteroids alone fails to achieve control.

2.3.3 Leukotriene Receptor Antagonists (LTRA)

In the airway leukotrienes cause smooth muscle contraction, lead to increased mucus production, reduced muco-ciliary clearance and enhanced eosinophil recruitment (114-116). LTRA have a variable response in asthma and BTS recommends a trial of LTRA if addition of LABA to ICS therapy does not provide adequate symptom control (24) (117).

2.3.4 British Thoracic Society Treatment Steps

Asthma treatment is increased to improve symptom control in a stepwise manner in accordance with the British Thoracic Society (BTS) guidelines (24). The steps are described in Table 3. Patients on step 1 are only treated with as required short acting beta agonist (SABA). ICS in a low dose is added at step 2. If symptoms persist ICS dose is increased, with addition of LABA and LTRAs. Patients on step 4 require high dose ICS and often multiple courses of oral corticosteroids (OCS), and on steps 5 are prescribed regular maintenance OCS. This approach is helpful in distinguishing patients with severe disease – on step 4 and 5, who may be eligible for new therapies such as monoclonal antibodies – which may reduce the use of OCS and achieve better asthma control with a reduction in exacerbations (118, 119). These guidelines were updated in 2019 and use of SABA is no longer recommended, instead low dose ICS therapy is advised as an initial treatment. The new guidance recommends referral to specialist centre for patients on high dose ICS with persistent uncontrolled symptoms (24).

2.4 Streptococcus pneumoniae

S.pneumoniae are gram positive cocci, with a polysaccharide capsule and a cell wall made up of peptidoglycans and teichoic acid (21). The capsule is an important determinant of the virulence for *S.pneumoniae*, with up to 90 different polysaccharides identified on the surface. These are different structurally and prevent phagocytosis of bacteria by generating a specific immune response, unique to the respective polysaccharide (5), with limited cross antigenicity due to shared polysaccharide epitopes (120). The 23-valent polysaccharide vaccine (PPV 23) is designed to cover serotypes causing the vast majority of infections (121).

The bacterial cell wall lies beneath the polysaccharide capsule and plays an important part in generating an inflammatory response by initiating recruitment of leukocytes, inducing cytokine production and increasing vascular permeability (120). It is a dynamic structure with embedded pneumococcal surface proteins (6), of which several are identified as potential vaccine targets.

Unlike the capsular polysaccharides, pneumococcal proteins are not serotype specific. Several proteins are identified on the surface of *S.pneumoniae,* with many found in all the clinically relevant strains (122). These factors make them a suitable target for novel vaccines, which would provide protection against more serotypes than the currently available vaccines and may help overcome the challenge of serotype replacement. An increase in antibody titres to pneumococcal proteins is seen in infants between the ages of 12 and 24 months naturally regardless of colonisation status, and in response to colonisation (123). These antibody titres do not provide protection against future colonisation episodes and are inversely related to age (123).

Pneumococcal choline-binding protein A (PcpA), genetically detoxified pneumolysin (PlyD1, PdA, PdB), chemically detoxified pneumolysin (dPly), and Pneumococcal histidine triad D (PhtD) and E (PhtE) have been widely studied. Phase II clinical trials have been performed with formulations of monovalent PhtD, PlyD1, PcpA or bivalent PhtD-PcpA proteins. Results from phase I studies show these formulations to be safe and immunogenic (124).

Choline binding proteins (CBP) help in adherence of the bacteria to the surface receptors such as the platelet activating factor receptor (PAFR) and provide assistance in the acute phase response (125).

Pneumococcal biofilms are structured cellular aggregates seen during nasopharyngeal colonisation and diseases such as sinusitis (126) . The cluster of cells in close proximity facilitates transfer of genetic material, this may help biofilms promote antimicrobial resistance (127, 128). Antibiotic resistance may be attributed to a change in phenotype of the bacteria, secondary to transfer of genetic information as well as reduced penetration. Biofilms may cause upward or downward regulation of genes with inclusion of competence stimulating protein leading to an increase in biomass. During interaction with the epitehlial cells pneumococci down regulate their capsule which is facilitated by biofilms Environmental factors such as nutrient availbilty and temperature are the major determinants of biofilm formation (129). Both ICS and the excipients in inhaled powders may

affect biofilm formation in people with asthma (130). Biofilm formation may lead to worse outcome secondary to genetic transformation and anti microbial resistance (126).

2.5 Pneumococcal Colonisation is More Common in People with Asthma

2.5.1 Early life pneumococcal colonisation and subsequent asthma

Infants colonised with *S. pneumoniae* have a two to four-fold increased risk of a first wheezy episode, persistent wheeze, development of asthma by the age of 5 years, and hospitalisations for wheeze. Early nasopharyngeal colonisation is associated with an increased eosinophil count, IgE and airway reversibility. This was demonstrated by the Copenhagen Prospective Study on Asthma in Childhood (COPSAC), an ongoing clinical, prospective, longitudinal birth-cohort of infants born to mothers with current or previous asthma. 321 of these infants had hypopharyngeal swabs collected at the age of 1 and 12 months, and bacteria were isolated using microbiological culture techniques. All the infants were asymptomatic at the time of sample collection. The overall prevalence of one or more organism was 71% including *S. pneumoniae, M. catarrhalis, H. influenzae* (35).

The prevalence of asthma in the overall cohort at 5 years of age was 14%, but 33% of those initially colonised were diagnosed with asthma. Blood eosinophil count and total IgE were significantly higher in colonised children at the age of 4 years (35). Early life nasopharyngeal colonisation of *S. pneumoniae* is associated with asthma later in life.

2.5.2 Pneumococcal colonisation and current asthma

An increased prevalence of *S. pneumoniae* colonisation has been seen in people with asthma in observational studies (23, 131, 132). An Italian study investigated *S. pneumoniae* colonisation in children and adolescents with asthma by collecting oropharyngeal swabs at one time point and analysing them using polymerase chain reaction (PCR) for the autolysin-A-encoding (lytA) and the wzg (cpsA) gene of *S. pneumoniae*. (132). The colonisation rate of 45% is similar to those seen in other observational studies in young children (133). Esposito *et al* (132) performed this study when

PCV-7 had been part of the immunisation schedule for five years in Italy and included children regardless of their vaccination status. There was no association between vaccination and nasopharyngeal carriage (48% vs 43% vaccinated compared to non- vaccinated), though time since vaccination was not studied. Parental smoking, recent use of systemic corticosteroids, and the severity of asthma did not affect carriage rates. A total of 423 children were enrolled with varying degree of disease severity. Results are described in the Figure 1:

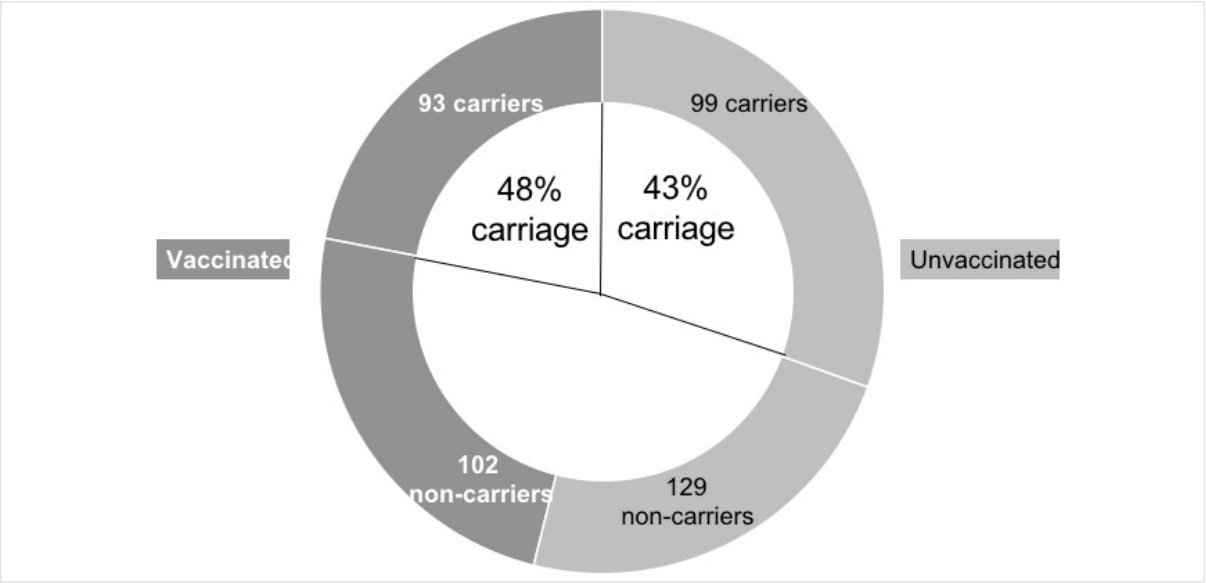


Figure 1: Nasopharyngeal carriage rates in children with asthma; comparison of rates in vaccinated and unvaccinated groups

(adapted from data Esposito et al (132).

A case control study of army recruits found a two-fold increased prevalence of *S. pneumoniae* colonisation in those with well-controlled, mild to moderate physician diagnosed asthma (n=224) in comparison to those without asthma (n=668) (134). Study participants were asked to complete a questionnaire on medical history and allergy status, and oropharyngeal swabs were collected within the first two weeks of service. Just under half of those with asthma were on medication for the condition (107, 48%), and this did not influence colonisation rates observed in this study (134). Microbiological culture was used to identify the organisms. No objective evidence of asthma or data on compliance with medication or smoking history was available.

Having an asthma exacerbation in the previous 12 months is associated with an increased risk of pneumococcal colonisation. A cross-sectional population-based study of over 1000 adolescents in Brazil found a nasopharyngeal colonisation rate of 19% (OR=2.89) in the 37 participants who had experienced an acute exacerbation of asthma in the previous 12 months (131). Sinusitis and rhinitis were associated with nasopharyngeal colonisation, both of which are common comorbidities in asthma (135).

In summary, pneumococcal colonisation appears more common in people with asthma, especially in individuals with a recent asthma exacerbation and possibly those with concurrent nasal symptoms.

2.5.3 Pathophysiological factors affecting colonisation rates in asthma

In this section, I discuss some aspects of the immune system which mediate airway inflammation in asthma and may influence pneumococcal colonisation and clearance in the nasopharynx.

2.5.3.1 Airway Inflammation

The hallmark of asthma is chronic airway inflammation mediated by innate and adaptive mechanisms (136). This causes differential damage throughout the airway epithelium (137), and biopsies show epithelial inflammation in patients across the spectrum of disease. Surface epithelial thickening, metaplasia, goblet cell hyperplasia, increased mucus secretion with altered density, and hypertrophy of smooth muscles can be present (138). Chronic inflammation secondary to eosinophils is seen in the nose and plays a part in development of nasal polyps (139). This is associated with higher pneumococcal colonisation due to increased sites for bacterial attachment (140) including the epithelial surface expression of platelet activating factor receptor (PAFR) (141). Pneumococcus binds to PAFR through phosphoryl choline in its cell wall (142). PAFR is upregulated during viral infections and secondary to other inflammatory stimuli, including asthma (143). It is itself an important pro-inflammatory mediator which contributes to asthma pathogenesis by activating immune cells, causes chemotaxis of eosinophils, increases vascular permeability, mucus production and bronchial constriction (144-146). PAFR antagonists represent potential therapies in

acute and chronic asthma (147). In vitro studies show that mice lacking PAFR are relatively protected against pneumococcal pneumonia (148). In another murine model, mice were sensitised by intraperitoneal injection of ovalbumin, and subsequently their sinuses were dosed with *S. pneumoniae* with concomitant administration of ovalbumin to induce local allergic inflammation. Infection with *S.pneumoniae* was increased in response to the allergic inflammation induced by ovalbumin (149). In summary, the type of airway inflammation seen in asthma appear to be associated with increased bacterial adherence and colonisation, and progression to infection.

2.5.3.2 Capsular Polysaccharide Specific and Pneumococcal Protein Antibody

Impaired antibody responses may account for increased rates of colonisation and risk of invasive pneumococcal disease in asthma.

Pneumococcal capsular polysaccharide immunity is partially protective against nasopharyngeal colonisation. In a human infection model with pneumococcus, capsular polysaccharide (CPS) IgG-mediated bacterial agglutination in the nasopharynx was observed in PCV-vaccinated adults and associated with protection against colonisation acquisition (150). PCV reduces serotype specific nasopharyngeal colonisation, probably resulting in herd immunity and protection from disease (151, 152). In an observational study more than two thirds of patients with severe asthma were found to have low baseline antibody levels to *S. pneumoniae* and *H. influenzae (153).*

In a cross-sectional study, levels of serotype-specific CPS antibodies were lower in children and adults with asthma compared with healthy volunteers. There was no difference in nasopharyngeal colonisation between the two groups (half of each group had been vaccinated) and their community exposure to *S. pneumoniae* is likely to have been equivalent. The median number of positive vaccine serotype specific antibodies in the PCV7 cohort was also lower in asthma when compared to healthy volunteers (5 vs 7 respectively p=0.046) (154). Additionally, an inverse relationship between interleukin 5/interferon gamma (IL5/IFN-γ) (Th2 immune profile) in response to house dust mite stimulation of peripheral blood mononuclear cells (PBMCs) and the number of serotypes to which

antibodies increased above a certain threshold is seen in the asthma group (154). The same research group undertook a similar investigation comparing serum IgG to anti-pneumococcal surface protein in people with and without asthma: anti-pneumococcal surface protein C (Psp C) levels were inversely correlated with Th2 immune profile. PspC is an adhesin to polymeric immunoglobulin receptor (PIgR). This receptor helps bacterial adhesion to the nasopharyngeal epithelium, initiates colonisation and facilitates invasive disease (155). Antibodies against pneumococcal surface proteins may also have a protective role against bacterial nasopharyngeal colonisation and subsequent disease (156). Hales *et al* identified lower levels of IgG1 titres PspC and *Haemophilus influenzae* antigens P4 and P6 in children with asthma (157). Taken together, these results suggest an impaired immune response in people with asthma which may predispose to an increased risk of pneumococcal disease (158).

2.5.3.3 Cytokine IL-17

Advances in our understanding of heterogeneity of asthma have led to more tailored use of therapies such as anti-IL5 monoclonal antibodies, which can be effective in patients showing Th2 cell-mediated inflammation. However, many people with asthma have at least some inflammation secondary to Th17 responses. This pathway appears more important in individuals who are older, obese and non-allergic (136). Th17 cells produce cytokines such as IL17 and IL22 (159). In mouse models of asthma, IL17 contributes to airway remodelling by promoting fibroblast proliferation (160) and opposing the anti-inflammatory features of T regulatory cells (161). It can also lead to direct contraction of smooth muscle leading to bronchial hyper responsiveness (162). IL17 secretion by Th17 cells is not inhibited by corticosteroids and this may explain the lack of improvement in symptoms of neutrophilic asthma with increasing dose of ICS (136, 163).

Although IL17 is an important pro-inflammatory cytokine which promotes airway remodelling in asthma, it facilitates timely clearance of pneumococcal colonisation. IL17 secretion leads to neutrophil and macrophage recruitment and activation which in turn facilitates clearance of the

bacteria (164, 165). Th17 responses have been associated with acquired immunity to colonisation in mice models and control of duration and density of pneumococcal colonisation (165).

Role of IL17 in mouse models suggests this may be a useful target for therapy in asthma. However, brodalumab a monoclinal antibody against IL17 receptor was not seen to improve either symptom control or lung function in patients with moderate to severe asthma on regular inhaled corticosteroids. A small subgroup of participants in the study with high reversibility (>20%) showed nominal improvement in Asthma control questionnaire score (ACQ). IL17 levels were not measured in the study, with atopy recorded as positive skin prick test seen in for 250 of the 302 participants. Therefore, it is plausible that the participants selected did not have underlying IL17 driven asthma (166). This would support further study of anti IL17 therapy in a selected subgroup of asthma patients, with more defined treatbale traits such as number of exacerbations and measurement of IL17 levels.

Allergic inflammation involving Th2 cytokines is associated with reduced antimicrobial activity and diminished IL17 expression at the epithelial surface secondary to antimicrobial peptide human beta defensin (167). This is also seen in other allergic conditions such as atopic eczema, and associated with multiple and often persistent skin infections (168). A murine model studying the effect of allergic inflammation on innate immunity found increased viable bacteria (*Pseudomonas aeruginosa,* used for infecting mice) in the lungs of sensitised mice after bacterial exposure along with reduced antimicrobial activity (168). Reduced immune responses in allergic airway inflammation may lead to an increased risk of pneumonia. In mice models, exogenous IL17 improves host defences against *S. pneumoniae*, and reduces pulmonary eosinophil recruitment and bronchial hyper reactivity (169).

The generalisation that Th17-driven asthma has better protection against pneumococcal infection, and allergic asthma predisposes to infections appears a useful construct. However it is evident that there is considerable overlap in these processes within individuals over time (136) and in the context of their differential response to corticosteroids (163). These findings emphasise that it is difficult to

make general statements regarding individuals with a clinical diagnosis of asthma without additional phenotypic investigation.

2.5.3.4 Therapy with Corticosteroids

Inhaled corticosteroids (ICS) are associated with an increased risk of pneumonia in patients with COPD (25). The commonly identified organisms in the study included *S. pneumoniae, H. influenzae, Klebsiella pneumonia and Pseudomonas aeruginosa.* They are the mainstay of treatment for asthma but could increase risk of infection by altering nasal colonisation or predisposing to infection if colonisation is present.

In a large retrospective analysis of the manufacturer's trial data, use of budesonide was not associated with increased risk of pneumonia (27). This study analysed double-blind placebo-controlled trials as primary data set, including studies of both ICS, and ICS with long-acting beta-agonist (LABA) as the active intervention. Pneumonia as an Adverse Event (AE) was observed in 0.5% of the budesonide cohort and 1.2% of the placebo group (95% CI 0.36–0.76; $p<0.001$) and was recorded as a Serious Adverse Event (SAE) in 0.15% of the budesonide group and 0.13% (95%CI 0.53–3.12, $p= 0.58$) in the primary set (only 22 occurrences in almost 15000 individuals). In a secondary data set of 70 placebo or active comparator-controlled trials, pneumonia as an AE occurred in 0.70% and as an SAE in 0.17%.

In contrast to controlled trial findings, a case-control study in the UK reported an increased risk of pneumonia and lower respiratory tract infection (LRTI) in asthma with any ICS (OR 1.96, 95% CI 1.15-1.33) and further increased if on high dose ICS (>1000μg BDP equivalent) (OR 2.04 (95% CI 1.59-2.64) (28). Patients with a diagnosis of pneumonia or LRTI in the preceding 3 years were identified from a primary care database and matched by age and gender to controls from the same geographical area. ICS dose and type were identified from prescriptions in the preceding 90 days (170). Adjustments were made for lung function and asthma severity along with oral steroid use. Of the commonly used

asthma treatments, fluticasone was associated with the greatest risk of pneumonia (OR, 1.64; 95% CI 1.50-1.79; P<0 .001), and budesonide with a modest risk (OR, 1.20; 95% CI 1.06-1.35; P<0.003) (28).

The differences in the risk profiles for ICS may be explained by the differing pharmacokinetics and pharmacodynamics of this class of medication. The addition of esters and cyclic esters increases the affinity for glucocorticoid receptors. Fluticasone is not a pro drug and its efficacy depends on intact molecule, and duration of action is dependent on the pulmonary transit or residence time which is high for lipophilic molecules such as fluticasone furoate and fluticasone propionate. The increased risk of pneumonia associated with fluticasone may be explained by these characrterstics at the molecular level (171).

An observational study from Brazil reported an increase in nasopharyngeal colonisation of *S. pneumoniae* in children with asthma taking ICS (172) compared with age-matched controls. The children were on treatment with ICS for at least 30 days (n=96) and the controls had no ICS for at least 90 days (n=96); rates of pneumococcal vaccination were similar in both groups (ICS group 19 (19.8%) and controls 21 (21.9%). The pneumococcal colonisation was higher in the ICS group (27%) as compared to the controls (8%), p = 0.001 (95% CI 1.72-8.18). A high dose and an increased duration of ICS was associated with increased prevalence of pneumococcal colonisation (31.7% for 400-800ug and >6 months treatment vs 19.4% for <100-300ug and <6 months). It is unclear whether higher dose ICS was causative or simply a marker of more severe asthma.

The discrepancy between the highly selected population in trials with their restricted ecology of care, and observations from practice warrants significant further study in humans given the prevalence of ICS use and the potential for harm.

When considering the effect of ICS on pneumococcal colonisation and infection, it should be remembered that inhaled corticosteroids are interacting with a broader airways' microbiome. Pneumococcal colonisation is linked to airway microbiome diversity in healthy adults (173). The diverse airway microbiome in asthma with predominance of proteobacteria may influence the

nasopharyngeal carriage acquisition secondary to interactions between different species (173) and certainly appears to influence corticosteroid responsiveness (174).

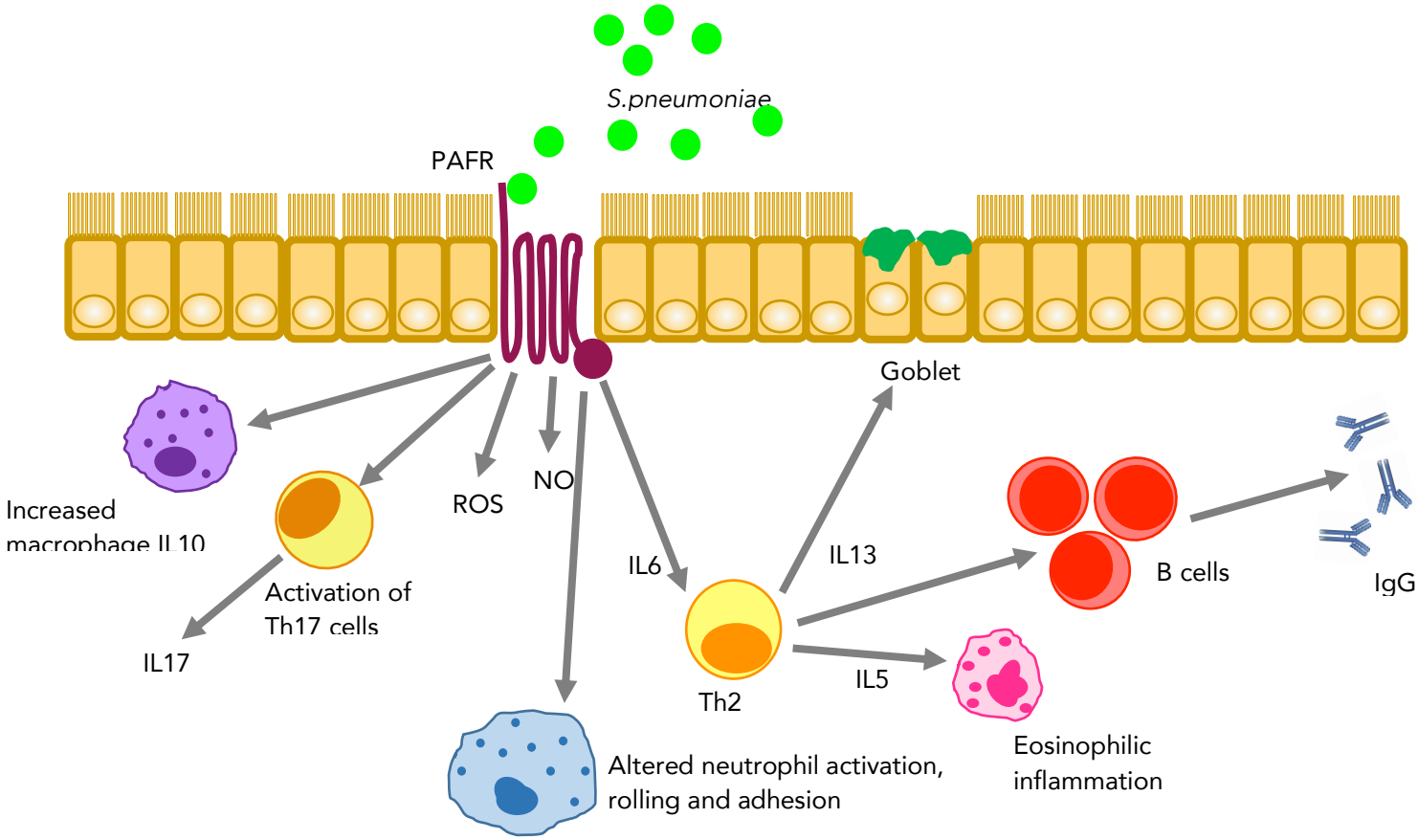


Figure 2: Schematic diagram depicting the molecular interactions between S. pneumoniae and immune cells at the nasopharyngeal mucosa.

Interleukin (IL) 17 and IL5 cause chemotaxis of inflammatory cells to the submucosa and mucosa causing airway inflammation. The Platelet activating factor receptor (PAFR) provides increased attachment sites for the bacteria. Inflammation driven by IL5 (Eosinophils), IL17 and PAFR all lead to bronchoconstriction, thereby playing a part in asthma pathophysiology. (NO – nitric oxide, ROS – reactive oxygen species)

2.6 Upper and Lower Respiratory Tract Immune Responses

In the upper airway, mucosal innate immune response is dominated by bacterial phagocytosis by macrophages and neutrophils. Adaptive immune responses occur when T cells present antigen to B cells which produce specific antibodies to opsonise bacteria, retain memory and initiate a targeted prompt immune response (175).

Immune responses seem calibrated to prevent *S.pneumoniae* colonisation and once established, its clearance. High levels of specific antibody in the mucosa lead to agglutination of bacteria, making it easier to be removed by mechanical mechanisms such as ciliary clearance (150). Anti-pneumococcal protein antibodies may help protect against colonisation, by opsonisation of bacteria (156). This forms part of the initial response preventing acquisition. Early degranulation of neutrophils is seen in nasal mucosa of non-colonised participants (176).

Once established, immune mechanisms work to control and clear colonisation. IL-17 secreted by CD4+ T helper cells (Th17) recruits and activates neutrophils and macrophages and may help with clearance (165, 177). Increase in IL17 is observed with age and may explain reduced rates of colonisation (178). This may be due to the presence of memory Th17 cells secondary to past exposure. An inverse relationship between T regulatory cells and IL17 is seen with increasing age.

Alveolar macrophages are closely adherent to alveolar epithelial cells and form an important part of immune response to *S. pneumoniae.* They are involved in phagocytosis of bacteria which leads to release of proinflammatory cytokines such as interferon γ (IFN γ), tissue necrosis factor β (TGFβ), and recruitment of neutrophils and (179). Following nasopharyngeal colonisation, bacteria reach the lungs and are a stimulus for activation of alveolar macrophages. The binding of bacteria to alveolar macrophages leads to phagocytosis and formation of phagolysosomes leading to intracellular killing. This internalisation is enhanced in the presence of opsonising antibodies (180).

2.7 Pneumococcal Vaccination in Asthma

Guidelines for pneumococcal vaccination in asthma differ between adult and paediatric patient populations and this often creates confusion in clinical practice (181). Current United Kingdom guidelines recommend vaccinating patients with chronic respiratory disease including asthma using the PPV (2). The Centres for Disease Control and Prevention (CDC) recommends vaccination according to disease severity in adults, with PPV for mild asthma and PCV for severe disease (requiring high dose corticosteroids) (182).

2.7.1 Pneumococcal Vaccines

Two types of vaccines are available: pneumococcal polysaccharide vaccine (PPV) and pneumococcal (polysaccharide) conjugate vaccine (PCV).

PPV provides serotype specific immunity, targeting capsular polysaccharides and induces short term immune response by stimulating a subset of B cells producing IgG2 subclass antibodies (183, 184). PCVs comprise of a polysaccharide in conjunction with a protein, stimulate a T cell dependent serotype specific immune response, and stimulate memory B cells (185, 186). The 23-valent PPV vaccine has been available since 1983 and the 7-valent PCV (PCV-7) was licenced in the USA and Europe in 2000 and 2002 respectively (186) followed by 10- and 13-valent vaccines in 2009 and 2010 respectively (187, 188). Serotype replacement by non-vaccine type strains has been observed after introduction of vaccination (16).

2.7.2 People with asthma have a blunted pneumococcal antibody response

Children with asthma who have been recurrently exposed to bacteria such as *S. pneumoniae* are observed to have low antibody levels, including those with recurrent clinical infection (189). In a study by Rose and colleagues, putatively protective levels were seen in 20% and 54% of young children depending on serotype considered. Vaccination of these children with PCV was apparently more effective than PPV in raising antibody levels to putatively protective levels (189). A further

study by this group investigating sequential immunization with PCV then PPV showed around 80% children did not have an initial protective level of 0.35ul/ml (190).

Similarly, a Korean study measured Ig levels to six serotypes (6B, 9V, 14, 18C, 19F and 23F) before and after vaccination in children aged 2-14 years. They studied healthy children and compared to those at clinical risk of invasive disease such as those with nephrotic syndrome, recurrent bronchitis due to asthma and post-splenectomy. The combined geometric mean (GM) for antibodies before and after vaccination with PPV were significantly lower in the high risk children compared to healthy (191).

2.7.3 It is uncertain if people with asthma are less likely to gain clinical benefit from pneumococcal vaccination

A retrospective observational study examined pneumonia episodes in patients with asthma before and after pneumococcal vaccination, and compared these to age, gender and region matched controls who did not have underlying airways disease. This study included over 2700 patients with asthma and did not find a significant difference in rates of hospitalisation for pneumococcal pneumonia pre or post vaccination when compared with the control group (192). However, the patient population had a mean age of over 50 so was perhaps not typical of asthma patients, and the infrequent events gave rise to wide confidence intervals around estimates.

Response to pneumococcal vaccination requires further investigation in asthma, as the bacteria has been identified in sputum and BAL samples in children and adults when stable and not experiencing an exacerbation (193-195). The response to pneumococcal vaccination in children with other allergic conditions appears lessened (196), and they may have an increased risk of invasive *S. pneumoniae* infection.

The paucity of evidence around the clinical effect of vaccination on people with asthma suggests further research is needed. As a “real-world” trial is unlikely to happen given the size of the study

required, experimental challenge in humans is an appropriate alternative, due to expedience, lower cost and more controlled nature requiring fewer participants.

2.8 Broncho alveolar Lavage in Asthma

Broncho alveolar lavage (BAL) sampling is used to study innate, cellular and humoral immune responses, determining cell population profiles that can facilitate the diagnosis of various diffuse lung diseases (180, 197-200).

Bronchoalveolar lavage samples from mice previously colonised with pneumococcus have demonstrated an enhanced inflammatory response to lung infection, with an increased recruitment of neutrophils, higher levels of cytokines TNFα, IL6, IL22 and IL17 (201). Antibody levels have also been studied in BAL samples.

In humans, bronchoalveolar lavage has been used in patients with pneumonia and experimentally colonised healthy controls to study mucosal immune response (180) (202). It is a useful investigation in asthma, helps to distinguish different phenotypes, and to study immune mechanisms in the lower respiratory tract (203-205). The safety is well established, and this can provide invaluable information on the alveolar response to pneumococcal colonisation in asthma with comparison to healthy controls. Research is ongoing to develop new techniques and tests for diagnosing asthma, with interest in understanding Particles in Exhaled Air (PExA ©) and volatile organic compounds (VOCs) (206). PExA © combines breathing manoeuvres with sophistaicated analysis instrumentation to collect biological samples from small airways. Differences have been identified in the breath of those with and without asthma in preliminary investigations with further studies ongoing.

Developing a safe and effective technique for people with asthma is important for future challenge studies to investigate the immune mechanisms in the lung. Preliminary work to establish an adequate sampling protocol is included in this thesis. However, subsequent sampling of challenged

asthma participants has not been undertaken. Table 2 describes the possible samples that can be obtained using bronchoalveolar lavage in people with asthma.

Table 2: Possible sampling methods and targets for diagnosis and management of asthma

	FeNO	Eosinophils	Microbiome	Inflammatory/ Immune cells	Antibodies
Venous Blood	X	√	x	√	√
Breath	√	X	x	x	X
Nasopharyngeal Samples (nasal wash and swabs)	X	√	√	√	√
Induced Sputum	X	√	√	√	√
Bronchoalveolar Lavage (BAL)	X	√	√	√	√
Bronchial Biopsies	X	√	√	√	√

2.9 Summary

Nasopharyngeal colonisation is the first step towards invasive pneumococcal disease (6), and inflammatory conditions lead to an increase in both the rate and density of *S. pneumoniae* (10). Damaged airway mucosa and use of ICS in asthma together may increase nasopharyngeal colonisation and its density leading to a higher risk of pneumonia in asthma (29).

Antibodies against capsular polysaccharides are an important defence against *S. pneumoniae*, and low levels are seen at baseline with a reduced response after vaccination in asthma. There is little information about the impact and responses of PCV and PPV vaccination in clinical practice.

There are cost implications in checking baseline antibody levels, and vaccinating everyone with asthma, particularly as effectiveness is uncertain. The antibody levels are only checked in patients who present with recurrent chest infections with asthma often requiring prolonged and multiple courses of antibiotics or in those who have radiological evidence of bronchiectasis (153). These patients are often referred and seen in tertiary severe asthma clinic for better characterisation of asthma phenotypes, as it may help in understanding the pattern of immune responses.

The immune mechanisms associated with nasopharyngeal colonisation and disease from *S. pneumoniae,* and the low immune responses at baseline and in response to vaccination require further investigation in asthma. In addition, pneumococcal virulence factors such as pneumolysin, which causes necroptosis of immune cells, may increase the risk of invasive disease and colonisation in asthma. Large prospective observational and vaccine intervention studies can address these gaps in our knowledge. These can be costly, time-consuming and challenging. Controlled human challenge models provide a more suitable option to study immune mechanisms in response to bacterial colonisation and eliminate the need to translate data from animal models into human trials whilst requiring a far smaller and more intensely studied cohort. Experimental human pneumococcal challenge (EHPC) has been shown to be well tolerated and informative in people without asthma (207). This type of approach could yield important pathological insight into the interaction between asthma, its treatment, and pneumococcus.

2.10 Experimental Human Pneumococcal Carriage Model (EHPC)

An overview of the principles of EHPC model are presented in this section, the basic principles are similar for all the studies performed using the EHPC and here the asthma study is used as an example.

Nasopharyngeal colonisation is the first step towards an infection with *S. pneumoniae (6),* and a source of immunity secondary to exposure (175). EHPC involves nasal inoculation with a strain of *S. pneumoniae* which elicits strong immune responses during colonisation and density levels close to those naturally detected with a low risk of causing invasive disease (175). There have been no cases of invasive pneumococcal disease to date in particpants of EHPC in Liverpool (208). Only one reported otitis media has been seen as an adverse event. It is reproducible and has been used safely since 2011 in Liverpool. It is well established to test efficacy for vaccines designed to reduce nasopharyngeal colonisation (152). The initial studies were done with healthy volunteers aged 18-50

years and now extended to study susceptible groups such as asthma, current smokers, patients with chronic obstructive pulmonary disease and healthy volunteers over the age of 50 years. A study looking at the effect of live attenuated influenza vaccine on colonisation has also been performed (209). Safety and ethical considerations are an essential part of any controlled human challenge model and require careful consideration.

2.10.1 Ethical considerations

2.10.1.1 Autonomy

Participation in the study was voluntary and it was ensured participants did not feel under pressure. All the participants were given written (Appendix A) and verbal information in simple, easy to understand language, enabling them to comprehend the purpose of the study, including any risks. The participant information sheet was sent at least twenty-four hours before consent to give time to read and consider the information. They were provided opportunity to ask questions individually before consent after an in-detail presentation. They could withdraw consent at any time if they wished, without providing any reason. Participants were compensated for their time and inconvenience. The payments were in keeping with the earlier EHPC studies and did not amount to a financial incentive.

2.10.1.2 Beneficence

Participants had relevant tests, examination and were offered information and education about their condition. There was an opportunity to ask questions and gain understanding of their medical condition by participating in clinical research. Control of asthma and severity was assessed during clinical screening and patients were investigated for any unexpected conditions identified as a result.

2.10.1.3 Non-maleficence

It was the responsibility of the research team to minimise the risk of harm to the participants. The research team had adequate knowledge and received appropriate training in accordance with current guidelines for the interventions and their potential risks. To ensure safety, study specific

inclusion and exclusion criteria were defined (see Table 5 and Table 6). Inoculation was performed in groups stratified by BTS treatment step, to ensure those on lower treatment were inoculated first. Experienced and trained staff performed all research procedures, including inoculation, venepuncture, nasal sampling and spirometry.

The risk to society of transmission of pneumococcus from study subjects to the wider community was minimised by selecting participants who reported no regular contact with vulnerable populations (including children aged under 5 years see Table 6) and administering bactericidal antibiotics to all participants who remained colonised at the end of the study (bacterial culture positive from nasal wash at the last or penultimate visit).

The specific risks to participants and their mitigation is discussed in section Safety considerations

2.10.1.4 Justice

This was balanced with non-maleficence. The research was open to all individuals, but important exclusion criteria were in place, primarily to protect individuals from undue risk.

2.11 Hypotheses

I hypothesise in people with asthma:

1. **Experimental pneumococcal nasopharyngeal colonisation**
    - is increased compared to healthy controls- A sample size of 52 was estimated from historic challenge study data in healthy volunteers (colonisation rate 49.6%) and assuming 50% reduction in colonisation rate in people with asthma (i.e. absolute risk difference 24.6%).
    - is of a shorter duration in asthma due to underlying inflammation – no prior literature from human data are available on this
2. **Clinical characteristics of the condition may affect experimental colonisation outcome**

- High FeNO and eosinophil count due to increased airway inflammation may be associated with increased colonisation rates, for example secondary to increased bacterial adherence sites and may also lead to rapid clearance secondary to mucosal immune activity both humoral and cell-based. .
- Disease severity as measured by low FEV_1 and PEFR may affect colonisation outcome - secondary to uncontrolled airway inflammation or treatment such as high dose of ICS.
- High ICS doses may be associated with increase in colonisation due to a reduced mucosal immune response secondary to their anti-inflammatory effect

3. **Systemic immune response to nasopharyngeal colonisation**
    - May be reduced due to the condition itself, secondary to ICS therapy or a reduced duration leading to a shorter exposure of antigen.
4. **To establish a safe and effective sampling method for performing research BAL in asthma**

    This may be used in be used in people with asthma to study mucosal immune responses post experimental pneumococcal colonisation.

3 Methods

3.1 Introduction

This chapter outlines the methodology used to set-up and perform the study, describes the process of study design, ethical issues, recruitment, implementation and study processes.

3.1.1 Experimental Human Pneumococcal Colonisation: Effect of Asthma on Immune Response to Pneumococcus

The Experimental Human Pneumococcal Carriage (EHPC) model was used to study experimental colonisation rates and mucosal and systemic immune responses in participants with asthma. I prepared the study protocol, and lead the process of ethical and governance approvals, along with recruitment, reviewing participants to determine eligibility and the analysis of samples.

This is a study of participants with mild to moderate well controlled, physician diagnosed asthma, aged 18-50 years, all on treatment with inhaled corticosteroids. They were inoculated with *Streptococcus pneumoniae,* serotype 6B, and were followed for four weeks to enable assessment of carriage acquisition and immune protection. The participants were at British Thoracic Society Treatment steps 2 and 3 according to the treatment prescribed. The BTS treatment steps are shown in Table 3 and discussed in detail in section 2.3.4 of chapter 2. All the participants were required to be on inhaled corticosteroid (ICS) up to a maximum dose of 800 micrograms (mcg) beclomethasone dipropionate (BDP) equivalent, conversion explained in Table 4. The risk categories were determined accordingly as described in Table 3, depending on the BTS treatment for asthma, higher risk individuals were postponed until 6 lower risk volunteers were inoculated, and safety demonstrated.

3.1.2 Ethical Approval

This study was approved by the Liverpool East NHS Research Ethics Committee (reference number NW/016/0124, Integrated Research Application System (IRAS) ID - 199884, ISRCTN 16755478) and was co- sponsored by the Royal Liverpool University Hospital (Research Development and Innovation

(RD&I) Study number 5173) and the Liverpool School of Tropical Medicine (RD&I Study number 16-010). The project was funded by MRC programme grant (MR/M011569/1).

Table 3: BTS Treatment steps and risk categorisation

*(modified from the BTS guidelines on asthma; *Risk categories as determined by the treatment step - shaded area represents the treatment participants)*

BTS Treatment Steps	**1**	**2**	**3**	**4**	**5**
	Mild intermittent asthma	**Regular Preventer Therapy**	**Initial add-on therapy**	Persistent poor-control	Continuous or frequent use of oral steroids
	Inhaled short acting β_2 (SABA)agonist as required	**SABA +** • **Inhaled Corticosteroids (ICS) 200-400 mcg**	**SABA+** • **Add in inhaled long acting β_2 agonist (LABA)** • **Increase ICS up to 800mcg** • **Consider other therapies – Leukotriene receptor antagonists (LRA) and slow release theophylline**	SABA+LABA • Increase ICS up to 2000mcg	SABA+LABA+2000mcg ICS • Regular ICS to control symptoms
Study risk stratum***Study Risk Category***		***1***	***2***		

Table 4 Inhaled Corticosteroid dose chart conversion

Inhaled Corticosteroid, Device and Brand	Equivalent Dose*
These dosage equivalents are approximate and will depend on other factors such as inhaler technique.	
Beclomethasone	200mcg
Clenil MDI	200mcg
Dry powder inhalers; Easyhaler, Pulvinal, Asmabec	200mcg
QVAR devices	100mcg
Fostair MDI / Nexthaler (with LABA)	100mcg
Budesonide	200mcg
Easyhaler, Turbohaler, Symbicort (with LABA), Duoresp (with LABA)	200mcg
Fluticasone propionate	100mcg
Flixotide Evohaler, Flutiform MDI (with LABA) Sirdupla MDI (with LABA)	100mcg
Fluticasone furoate	
Relvar®	1000mcg

*(*these values represent clinical equivalence only and are not similar pharmacologically)*

3.2 Recruitment and selection

3.2.1 Study Promotion

I recruited participants with physician diagnosed asthma at BTS treatment steps 2 and 3, by advertising on the University of Liverpool website, promotion on specific events such as fresher's fair, Liverpool School of Tropical Medicine Open day and in the library. Advertisements inviting volunteers to participate were placed on physical notice boards in public areas within the hospital and university and on social media of these institutions.

Primary care recruitment was done in collaboration with the National Institute of Health Research (NIHR), who approached all the practices within the Liverpool Clinical Commissioning Group, including some from the Wirral Commissioning Group. The practices were sent out a standard email with details of all the studies being supported by the NIHR. An ethically approved participant invitation letter was sent out to eligible patients from participating practices (Appendix B).

Interested volunteers contacted the research team by phone or email for further information. The Royal Liverpool and Broadgreen University Hospital (RLBUHT) database (consent4consent) was also

used to approach potential participants. They were invited to discuss the study as a group and were given the opportunity to ask questions individually. Participants were given as much time as required to decide and consented once it was ensured they understand the study objectives, associated risks and potential benefits. Further clinical information was sought from their GP.

3.2.2 Recruitment:

3.2.2.1 Inclusion criteria

These are described in Table 5.

Table 5: Inclusion Criteria

General Inclusion Criteria:	**Rationale**
World Health Organisation 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)	To reduce the risk of including participants with multiple other co-morbidities
Fluent spoken English	To ensure a comprehensive understanding of the research project and their proposed involvement
Access to their own mobile telephone	For safety and timely communication
Capacity to give informed consent	Ethical requirement to ensure autonomy
Specific Inclusion Criteria:	
Adults aged 18-50 years, with a diagnosis of asthma as described in Figure 9	To include participants with mild asthma on preventive medication
No exacerbations requiring antibiotics or oral steroids within the last 28 days	To ensure safety and scientific integrity of results as steroids may affect the immune response and antibiotics can alter nasopharyngeal colonisation

Spirometry: Forced Expiratory Volume in one second >70% predicted	To include only those with mild disease

3.2.2.2 Exclusion criteria

These are explained in Table 6.

Table 6 : Exclusion Criteria

	Exclusion Criteria	Rationale
1.	Close physical contact with at-risk individuals (children under 5yrs, immunosuppressed adults)	To reduce the risk of pneumococcal transmission in vulnerable groups
2.	History of drug or alcohol abuse - frequently drinking over the recommended alcohol intake limit: men and women more than 14 units of alcohol per week.	Alcohol excess can lead to impaired immune response and may increase the risk of pneumococcal disease
3.	Current Smokers	Smoking is an independent risk factor for pneumococcal disease
4.	Ex-smoker with a significant smoking history (>10 pack years – defined as someone smoking 20 cigarettes a day for 10 years) – minimise risk of pneumococcal disease (For loose tobacco: ounces per week × 2/7 × number of years smoked = pack years (210))	These participants might have obstructive airways disease other than asthma
5.	Taking medications that may affect the immune system e.g. *oral steroids, steroid nasal spray*, antibiotics, and disease-modifying anti-rheumatoid drugs.	Such medication may affect immune responses
6.	Any acute illness (new symptoms within preceding 14 days which are unexplained by the known past medical history)	This ensured participant's safety as they may have had an underlying undiagnosed condition or could be suffering from an infection
7.	Having received any antibiotics, oral steroids or nasal steroid spray in the preceding 28 days	Antibiotic treatment may alter the normal nasopharyngeal flora and may affect primary outcome. Nasal steroids can alter the mucosal immunology.
8.	More than 1 asthma exacerbation in the last twelve months (Asthma exacerbation defined as an acute episode of progressive worsening of symptoms of asthma, including shortness of breath, wheezing, cough, and chest tightness, or a decline in objective measure such as peak expiratory flow rates of more than 30 percent requiring treatment with oral corticosteroids for a period of 3 days or more) (211)	This helped us include volunteers with well controlled disease, therefore ensured safety.
9.	Taking medication that affects blood clotting e.g. aspirin, clopidogrel, warfarin or other oral or injectable anticoagulants	To avoid risk of excess bleeding post nasal cell sampling

10.	History of culture-proven pneumococcal disease	This ensured safety and scientifically they may have previous antibody response.
11.	Allergy to penicillin/amoxicillin	Colonised participants were treated with amoxicillin at the end of the study.
12.	Currently involved in another clinical trial unless observational or in follow-up (non-interventional) phase.	To avoid any interactions or adverse events due to other trial medication/intervention
13.	Have been involved in a clinical trial involving EHPC and bacterial inoculation in the past three years	This may confer protection from previous exposure
14.	Significant cardiorespiratory disease (excluding stable hypertension, and asthma at treatment step 2 and 3)	Respiratory disease may affect nasopharyngeal colonisation
15.	Disease associated with altered immunity, including diabetes, alcohol abuse, malignancy, rheumatological conditions	These can impair immune responses
16.	Pregnancy	For safety and due to immune changes in pregnancy
17.	Taking any medications except those on the "allowed list": • statins; • anti-hypertensives; • antidepressants; • bisphosphonates; • hormone replacement therapy • vitamin supplements (including multivitamins, iron) • anti-acid medications • nicotine replacement therapy (NRT) • inhaled steroids up to 800 micrograms BDP equivalent per day • inhaled beta 2 agonists • leukotriene receptor antagonist	These comprised of medications which do not cause significant alteration of immune function and ensured safety of our participants.

Previous pneumococcal vaccination was not an exclusion criterion, as pneumococcal polysaccharide vaccination is currently recommended in asthma (as per *The Green Book*, accessible on https://www.gov.uk/government/publications/pneumococcal-the-green-book-chapter-25). Vaccine status of volunteers was recorded where available.

3.2.3 Assessment for participation

Assessment was completed in 2 stages -consent and pre-screening. Both are explained in detail below.

3.2.4 Consent

This was done as explained in 3.2

3.2.5 Pre-screening

After the completed General Practitioner Questionnaire (GPQ) (Appendix C) was received and no exclusion criteria were identified, participants were invited for the first appointment. At each visit consent was confirmed and their identification checked with previous obtained copy in the volunteer master file. The study nurse or doctor took a brief history to ensure the volunteer met all the screening criteria. This was followed by targeted clinical examination involving auscultation of the lungs and heart sounds, general observations and the participants were weighed and measured to perform spirometry with reversibility. The tests were done in the following order: Fractional Exhaled nitric oxide (FeNO), spirometry with reversibility, peak expiratory flow rates (PEFR) and bloods. FeNO was performed before spirometry, as the reading can be altered if measured after. All the procedures were performed according to the standard operating procedure and these are described in detail below. Any abnormality identified was explained to the participant, and all relevant results were forwarded to their GP to arrange appropriate investigations and follow-up as required. The GPs also received copy of spirometry results for all the participants.

3.2.5.1 Asthma Action Plan

This is usually done in primary care by the nurses for all patients with asthma. This was completed by a member of the research team for participants who did not have one. Only 4 of the particpants had an asthma action plan from primary care at the time of enrolment into the study. The plan supported by Asthma UK was used (212) see Appendix D. Further participation was determined according to the stop criteria in Table 7. Eligible participants were asked to maintain a PEFR diary

(Appendix E) and further appointments booked. Final confirmation was sent when the results of the blood tests were available.

Table 7: Baseline assessment and Stop criteria

	Stop criteria
Age	STOP if <18 or >50 years
Clinical history + 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 + safety checks
Full blood count (FBC)	STOP if Haemoglobin (Hb)<10g/l STOP if total White Cell Count (WCC) <1.5 $x10^9$/l or neutrophils <1.0 $x10^9$/l STOP if total WCC >10 $x10^9$/l STOP if platelets <75 $x10^9$/l
Resting SpO_2	STOP if < 95%
Spirometry	STOP if FEV_1 < 70%
PEFR	<75% of predicted
BTS Treatment Step	<2 or > 4

An interim safety report was sent to the Data Safety and Monitoring Board (DSMB) after 6 participants on step 2 were completed. Safety was defined as per previous EHPC studies: ≥6 uneventful completed participants per group and no reservations among the clinical team and data monitoring and safety committee about proceeding.

Figure 3: Asthma Diagnostic Tests- Protocol V 1-4

This flow chart explains the inclusion criteria for participants up to protocol version 4 – where an objective evidence for the diagnosis of asthma in the form of FEV_1 reversibility post salbutamol, FeNO >40ppb or documented evidence of these by the GP was sought. If neither were present participants were placed in a waiting group pending further tests

3.2.5.2 Tests to confirm diagnosis of asthma:

These were performed on the pre-screen appointment (up to 7 days before collecting nasal and blood samples on the screen visit).

3.2.5.2.1 Fractional Exhaled Nitric Oxide (FeNO)

This was measured using Niox Vero® Figure 4 and involved blowing into the machine at a steady rate. FeNO measures airway inflammation and is routinely used for diagnosis and monitoring of asthma in clinical practice. The measurements are recorded in parts per billion (ppb) (213) Table 8 and is very sensitive with several substances altering the result (50, 214) as described in Table 9.

Table 8: Interpretation of FeNO Values

FeNO levels and inflammation			
FeNO (ppb)	Normal	Elevated	High
Adults	<20-25	20/25-50	>50
Th2 driven Inflammation	Unlikely	Likely	Significant

Table 9: Factors Disturbing FeNO Measurement

Factor Increasing FeNO measurements	**Factors Reducing FeNO Measurements**
Age/Gender-levels increase with age in children	Respiratory Manoeuvres
Airway calibre - variable with degree of obstruction	Reduced after alcohol ingestion
Ingestion of nitrate or nitrate containing-foods such as lettuce	Smoking - but increased in smokers with asthma
drinking water	Oral and Inhaled corticosteroids
Caffeine	NO synthase inhibitors
Respiratory tract infection	Leukotriene receptor antagonists
	Hypoxia

FeNO was measured before spirometry and PEFR. The machine was set up in advance and a new filter placed. Participants were explained to breathe out completely, then form a seal around the mouth piece as shown in Figure 4, and breath in until the machine indicated. Then they had to breathe out at a steady rate for at least 10 seconds. Only adequately performed tests were accepted and a result generated.

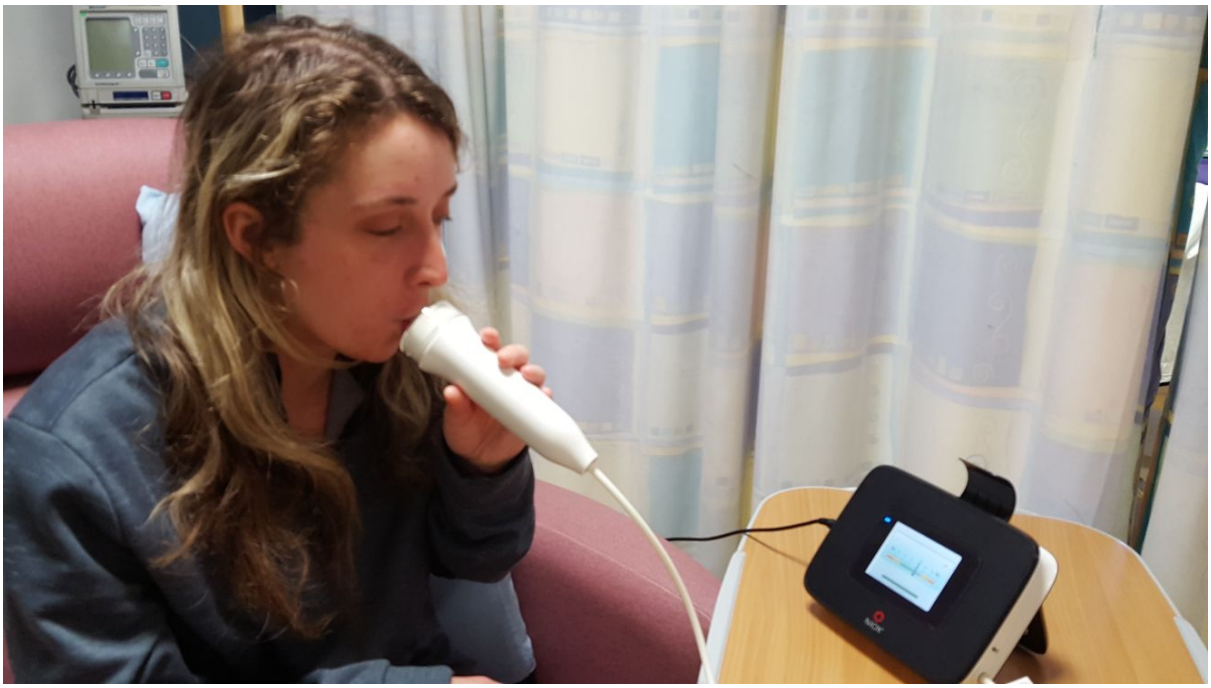


Figure 4 Niox Vero®

A participant performing the FeNO test using Niox Vero® (consent was sought for taking pictures to use for the thesis and study promotion)

3.2.5.2.2 Spirometry with reversibility

This was measured using the EasyOne® Plus Diagnostic Spirometer Figure 5, and following parameters were recorded:

A. **Forced Vital Capacity (FVC)** is the total volume of air exhaled in a forced expiratory manoeuvre.

B. **Forced Expiratory Volume at One Second (FEV_1)** is the amount of air blown out during the first second of a forced expiratory manoeuvre.

C. **The ratio of FEV1 to the FVC (FEV_1/FVC)** is the most sensitive and specific index of airways obstruction measured by a spirometer. It is obtained by dividing the FEV_1 by the FVC, and is usually expressed as a percent (i.e., 100 x FEV_1/FVC).

3.2.5.2.3 Medications prior to testing

Participants were asked to withhold their inhaled medication both short and long acting prior to attending the clinic for the test and any bronchodilator medications taken during the 24 hours prior

to the test were recorded. Medication used prior to testing can influence the pre-bronchodilator measurement Table 10.

Table 10: Medication examples and duration to withhold prior to test

Types of Medication	Examples	Withhold Prior to Test
Short Acting Beta Agonist	Salbutamol, Bricanyl	6 hours prior to clinic visit
Long acting Beta 2 Agonist	Formoterol, Serevent,	12 hours prior to clinic visit
Inhaled corticosteroids	Beclomethasone, Budesonide Fluticasone	

3.2.5.2.4 Contraindications for spirometry testing

In addition to the exclusion criteria for this study, following conditions were considered before performing the test.

- chest or abdominal surgery in the past three months
- a cardiac event in the preceding three months
- detached retina or eye surgery in the past three months
- hospitalization for any other heart problem in the past month
- a resting pulse rate more than 120 beats/minute measured at least 5 minutes after sitting down.

3.2.5.2.5 Performing the test

Participant details were entered in the spirometer and this was connected to a laptop with software pre-installed to enable reading the results. A mouth piece (spirette) was attached and the manoeuvre explained. The participants were asked to take a deep breath in and blow into the spirometer as hard and fast as possible, until their lungs were completely empty. This routine was repeated 3 times to ensure the results were consistent, then they were given 400 mcg salbutamol inhalers with an aero chamber spacer and the test was repeated 15-20 minutes later to determine

reversibility. This test forms part of standard asthma care (215). American Thoracic Society (ATS) criteria were used for quality assurance (70).

Figure 5: EasyOne® Plus Diagnostic- the device used for measuring spirometry during the study

3.2.5.2.6 Peak Expiratory Flow Rates (PEFR):

PEFR is a measure of the fastest rate of air (airflow) blown out. It was measured using a Mini Wrights® peak flow meter in litres per minute (L/min) Figure 6. Participants were shown how to take a peak flow reading (i, put the marker to zero, ii, take a deep breath, iii, seal lips around the mouthpiece, and blow as hard and as fast as possible into the PEFR meter). They were asked to perform three readings and record them on the chart provided to maintain a diary (Appendix E) from this visit onwards. The PEFR variability was calculated by using a tool developed in house with Microsoft Excel (Appendix F). A PEFR variability of 12 percent was accepted for this study.

Figure 6: Mini Wright's Peak Expiratory Flow Rate Meter

3.3 Subjects and timelines

Participants aged 18-50 years with asthma were inoculated with pure culture of a well-characterised, fully sequenced penicillin-sensitive pneumococcal serotype 6B (strain BHN418). They were observed for the development of pneumococcal colonisation.

Study visits took place within 7 days of the proposed date to accommodate for issues/events and personal commitments of participants that prevented them from attending (e.g. illness, bereavement, exams, professional and travel issues) Figure 7. The samples collected at each time point are described in the text and in Table 11.

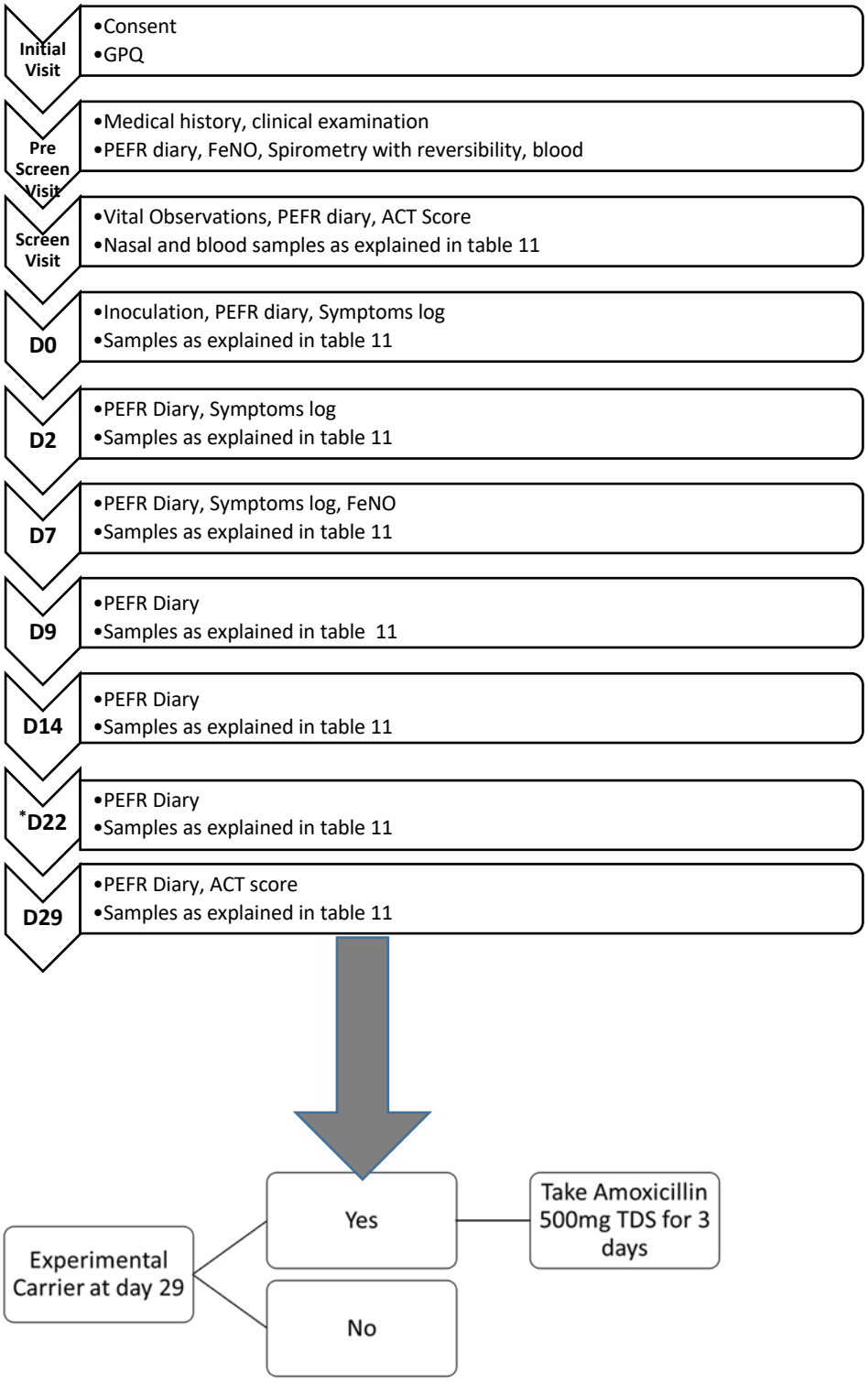


Figure 7 Study Visits – Flow chart detailing study visits and time points

General Practice Questionnaire (GPQ), Peak Expiratory Flow Rate (PEFR), Fractional Exhaled Nitric Oxide (FeNO), Asthma Control Test (ACT), Day (D), Three times per day (TDS)

**carriage positive participants only*

3.3.1 Visit 1: Pre-Inoculation screening visit

The consent and eligibility were checked, and all the participants were asked to complete the Asthma Control Test questionnaire.

3.3.1.1 Asthma Control Test (ACT)

This is a simple questionnaire, designed to assess patients' symptoms of asthma. A score of 25 indicates good and that of less than 20 poor control. This is used as part of standard asthma care in secondary care (216) (Appendix G). I was unable to use Asthma Control Questionnaire (ACQ) score as this is copyrighted and would have rquired permission before use. However, both ACT and ACQ scores are validated and widely used in clinical practice to asses asthma control which was the purpose of administering the test in my study (217).

3.3.1.2 Pregnancy Test

A urine β HCG test was performed on all female participants.

3.3.1.3 Throat swab

The participant's tongue was depressed using a tongue depressor exposing the palatopharyngeal arch. Two samples were obtained: one for bacterial identification (blood agar), and one for detection of pathogens by molecular techniques, each by making 5 small circular motions of the palatopharyngeal arch in contact with the mucosa whilst avoiding the tongue. Throat swabs were collected prior to nasal wash to ensure the oropharynx was not contaminated with nasal pathogens.

3.3.1.4 Nasal Samples

3.3.1.4.1 Nasal wash

This was performed using a modified Naclerio method (218). This has been used for more than 8 years for pneumococcal carriage studies, is a sensitive method for bacterial detection and quantification, being more comfortable than nasal swabs for participants (219). Five millilitres of saline were introduced using a syringe and held for a few seconds in the nose before being expelled into a sterile container. This was repeated twice in each nostril using 20ml saline in total. In the

event of nasal wash loss (defined as cough/sneeze/swallow) the procedure was repeated to obtain an adequate specimen (defined as ≥10ml saline recaptured). The sample was transported to and processed at the laboratory in Liverpool School of Tropical Medicine within 60 minutes of collection. If more than 20ml of saline was used, this was documented on the form for information of the laboratory team. The sample was then spread onto agar plates as described below in section 3.5.2 to determine nasopharyngeal colonisation of bacteria.

3.3.1.4.2 Rhino probes

Nasal cells were collected using a naso-sampling method where cells are obtained through minimally-invasive superficial nasal scrape biopsies (Rhino-pro nasal curette) with no significant side effects (220). Up to 4 samples were obtained at each nasal sampling visit. These were used to obtain a higher density of cells from the mucosa and submucosa compared to that achieved with nasal wash.

3.3.1.5 Blood

Up to 50mL of blood was collected by venepuncture to measure full blood count (for safety), and immune responses such as serum immunoglobulins. The serum samples were stored for each participant at -80˚C, in 4 separate aliquots.

3.3.2 Visit 2 – *S.* pneumoniae Inoculation (D0)

After identification and safety check (including PEFR diary), nasosorption samples were collected from participants.

3.3.2.1 Nasosorption

Concentrated nasal lining fluid was obtained for cytokine analysis using nasosorption strips (like blotting paper) developed by Hunt Developments Ltd (UK). Strips were held inside the nose for 1—3 minutes then stored at -80°C pending cytokine analysis.

3.3.2.2 Safety instructions prior to nasal bacterial inoculation

Following this all the participants:

- were shown how to use the thermometer and record their temperature.
- given an emergency pack containing:
    1. a 3 day course of oral antibiotics (amoxicillin),
    2. a safety information sheet (Appendix H),
    3. symptom log (Appendix I)
    4. a participant study card with details of the study participant number (Appendix J), date of inoculation, the clinical team contact number and a new PEFR diary
- Participants were explained and shown how to complete the symptom log for the next 7 days and the day 0 was done in clinic prior to inoculation.
- Contact numbers for the participants and a close friend (secondary contact) who was likely to see them every day (e.g. flat mate) were saved in the emergency study phone. They were all sent a text message from this study phone to ensure they had our number saved in their phones.
- Instructions were given to record their temperature and three readings of PEFR and send these daily via text message before 12 noon to the research team. All the participants were asked to set a reminder in their phones.
- They were explained the pack contained 9 capsules of amoxicillin and the reasons when they may have to be taken were explained as below:
    1. At the end of the study - if they were still colonised with the bacteria (unless they had two negative nasal washes)
    2. If they were unwell during the study and the research team asked them to take these.
    3. If they had any of the symptoms explained on the safety sheet and were unable to contact the research team, a GP or any other emergency department. Study team members were on call 24 hours a day seven day a week.

3.3.2.3 Inoculum Preparation

The inoculum was prepared in the laboratory at the Liverpool School of Tropical Medicine, within a dedicated safety cabinet. Bacterial stock purity and penicillin sensitivity was confirmed by Public Health England laboratories. Frozen aliquots of *S. pneumoniae* were thawed and checked for bacterial number (colony forming units [CFU] per ml), and purity. Two identical tubes were prepared, one for inoculation and a second as a backup. The inoculum was immediately transferred to the clinical research unit and participants were inoculated with 80,000 CFU/100µl serotype 6B pneumococci administered to each nostril as explained below.

3.3.2.4 Inoculation

Participants were asked to lie down at a 45-degree angle in an adjustable chair. They were advised not to wipe their nose and remain in this position for 15 minutes after the inoculation. Using a P200 micropipette 0.1ml saline containing the desired dose of *S. pneumoniae* was instilled into the nose. The target dose was 80000 CFU/100µl, with an allowable margin of error of half or double of this dose based on our dose-ranging studies with similar colonisation and safety outcomes (rates and density recovered from the nasopharynx) (221).

Table 11: Study Visits and sampling schedule

Visit	Blood samples	*Throat Swab	Nasosorption	Nasal Wash	Nasal Cells	Other tests
Pre-screen	FBC					Spirometry PEFR meter to be given, FeNO
Screen	✓	✓		✓	✓	ACT score Pregnancy Test
D0			✓			PEFR, Symptom log
D2	✓	✓	✓	✓	✓	PEFR
D7	✓	✓	✓	✓	✓	PEFR FeNO
D9	✓	✓	✓	✓	✓	PEFR
D14	✓	✓		✓		PEFR
(D22) +	✓	✓		✓		PEFR
D29	✓	✓	✓	✓	✓	PEFR ACT score

*Throat swabs were collected for viral and bacterial culture on the screen visit, both were stored on ice immediately, and bacterial was transferred to the laboratory within 40 minutes of collection. Nasal Wash was collected at all timepoints except on the day of inoculation and was transported to the laboratory for processing within 40 minutes of collection, Nasal Cells were collected on screen visit, days 2, 7, 9 and 29. Nasosorption was collected on days 0, 2, 7, 9 and 29. Peak Expiratory Flow Rate (PEFR), Fractional Exhaled Nitric Oxide (FeNO), Asthma Control Test (ACT). + Carriage positive participants only

3.3.3 Monitoring of participants and colonisation

Colonisation was determined by bacterial culture of nasal washes. Participants were provided a clear flow chart of the necessary intervention should any symptoms develop (Appendix H participant inoculation information sheet). A three-day course of amoxicillin, a digital thermometer and a PEFR meter were provided and participants asked to send text messages daily for the first seven days with their temperature and three PEF rate readings. These were recorded by a member of the research team. If they did not text by the specified time, a member of the research team contacted the

participant. If no response was received the prior defined 'secondary contact' was phoned. Participants had access to the research team 24/7 on-call telephone service until the end of the study after inoculation.

3.4 Study Amendments

The protocol described above was the original submission to the ethics committee on the 3rd February 2016. However, during the study due to practical implications several amendments were made. These were submitted to REC using the IRAS system, and were approved by the committee, the Health Regulatory Authority and the sponsor before implementation. These are detailed in Table 12, and substantial amendment 2 is discussed in detail as this changed the eligibility criteria significantly.

3.4.1 Substantial Amendment 2

This amendment was submitted after discussion with the principal investigator (PI) due to difficulties with recruitment. The reasons and changes are explained below:

1. Inclusion criteria changed from an objective diagnosis of asthma to physician diagnosed asthma. The reasons for this were:

- We were looking for participants with mild well controlled asthma on low dose ICS at British Thoracic Society treatment steps two and three. These participants as per the BTS guidelines were on appropriate treatment to be symptom free. Therefore, the participants may not always have the features of airway inflammation required as per current diagnostic tests if well controlled.
- This change enabled us to recruit participants with a physician diagnosis of asthma, on regular treatment with ICS based on historic symptoms.

2. I removed histamine challenge test from the diagnostic pathway, as with a change in inclusion criteria this was not necessary. The patient information sheet and protocol were amended accordingly.

3. I included a reminder letter to the participants through the GPs, to increase response rate Appendix K.

4. An approved text for advertising our study on social media was added, to improve the publicity and advertisement of the study.

Following approval of this amendment our study recruitment improved significantly and all those participants who were pre-screened and awaiting a histamine challenge test were included.

Table 12: Study Amendments

Amendment Type	Date	Document Versions	Changes
Minor Amendment 1	9th May2016	Protocol V 3	Montelukast added to the list of allowed medication
Substantial Amendment 1	10th May 2016	Protocol V4 Website text V1 Co-sponsorship	Added a nasosorption test at day 29 Text for website approved Study approval for co-sponsorship
Minor amendment 2	10th May 2016	GPQ V2	GPQ changed to add "please provide a relevant summary or fill in the form" previous records of past 12 months asked for instead of 3 years
Minor Amendment 3	22nd June 2016	Protocol V5	Diagnostic flow chart simplified to help determine eligibility (see Figure 8)
Substantial Amendment 2	26th July 2016	Protocol V7	Eligibility criteria changed from objective evidence to physician diagnosis a histamine challenge test removed (Figure 9 for eligibility criteria changes and Figure 10 for study visit Reminder letter from GPs added social media statement

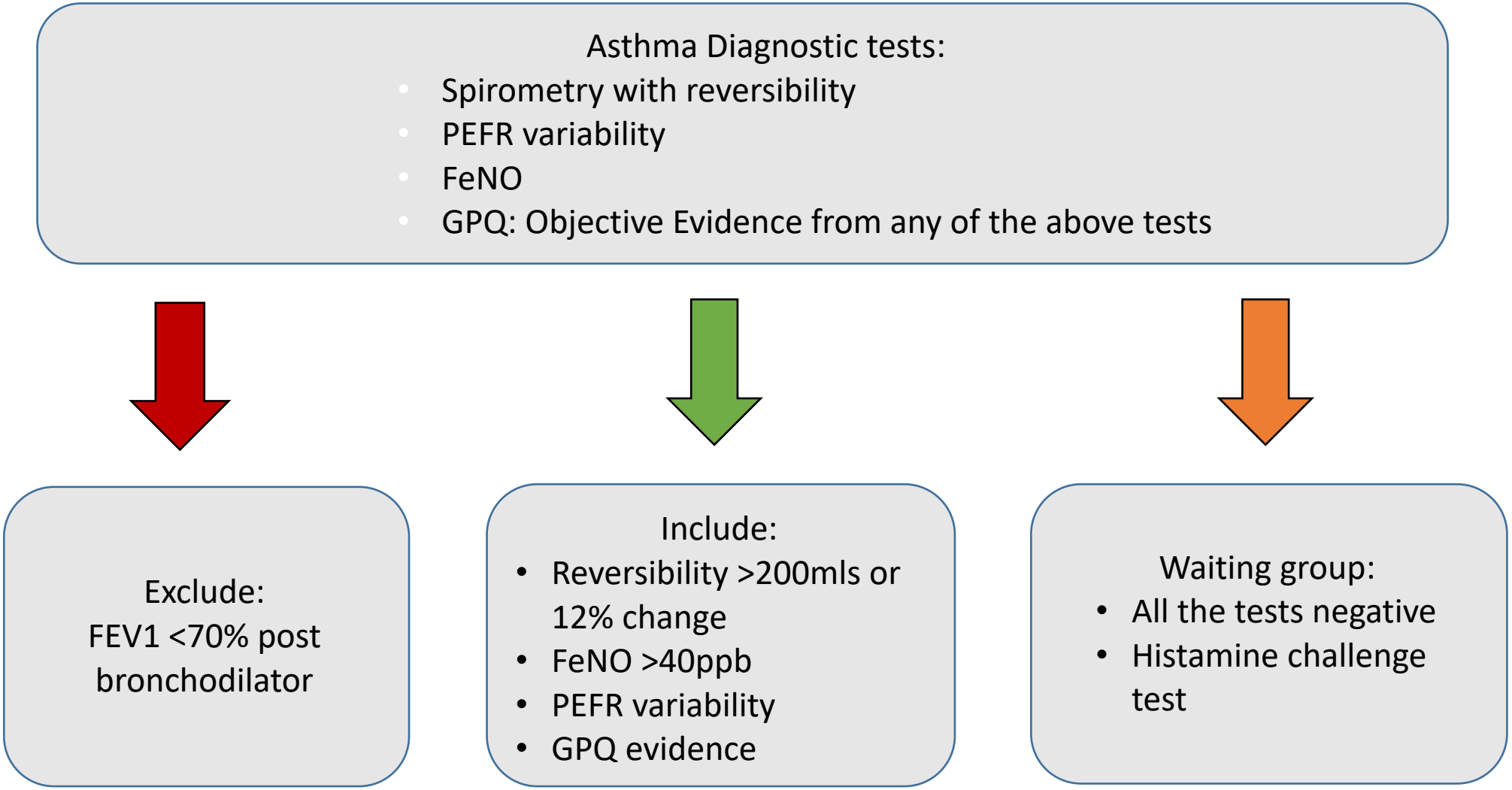


Figure 8: Asthma Diagnostic Tests – Minor Amendment 3 Protocol Version 5

Flow chart explaining inclusion criteria for asthma diagnostic tests

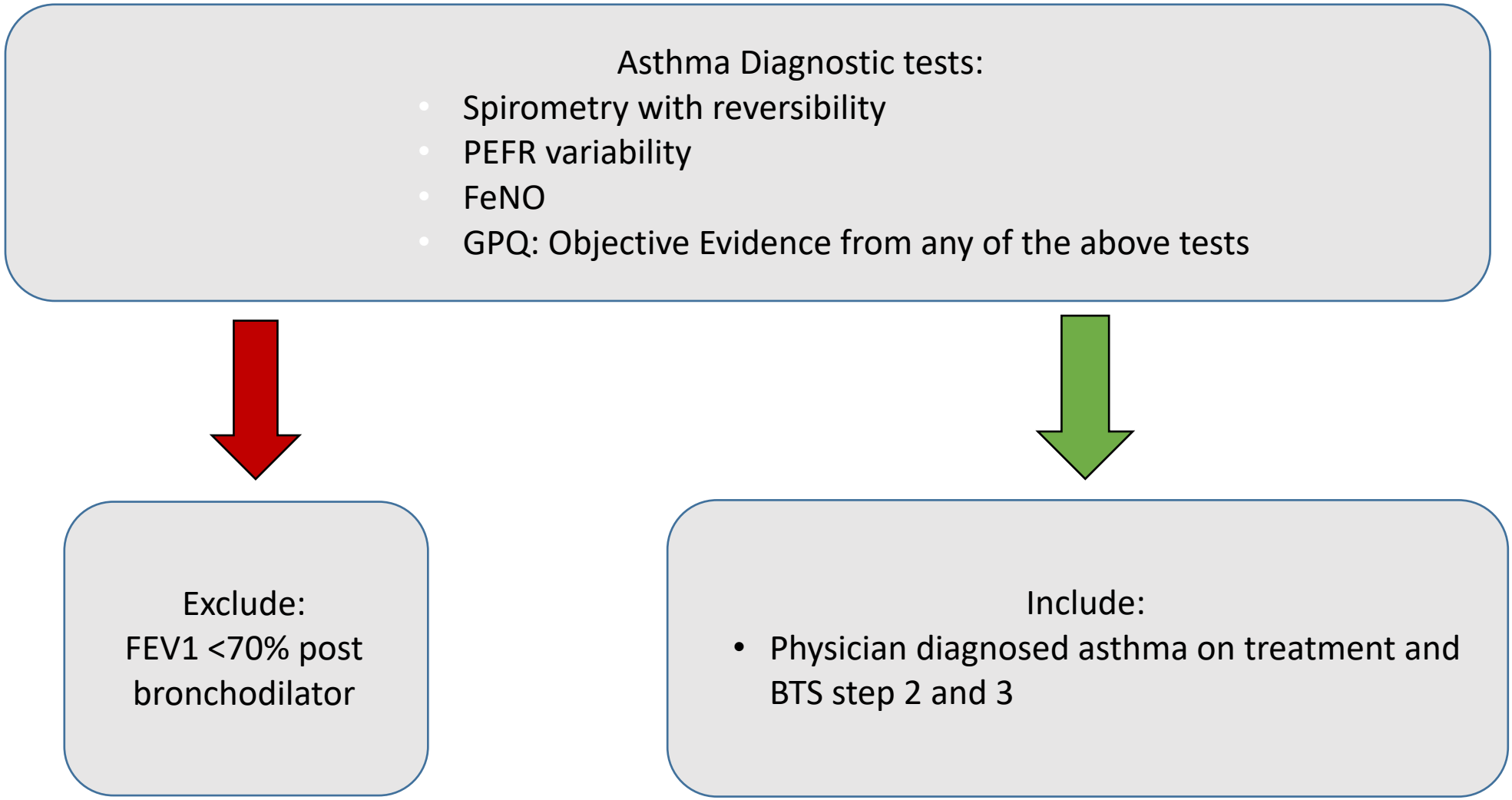


Figure 9 Asthma Diagnostic Tests – Substantial Amendment 2 Protocol Version 7

Flow chart explaining inclusion criteria for asthma diagnostic tests following Substantial Amendment 2, these are further explained in Table 12

3.5 Laboratory Methods: *S. pneumoniae* Inoculation

3.5.1 Preparation of bacteria for inoculation

As explained in section 3.3.2.3 the inoculation section

3.5.2 Nasal Wash Processing and determination of colonisation

Colonisation was defined by the result of nasal washes taken at days 2, 7, 9, 14, 22 and 29 post inoculation. The colonisation status was determined by classical microbiology, as described below: Nasal washes were plated on culture media (blood agar plates with and without gentamicin) and incubated overnight at 37°C in 5% carbon dioxide (CO_2). Colonies were confirmed as *S. pneumoniae* using classical microbiological techniques including (i) typical draughtsman-like colony morphology for gram-positive diplococci ii) the presence of α-haemolysis, (iii) optochin sensitivity, and (iv) solubility in bile salts. Typing by latex agglutination was done using a commercial kit to confirm pneumococcal serogroup. Isolates were frozen at -80ºC for storage.

3.5.2.1 Materials and Reagents Required

- Blood plates – Oxoid PB0122A
- Skim milk, tryptone, glucose, glycerine (STGG) medium
- Saline
- Pipettes
- 96 well plate – for dilutions
- Eppendorf tubes
- Cryotubes
- Heraeus Megafuge 1.0 in the Microbiology lab CT350
- Latex agglutination Kit
- Gentamicin 1mg/mL

3.5.2.2 Preparation of STGG medium used for nasal wash pellet dilution and storage:

3.5.2.2.1 Materials:

- Oxoid tryptone-soya broth (CM 129) 3.0ml
- Glucose 0.5g
- Oxoid skim milk powder (CM L31) 2.0g
- Glycerol 10.0ml
- Double distilled water 100 ml

Method: 1ml of each of the above materials were added into bijoux's and autoclaved for no more than 10 minutes. The tubes were stored at 4-6°C. The pellet was re-suspended vortexing for 10 to 15 seconds.

3.5.2.2.2 Preparation of Plates

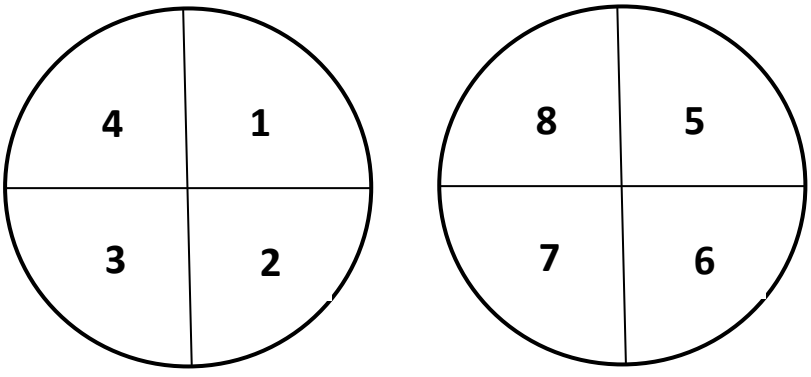
Two different types of plates were required: Gentamicin plates, and dilution plates. Blood agar plates were purchased from Oxoid and labelled with volunteer ID, and study day. On the day of sample collection, the dilution plates were placed under a sterile hood and left to dry out.

3.5.2.2.3 Gentamicin Plates:

80µL of 1mg/mL gentamicin was plated on a blood plate and spread until dry. These were stored in the fridge until use.

3.5.2.2.4 Dilution plates:

These were blood agar plates, divided into four sections and labelled as below. 2 plates were made for each volunteer at day 2. From then on two plates were made for only colonised participants.



3.5.2.2.5 Sample Processing:

- The nasal wash was collected within one hour from the clinical research unit, and centrifuged for 10 minutes at 4000 rpm, to separate the supernatant and the pellet
- The volume of nasal wash was recorded on the inventory form
- 1ml aliquots of the supernatant was transferred into each of 5-11 pre-labelled supernatant tubes (subject to volume collected from the participant)
- 100µl of STGG was added to the pellet, and this was pipetted up and down
- The volume of STGG in the tube was determined and recorded on the inventory form (usually >100µl)

3.5.2.2.6 For Gentamicin Plates

- 20µl was plated onto the blood agar with gentamicin (4µg/mL) and streaked
- A 96 well plate was prepared for Miles and Misra (serial dilution) by adding 90µl of sterile saline into wells to 4 (or 8) wells
- Then 10µl of the STGG pellet was added into well A and mixed by pipetting up and down
- Serial 4 (or 8) 1:10 dilutions of the STGG pellet were made by starting to mix contents of well A 3-5x and transferring 10µl to well B. The top was changed, and process repeated using contents of wells B-C (or B-G) to reach well D (or H)
- One 10µl drop from each well was placed into the corresponding section on the plate: Well A = section 1, Well B = section 2, well C = section 3, well D = section 4, well E = section 5, well F = section 6, well G = section 7, well H = section 8. This was repeated twice and a total of 3 drops were added to each section.
- The sample was left to dry with the lid closed

3.5.2.2.7 For Blood Agar-dilution plates

- 800µl of STGG was added to the nasal wash sample, and pipetted up and down
- 25µl was plated on blood agar and streaked
- The remaining amount of the cell pellet was divided into 3 labelled cryovials for collaborators for other experiments
- The plates were incubated at 37°C for 9 to 16 hours in 5% CO_2
- The temperature and CO_2 level were checked the following morning and recorded on the form
- The plates were then examined the next day for presence of pneumococcus
- If pneumococcus was detected, the colonies were counted on all the plates by two research assistants (blinded for each other's count):

Dilution	Dilution Factor	# of visible colonies	CFU/100µl
1	**10**		
2	**100**		
3	**1000**		
4	**10000**		

- The colonies were streaked for purity and frozen the next day to be stored at -80°C
- The pneumococcus serotype was tested using the latex agglutination kit Oxford Biosystems, United Kingdom, as per manufacturer's instructions.

3.5.3 ELISA protocol to measure anti-capsular polysaccharide antibodies (CPS) antibodies

These were all performed by myself in the LSTM laboratory with guidance from my laboratory scientist colleagues. I performed these tests using the protocol below over a 2 week period.

3.5.3.1 Materials required

- 96 well immuno sorp plates - Nunc Maxi®
- 96 well deep well plate - Star Lab®
- Purified 6B polysaccharide and cell wall polysaccharide (CWPS) - Oxford Biosystems
- WHO international pneumococcal reference standard 89SF – supplied by Food and Drug Administration (FDA), United States
- Goat anti-human IgG conjugated to alkaline phosphatase (secondary Ab): - Sigma Aldrich, St Louis, Missouri, USA
- Phosphate Buffered Saline tablets (PBS)
- Dilution Buffer: 2ml Heat-Inactivated foetal bovine serum (HI-FBS) in 20ml PBS
- Absorption Buffer: 10µl CWPS (stock 10mg/ml) in 10ml of dilution buffer (final concentration 10µg/ml)
- Polooxyethylenesorbitan (Tween 20) - Sigma-Aldrich
- Washing Buffer: 500µl Tween20 in 1L PBS
- 1 tablet of p-nitrophenylphosphate (pNPP) (5mg) and 40ml of distilled water

3.5.3.2 Method

1. Each ELISA well of the Nunc Maxi® immuno sorp plate was coated overnight with 100 microlitre 6B polysaccharide (10µl of purified polysaccharide (stock 5mg/ml) in 10ml PBS – final concentration 5µg/ml) and incubated at 4° C.

2. Following morning, in a separate star lab® deep 96 well plate, standard was prepared in columns 1 and 2 (wells A-H) using absorption buffer and 89SF in a dilution of 1 in 500). The samples were prepared in a 1 in 100 dilutions in rows A3 to A12 and E3 to E10. Each sample was put in two wells simultaneously for duplicates. 597 µl of absorption buffer with 3 µl of the sample was added to each well. Samples from different time points (day -5, day 7 and day 29) were all processed on the same plate. The plates were incubated for 30 minutes. The blank was prepared separately in wells H11 and H12 with 200 microliters of absorption buffer.
3. The Nunc Maxi® plates which had been incubated overnight were washed 3-times with the washing buffer (PBS with tween) washed. 100 µl of the pre-absorbed standard serum was transferred from star lab® deep well plate to columns 1 and 2 (A-H) to the washed Nunc Maxi® plate. Similarly, 200 µl of the samples were transferred to row A3 - A12 and E3-E10. 100 µl of dilution buffer were added to the remaining wells (B3-D13, and F3 to H10). Then 100 µl of the sample were transferred from A to B, mixed then from B to C and C to D, the tips on the pipetted were changed with each dilution. From row D 100 µl was discarded, leaving each well with 100 µl of sample. This process was repeated for rows E to H. The plates were incubated at room temperature for 2 hours.
4. The plates were then washed 3-times with the washing buffer and 100 µl of secondary antibody was added to each well and incubated for 1 hour and 30 minutes.
5. After washing the plates 3-times with the washing buffer 100 µl of pNPP (prepared by adding one tablet to 40 ml of distilled water) was added to each well. The plates were developed for ten minutes in the dark and read using the Omega software® ELISA reader at 405nm.
6. Samples with coefficient of variation>15% between duplicates were repeated and one sample due to a very high signal (outside standard curve) was repeated using a 1 in 800 dilutions.

3.5.4 Meso Scale Discovery (MSD) for anti-pneumococcal protein antibodies

This was performed at the WHO reference laboratory at the Institute of Child Health, University College London (Prof David Goldblatt).

3.5.4.1 *Materials Required:*

- MULTI-SPOT® 96 10- Spot High Binding plates. Customised order from Meso Scale Discovery, Gaithersburg, MD
- 96-well Microplate imager - MSD Sector Imager, Model #1300, MSD, Gaithersburg, MD
- MSD Discovery Workbench 4.0.12
- Deep well plate, 96 well 1.2 ml - Anachem
- Pneumococcal Capsular Polysaccharide (CPS)- American Type Culture Collection (ATCC)
- Pneumococcal Cell Wall Polysaccharide CWPS- Statens Serum Institute
- Pneumococcal Proteins – 27 measured named below

CbpA PP01	SP0609 PP31	PcpA PP13	RrgA-T4 PP22	Ply PP17
LytC PP02	SP2027 PP07	PhtD PP14	RrgB-6B PP19	PspA F1 PP16
PcsB PP06	SP2194 PP30	PhtE PP10	WtPly PP12	RrgB-23F PP20
PhtD PP03	StkP PP05	PiuA PP08	NanA PP33	RrgB-T4 PP18
PsaA PP04	LytB PP11	PspA F2 PP15	PiaA PP09	Spr0057 PP29
Spr0096 PP24	Spr2021 PP32			

  - WHO international pneumococcal reference standard 007sp - supplied by Food and Drug Administration (FDA), United States
- Southern Biotech Goat Anti Human IgG – purified -
- MSD SULFO -TAG NHS-ESTER
- Human control sera in house control sera 96/570 - National Institute of Biological Standards and Controls (NIBSC)
- Polooxyethylenesorbitan (Tween 20) - Sigma-Aldrich
- Sterile non-pyrogenic water – Baxter Healthcare

- Phosphate Buffered Saline tablets (PBS)
- Bovine serum albumin (BSA) - Sigma
- Read Buffer T with surfactant (4X to 1X in sterile water)
- Blocking Solution (5% Bovine Serum Albumin (BSA) in 1XPBS- 2.5g BSA in 50 ml PBS)
- Antibody Buffer (DAB) – 1% BSA in wash buffer (1X PBS in 0.05% Tween) with CWPS (10ug/ml) with 22F (5ug/ml)

3.5.4.2 Method

1. The plates were coated with proteins by MSD according to pre-defined specific requirements and stored at 4°C
2. Standard was prepared using 007sp in a 1:100 dilution and a fourfold serial dilution was performed
3. The samples were prepared in 1:100 dilution using DAB and added to the dilution block containing the reference standard in replicate
4. Separately, two dilutions were prepared for the quality control (QC) sera – Low 1:1000 and high 1:2000. These were added to the dilution block containing the reference standard and samples in replicate according to plate layout 1 (example shown below):

	1	2	3	4	5	6	7	8	9	10	11	12
A	REFERENCE STANDARD007sp 1:100 4FOLD		Sample 1		Sample 2		Sample 3		Sample 4		Sample 5	
B			Sample 6		Sample 7		Sample 8		Sample 9		Sample 10	
C			Sample 11		Sample 12		Sample 13		Sample 14		Sample 15	
D			Sample 16		Sample 17		Sample 18		Sample 19		Sample 20	
E			Sample 21		Sample 22		Sample 23		Sample 24		Sample 25	
F			Sample 26		Sample 27		Sample 28		Sample 29		Sample 30	
G			Sample 31		Sample 32		Sample 33		Sample 34		Sample 35	
H	BLANK		Sample 36		Sample 37		Sample 38		Hi QC		Lo QC	

5. Then 150 µl of the blocking solution was added to each well, sealed and incubated while shaking at 700rpm for 1 hour at room temperature
6. The plates were washed 4-times. 30 µl of detection antibody was added to each well, sealed and incubated at room temperature for 1 hour on the shaker as before
7. The plates were washed and 150 µl of diluted read buffer was added to each well
8. The plates were read immediately after

3.6 Safety considerations

Safety of the participants and of the researchers was taken into consideration while planning the study. *S. pneumoniae* can cause infections such as otitis media (OM), sinusitis, pneumonia, bacteraemia and meningitis.

3.6.1 Study design:

Participants were provided with detailed information of the study before consent and information was given about their condition. A personal asthma action plan was completed based on peak expiratory flow rate (PEFR) readings explaining how to increase their inhaled medication and whom to contact if PEFR dropped. A daily PEFR diary was maintained by participants and checked at each visit by the study team. There was a process in place to ensure daily contact with the participants for the first 7 days including weekends.

3.6.2 Clinical on call cover

A member of research team was on call 24 hours, seven days a week and was contactable via phone. The contact details were provided to the participants on a business card and they were advised to store these in their phones. The hospital switch board also had details of the on-call team.

3.6.3 Participant Selection:

The inclusion and exclusion criteria as described in Table 5 and Table 6, were determined by experienced chest physicians with specialist interest in asthma, to ensure only those with mild, well controlled disease were included. Participants on BTS treatment step two with mild disease were inoculated first and after successful completion of six participants, those on step 3 were included. Table 3 explains the BTS treatment steps and risk categorisation.

3.6.4 Laboratory Safety Procedures

S.pneumoniae serotype 6B approved by public health England was used for inoculation. A laboratory manual was formulated with all the procedures in accordance with good clinical practice. This highlighted risks such as spill of bacteria, temperature regulation to store samples and bacterial stock along with a standard operating protocol to follow in an emergency. All laboratory staff were adequately trained in laboratory safety procedures. This reduced biological and chemical hazards within the laboratory. Health and safety regulations for research with human tissues / infectious agents were always followed.

3.6.5 The Data Monitoring and Safety Committee (DMSC)

A DMSC has monitored EHPC studies for 8 years and provided advice to the PI and asthma study team, ours consisted of two professors – one internal at LSTM and one external was available to provide advice and guidance to the principal investigator (PI) and the research team on any serious adverse events. An interim safety report was sent to the DMSC on completion of the first six step two participants, and a full the safety report containing information on all participants enrolled and inoculated was provided on completion of the study. An annual safety report and end of study report with number of participants recruited and inoculated was sent to the NHS ethics committee also.

3.6.5.1 Adverse Events (SAEs)

These were documented and reported to the sponsor and the DMSC as soon as possible and serious adverse events were reported within 24 hours. There were no serious adverse events during the study.

3.6.5.2 Serious Adverse Events (SAEs)

An SAE is an adverse event or reaction that: (a) results in death (b) is life-threatening (c) requires hospitalisation or prolongation of existing hospitalisation (d) results in persistent or significant disability or incapacity (e) consists of a congenital anomaly or birth defect. Serious adverse events (SAEs) were reported to the DMSC within 24 hours and adverse events within a week of occurring. All research staff were aware of the relevant paperwork to complete in any such event. Any SAE would have been reported to the research ethics committee (REC) and study stopped pending their review and guidance from the DMSC.

3.7 Data Management and Analysis Methods

The data was collected using specifically designed case report forms. For analysis Statistical Package for Social Sciences (SPSS) version 22 and Graph Pad Prism version 5 were used. The statistical tests are described in each section in detail.

3.8 Comparative data from healthy controls

The comparative data from healthy controls came from 4 different studies as described below.

3.8.1 Microbiology Comparisons – colonisation rates and density

For comparison I used age-matched healthy controls from an influenza vaccine study (Sep 2015- Mar 2017), with identical methods and sampling, although participants received intramuscular tetravalent inactivated influenza vaccine either 3 days before or after inoculation with pneumococcus (209). This study was conducted simultaneously with the asthma study. Ideally I should have included an arm of healthy controls in parallel to the asthma study, as the flu vaccination may have affected the immune response to nasopharyngeal colonisation. However, due to time constraints and limited resources this was not possible.

3.8.2 Anti-capsular Polysaccharide IgG

The data for healthy controls came from the control arm of an earlier EHPC study, with a similar protocol and identical general inclusion and exclusion criteria except for defining history of respiratory disease in asthma and its absence in healthy controls. This study looked at the effect of PCV 13 on nasopharyngeal colonisation in healthy volunteers. This was double blind randomised controlled trial and participants were either assigned to PCV 13 or hepatitis A vaccine group. They were subsequently inoculated with *S.pneumoniae* 6B and nasal wash samples collected at different time points to look for colonisation by classical microbiology culture techniques (152). The comparative data is from healthy controls who received hepatitis A vaccine and serum samples for IgG to 6B polysaccharide were processed using the same ELISA protocol in our lab as described in section 3.5.3.

3.8.3 Anti-pneumococcal protein IgG

The comparative data is from a dose ranging study performed by our group designed to examine the immunising effect of a single colonisation episode in healthy volunteers (175). The study protocol and general inclusion and exclusion criteria were similar to our study; the only difference being the presence of asthma in our cohort and lack of any underlying medical condition for the healthy controls. Participants

were inoculated with an increasing dose of *S.pneumoniae* ranging from 10000 cfu to 160000 cfu. Nasal and serum samples were collected before and after inoculation.

4 Results – Experimental Colonisation rates, density and antibody levels in Asthma

4.1 Introduction

In this chapter I discuss the results from the study. The research questions were:

- do people with asthma have higher experimental colonisation rates compared to healthy controls?
- is experimental colonisation affected by clinical characteristics of asthma?
- is the systemic immune response to experimental colonisation different in asthma compared to healthy controls?

Clinical features may help identify asthma control and compliance with medication such as high FeNO suggests poor compliance or inadequate dose of ICS (222).

Eosinophils and FeNO are important biomarkers of asthma diagnostic pathway (222), and if found to determine colonisation may help identify patients at risk of colonisation and subsequent disease.

Forced expiratory volume in one second (FEV_1) and PEFR variability are used to define asthma severity with low FEV_1 indicating severe disease and PEFRs to monitor the condition and describe exacerbation severity (<33% predicted life threatening, 33-50% very severe, 50-75% moderate) as well as recovery from an exacerbation (223). Both these are clinically important indicators of disease prognosis and it is important to understand if they influence colonisation in asthma to guide vaccination and determine risk of pneumococcal disease.

Pneumococcal colonisation in the nasopharynx generates an inflammatory response (177) and acquisition is increased in pre-existing inflammatory conditions such as co-existing viral infection (224). The anti-inflammatory action of ICS is important as it may affect colonisation acquisition, density, duration and subsequently its immunogenic response. Increased risk of pneumonia is seen in COPD patients on ICS (25), with conflicting evidence in asthma (27, 28).

Asthma and an exacerbation in previous 12 months are risk factors for nasopharyngeal colonisation (30, 131). A high bacterial density in the nasopharynx is seen in inflammatory conditions such as viral co-infection and is associated with pneumonia (225). Both these factors are important for people with asthma as they not only have an increased risk of being colonised, but the underlying airway inflammation may increase the bacterial density during colonisation. We have previously shown that pneumococcal colonisation is immunogenic (175), and duration of colonisation is important to study in asthma as if reduced, it may not be immunogenic with possible impaired protection against future colonisation. An inability to clear colonisation may suggest increased propensity to disease.

Antibodies (IgG) to capsular polysaccharide (CPS) capsule are serotype specific and play an important part in prevention of pneumococcal disease (5). They opsonise the polysaccharide capsule increasing susceptibility of the bacteria to be attacked by the host immune cells. They may have a role in agglutination of bacteria at the mucosal surface and lead to a reduction in nasopharyngeal colonisation (150). Polysaccharide vaccine (PPV 23) is widely used globally and provides serotype specific immunity by stimulating a subset of B cells to produce serotype specific antibodies. In contrast, conjugate vaccines (PCV) produce T cell dependent serotype specific immunity and stimulate memory B cells (226, 227). PCV reduces serotype specific nasopharyngeal colonisation and hospitalisations from pneumonia (228).

Functional antibodies to 12 pneumococcal serotypes are routinely measured in clinical practice in patients with chronic respiratory diseases, prone to recurrent chest infections. Often the results show suboptimal levels (low baseline levels if >6 of the12 checked <0.35 mg/dl), and patients are offered a polysaccharide vaccine. The response is measured 8 weeks post vaccination which if low a referral to an immunologist for further assessment is considered.

I studied the levels before and after inoculation in people with asthma and compared to healthy controls, to determine if people with asthma have increased antibody levels in response to experimental colonisation, if baseline levels affect colonisation outcome and whether this is different to that seen in

healthy controls. The data for healthy controls came from the control arm of an earlier EHPC study, with a similar protocol and identical general inclusion and exclusion criteria except for defining history of respiratory disease in asthma and its absence in healthy controls. This study looked at the effect of PCV 13 on nasopharyngeal colonisation in healthy volunteers. This was double blind randomised controlled trial and participants were either assigned to PCV 13 or hepatitis A vaccine group. They were subsequently inoculated with *S.pneumoniae* 6B and nasal wash samples collected at different time points to look for colonisation by classical microbiology culture techniques (152). The comparative data is from healthy controls who received hepatitis A vaccine and serum samples for IgG to 6B polysaccharide were processed using the same ELISA protocol in our lab as described in Chapter 3.

Anti-pneumococcal protein antibodies are serotype non-specific and an increase in titres is observed both post natural and experimental colonisation and infection (123, 175). 27 pneumococcal proteins are identified and their role in protection from colonisation and disease is under evaluation as potential targets for vaccine development, titres to several of these have been studied with varying results (229, 230).

The serum samples were collected before and after inoculation same as for anti CPS IgG. The comparative data is from a dose ranging study performed by our group designed to examine the immunising effect of a single colonisation episode in healthy volunteers (175). The study protocol and general inclusion and exclusion criteria were similar to our study; the only difference being the presence of asthma in our cohort and lack of any underlying medical condition for the healthy controls. Participants were inoculated with an increasing dose of *S.pneumoniae* ranging from 10000 cfu to 160000 cfu. Nasal and serum samples were collected before and after inoculation.

4.1.1 Description of the study

The study visits schedule and protocol are described in the methods chapter. Participants at BTS treatment steps 2 and 3 (described in Table 3) were all on preventive therapy with inhaled corticosteroids (ICS) at a minimum dose of 200 µg up to 800 µg beclomethasone equivalent (BDP). Steps 4 and 5 were not included

due to more severe disease, high dose ICS and/or frequent courses of oral corticosteroids in whom bacterial inoculation may have been associated with an increased risk of exacerbation. Study Overview is described in Figure 10.

Timepoint	*Pre-screen*	*-5*	*2*	*7*	*9*	*14*	*22**	*29*
Baseline investigation	✓							
Nasal wash		✓	✓	✓	✓	✓	✓	✓
Nasal cells		✓	✓	✓	✓			✓
FeNO	✓			✓				

Figure 10: Study Overview: Study design showing timepoints for follow up visits.

*Participants were pre-screened up to 14 and at least 7 days before screening; at baseline full history, including relevant examination was done along with Spirometry, Peak Expiratory Flow Rate (PEFR), and baseline bloods All female participants had a pregnancy test before inoculation. Participants were asked to maintain a PEFR diary from then onwards throughout the study. After inoculation they were asked to send text messages with PEFR recordings and temperature (PEFR meter and thermometer were provided). *Only colonised participants. Fractional Exhaled Nitric Oxide (FeNO)*

4.1.2 Recruitment

See methods chapter for details. I consented 95 participants of which 50 were inoculated (Figure 11 Panel A). The reasons for withdrawal are described in Figure 11 Panel B.

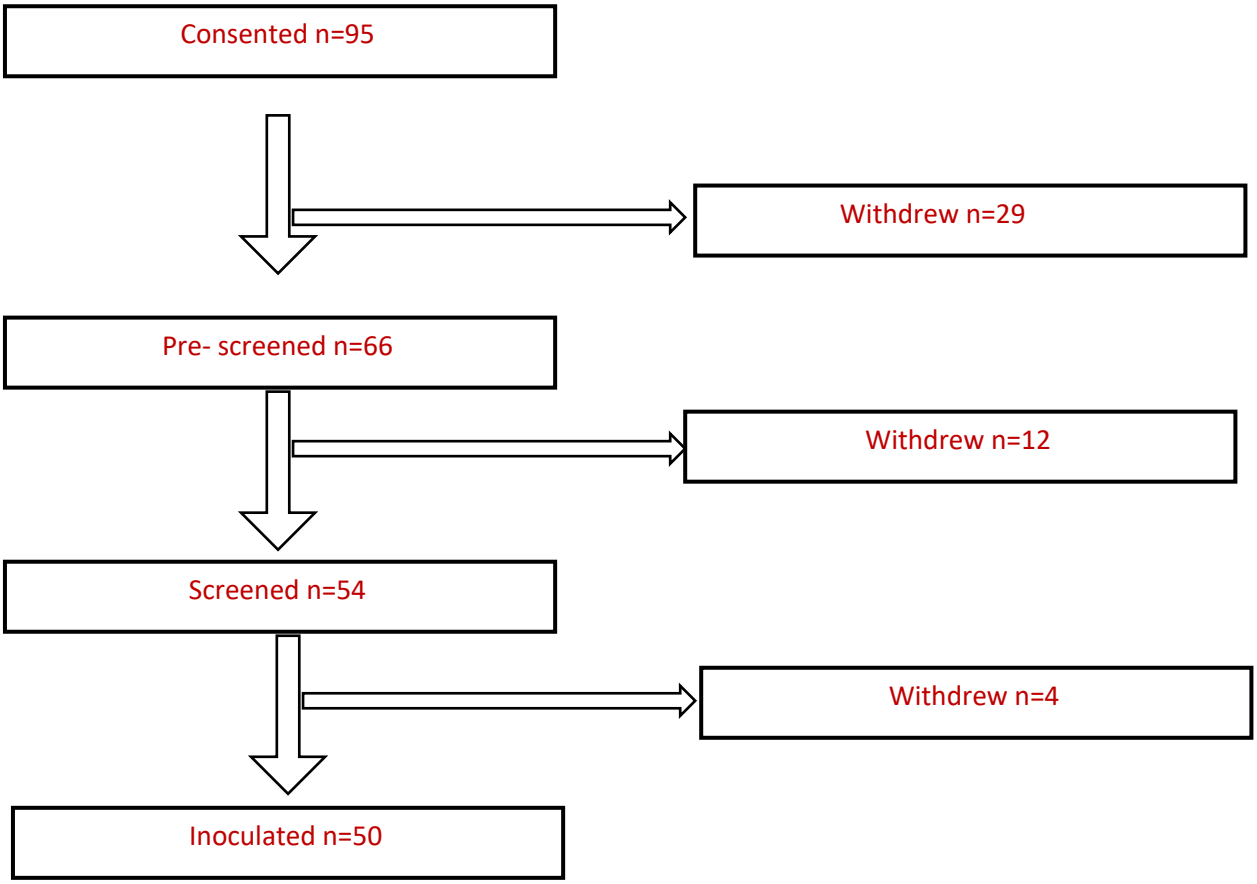
Overall from June 2016 to April 2018, I recruited more than fifty percent participants from the university students, and in small numbers from other sources, such as information received from their GP, sensitisation at the LSTM's open day, RLUH's newsletter and word of mouth from volunteers participating in asthma and other research studies running simultaneously.

After receiving completed questionnaires from primary care some participants were excluded due to other underlying conditions such as bronchiectasis, using CPAP therapy and recurrent exacerbations for asthma Figure 11. One participant was excluded for previous proven pneumococcal pneumonia.

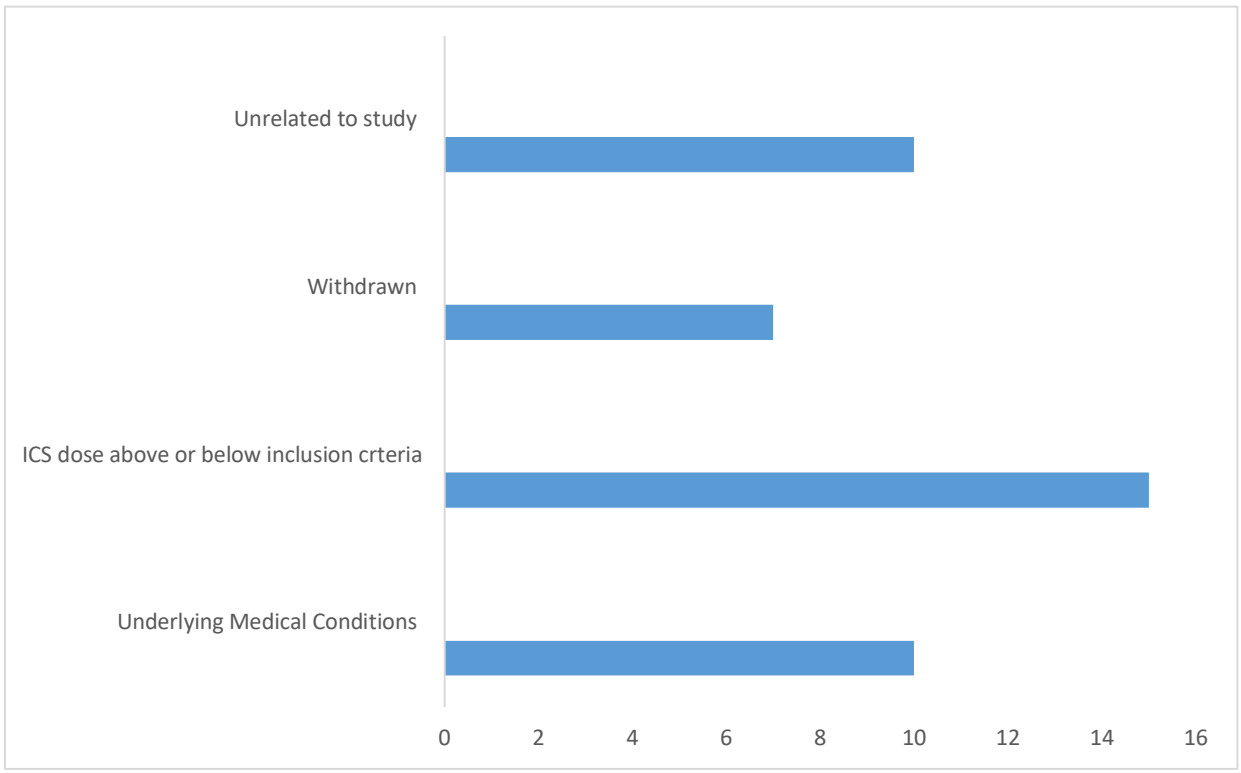
4.1.2.1 Recruitment Challenges and Reasons for Withdrawal:

Our most successful recruitment was from the university, which had its own challenges; the students had changed their primary care provider in the previous 12 months, meaning the records were fragmented. Our commonest reason for exclusion from the study was lack of therapy with ICS (n=13) (Figure 11 Panel B).

Participants on step 3 treatment and those with lack of reversibility on spirometry withdrew due to change in personal circumstance, whilst awaiting approval of study amendments and completion of six step 2 participants to demonstrate safety.


Consented n=95
Withdrew n=29
Pre- screened n=66
Withdrew n=12
Screened n=54
Withdrew n=4
Inoculated n=50

Panel A



Panel B

Figure 11: Study Recruitment:

Panel A) Flow chart showing recruitment and retention numbers; pre-screen visit – performed up to 14 days before inoculation for detailed history, examination, tests including FeNO, spirometry and full blood count (FBC); screen visit – up to 7 days before inoculation for baseline samples including nasal wash, nasal cells and bloods. Panel B) Graph with information on reasons for withdrawal and ineligibility – Underlying medical conditions include bronchiectasis, previous pneumococcal pneumonia, headaches, poorly controlled asthma; Unrelated to study – change in personal circumstance, moving out of the area, new job with time commitments; ICS dose above or below the inclusion criteria – commonest not prescribed any in the preceding 3 months or not using as prescribed. Withdrawn – no reason given

4.1.2.2 Study Completion

Only one participant did not complete the study. They required an increased dose of ICS due to low PEFRs (below 80% predicted), which was more than the protocol defined 800µg per day and was subsequently excluded from the study. Four participants withdrew after the screen visit, all due to a change in personal circumstances.

I was unable to collect blood from one participant on the final visit. Nasal wash samples were available for all the time points for all the participants who completed the study.

4.1.2.3 Statistical Analysis

SPSS version 22 and GraphPad prism version 5 were used for analysis. Area under the bacterial density-time curve were calculated by trapezoid method of $\log_{10}$(value+0.01). This addition was necessary as some values were 0 for density when participants were not colonised and these could not be log transformed. Colonisation rates were compared by chi square test. Continuous measures of colonisation by Mann Whitney U test and independent samples t test for non-normal and normal distributed data, respectively. All tests were two-tailed, with significance level of $p<0.05$. Spearman and Wilcoxon tests were used for correlation and paired-sample analysis respectively. Anti CPS and protein IgG values were log transformed before statistical comparison for normal distribution.

4.2 Clinical characteristics and demographics

The demographics and baseline clinical characteristics of the 50 participants inoculated are described in Table 13 stratified by BTS Treatment category. The demographics were similar for participants on step 2 and 3 treatment. Overall there were more female participants in our study making up 38 (76%) of the cohort. The percentage predicted of the Forced Expiratory Volume in 1 second (FEV1% predicted), blood eosinophil count and Fractional Exhaled Nitric Oxide (FeNO) were all similar with no statistically significant difference in step 2 and 3 participants. The baseline asthma control was good with median Asthma Control Test (ACT) score of 22 for both the groups. Participants on Step 3 treatment were on a higher dose of ICS

(Median [IQR] step 2 400 [400-400] vs step 3 450 [400-800] p=0.001). The importance of these clinical characteristics is described for asthma phenotyping in chapter 1.

Table 13: Demographics and clinical characteristics for the asthma cohort according to BTS treatment step

	Whole Group	**BTS Step 2**	**BTS Step 3**	**P value**
Total participants n (%)	50	36 (72)	14 (28)	
Age (years) median (IQR)*	22 (19.75-26.0)	21 (19.25-25.7)	22(19.75-27.0)	0.632
Gender Female n (%) †	38 (76)	28 (77.7)	10 (71.4)	0.637
BMI (kg/m2) Mean (95 CI, SD) ‡	25.2 (23.5-26.9, 5.8)	25.3 (23.1 - 27.6, 6.7)	24.9 (22.9 - 26.8, 3.3)	0.821
Blood eosinophils Median (IQR)*	0.2 (0.1-0.4)	0.2 (0.1-0.4)	0.3 (0.1-0.4)	0.596
FEV1% predicted Mean (95 CI, SD) ‡	95 (91-100, 13.28)	95 (91-100, 13.48)	94 (86-101,13.19)	0.752
FeNO-ppb baseline Median (IQR)*	25.5 (13.8-49.2)	27 (12.0-50.5)	20.5 (14.8-49.2)	0.936
PEFR variability% Median (IQR)*	14.6 (9.6-21.1)	14.3 (9.5-21.9)	14.8 (10.0-26.7)	0.756
ACT score baseline Median (IQR)*	22 (20-24)	22 (20-24)	22(20-23)	0.278
Inhaled Corticosteroid (mcg) median (IQR)*	400 (400-400)	400 (400-400)	450 (400-800)	0.001

Colonised n (%) †	28 (56%)	19 (53%)	9 (64%)	0.462

BTS Treatment Steps are part of the British Thoracic Society (BTS) guidelines on management of asthma, where treatment is increased in stepwise manner -starting from short acting beta agonist at step 1, to oral steroids at step 5. *Mann Whitney U Test, †Pearson chi square, ‡Independent Samples T test

4.3 Experimental Colonisation Rates in Asthma

Our primary research question was to determine experimental colonisation rates in people with asthma. Overall 28 (56%) of the 50 inoculated participants were experimentally colonised (defined as positive bacterial culture from nasal wash at any time point). The demographics and clinical characteristics of the asthma cohort according to colonisation are described in Table 14. Only 2 participants of the 54 screened were naturally colonised, one withdrew and the other colonised experimentally with serotype 6B.

Table 14 Demographics and clinical characteristics for the asthma cohort according to colonisation status

	Whole Group	Colonised	Non-Colonised	p value
Number of Participants	50	28 (56)	22 (44)	n/a
Age (years) median (IQR)*	22 (19.8-26.0)	22 (20-26)	21(19-24)	0.432
Gender Female n (%)†	38 (76)	23(82)	15(68)	0.251
BMI (kg/m2) Mean (95% CI, SD)‡	25.2 (23.5-26.9, 5.8)	26.9 (24.7-29.1, 5.7)	23.5 (20.6-25.5, 5.4)	0.019
Blood eosinophils Median (IQR)*	0.2 (0.1-0.4)	0.2 (0.1-0.45)	0.25 (0.1-0.4)	0.933
§Blood Eosinophils >0.3 n (%)†	n/a	16 (59%)	11 (41%)	0.615
FEV_1% predicted Mean (95 CI, SD)‡	95 (91-100, 13.28)	96 (91-101, 12)	93 (86-99, 14)	0.340
^PEFR variability at baseline >12% † n (%)	28 (66%)	15 (62.5%)	13 (72%)	0.508
^PEFR variability post inoculation >12% † n (%)	25 (62.5%)	17 (71%)	8 (50%)	0.182
FeNO-ppb baseline Median (IQR)*	25.5 (13.75-49.25)	20.5 (13.5-37.5)	29.5 (13.5-54.75)	0.287
FeNO ppb Day 7 median (IQR)*	25 (11-41.5)	25 (10-37)	26.5 (13.75-54.75)	0.526
FeNO fold change (FC) Mean (SEM, SD)	1.01 (0.062, 0.43)	1.079(0.093, 0.48)	0.925 (0.078, 0.36)	0.225
‖FeNO >40 ppb n (%)†**	n/a	6 (37.5%)	10 (62.5%)	0.071
ACT score baseline Mean (95 CI, SD)‡	22 (21-22, 2.0)	22 (21-22, 2.1)	22 (21-23, 1.9)	0.129
ACT score Day 29 Mean (95 CI, SD)‡	22 (22-23, 1.8)	23 (22-23, 1.9)	22 (22-23, 1.7)	0.816
Inhaled Corticosteroid (µg) median (IQR)*	400 (400-400)	400 (400-500)	400 (350-400)	0.208

* Mann Whitney U Test, † Pearson chi square, ‡ Independent Samples T test, § missing values **n = number of participants with a raised FeNO in each cohort. ‖ Pearson's chi square p=0.071 for a raised FeNO of >40 at baseline for colonisation and for change in FeNO from baseline * Pearson's chi square p=0.367.

§Pearson's Chi square p=0.615 for a raised eosinophil count of >0.3 at baseline, Pearson's chi square test p=0.274 for colonisation positive with steroid dose >400mcg, and for steroid dose >500mcg p=0.288, ^data available for 42 participants for PEFR variability at baseline, and 40 participants for variability after inoculation

4.4 Clinical Factors associated with experimental colonisation in Asthma

The colonised and non-colonised groups were similar in demographics with the body mass index (BMI) higher in colonised participants (mean [95% confidence interval, 95 CI] positive 26.9 [24.7-29.1] vs negative 23.0 [20.6-25.5] Independent samples t test p=0.019). There was no difference in any other clinical parameters measured.

4.4.1 Fractional Exhaled Nitric Oxide (FeNO) and Blood Eosinophils

The FeNO levels >40 parts per billion (ppb) were used as a cut off for high levels as in clinical practice and blood eosinophil of >0.3 accepted as increased. The values were similar in the two groups as described in Table 15.

Table 15 Colonisation status according to raised Fractional Exhaled Nitric Oxide at baseline, and increase from baseline and Blood Eosinophils 0f >0.3

	Colonised (n=28)	**Non-Colonised (n=22)**
⁺FeNO >40 ppb n (%)	6 (37.5%)	10 (62.5%)
FeNO <40 ppb	22 (64.7%)	12 (35.3%)
***Participants with an increase in FeNO from baseline**	12 (63.2%)	7 (36.8%)
^Blood Eosinophils >0.3 n (%)	16 (59.3%)	11 (40.7%)
Blood Eosinophils <0.3 n (%)	12 (52.2%)	11 (47.8%)

n = number of participants with a raised FeNO in each cohort. The ⁺Pearson's chi square p value =0.071 for a raised FeNO of >40 at baseline for carriage and for change in FeNO from baseline * Pearson's chi square p value=0.367. The ^Pearson's Chi squares p value for a raised eosinophil count of >0.3 at baseline is 0.615

I looked at participants with FeNO levels >40 and with eosinophils >0.3 to determine whether they influence experimental colonisation outcome. Out of our 50 participants 14 had a FeNO >40 ppb at baseline, of these 6 (37.5%) were colonised. This was not statistically different Pearson's chi square p=0.07. I also looked at a change in FeNO level from baseline following inoculation, as this was measured at two

time points Table 15. Overall, 19 participants showed an increase in FeNO from baseline with 12 (63.2%) colonised, not statistically significant (Pearson's chi square p=0.367). Fold change (FC) in FeNO values were calculated by dividing the value at day 7 by the baseline value was not statistically different in the colonised and non-colonised participants.

In our cohort of participants 27 (54%) had a blood eosinophil count more than 0.3, of these 16 (59.3%) were colonised and 11 (40.7%) non-colonised (p=0.615). Within the entire asthma cohort, there was a positive correlation between FeNO levels and blood eosinophil levels (p<0.0001, r= 0.573).

4.4.2 Forced Expiratory Volume in 1 second (FEV1) and Peak Expiratory Flow Rate Variability

The median [IQR] FEV_1% in our cohort was 95.5 (87.5-105), with no difference in colonised and (98.5 [88.25-105]) and non-colonised (94.5 [84.5-103.5] p=0.340 independent samples t-test) Table 14. No significant reversibility (increase in FEV_1% post salbutamol) was seen in colonised or non-colonised mean [SEM, SD] 6.15 [1.08, 5.55] vs 6.2 [1.51, 6.78] p=0.980, as described in Table 14.

I asked participants to measure and record PEFRs for a period of six weeks. Two weeks prior to inoculation and for four weeks afterwards follow up. A PEFR variability calculator developed in house using excel (Appendix F) was used to calculate this. All the participants recorded 3 readings, morning and evening before taking their usual inhalers – the best reading was entered, and a value generated. Our cohort had a median of 14.6% PEFR variability [IQR] [9.6-21.1], and this was similar in BTS treatment Step 2 and 3 (14.3% [9.5-21.9] vs 14.8% [10.0-26.7] p=0.756 Mann Whitney U test) Table 13.

I accepted a PEFR variability of 12% for the study, and this was not significant in colonised vs non-colonised participants median [IQR] 13.8 [9.5-22.6] vs 14.8 [10.4-21.1] p=0.684 Table 14. The number of participants with a PEFR variability of 12% colonised and non-colonised was similar (n, (%) 15 (62.5%) colonised vs 13 (72%) p=0.508) non-colonised Table 16.

Table 16 Colonisation status according to change in FEV1 post salbutamol >10% and PEFR variability of >12%

	Colonised (n=28)	Non-Colonised(n=22)	P value
Change from baseline in FEV_1 >10% n (%) †	4 (18%)	2(11%)	0.591
Change in FEV_1% Mean (SEM, SD) ‡	6.15 (1.08,5.55)	6.2 (1.51, 6.78)	0.980
PEFR variability >12% n (%) †	15 (62.5%)	13 (72%)	0.508
PEFR variability % Median (IQR)*	13.8 (9.5-22.60	14.8 (10.4-21.1)	0.684

†Pearson's chi square, ‡ Independent Samples T test, * Mann Whitney U Test

4.4.3 Inhaled Corticosteroid Dose (ICS)

The median dose of ICS in our cohort was 400 µg beclomethasone (BDP) equivalent IQR (400-400), with those on step 3 median (IQR) 450 µg (400-800) p=0.001 Mann Whitney U test. The dose of ICS was not associated with experimental colonisation outcome Table 17. I looked at participants on a dose of =<400 µg or on >400 µg, and those on =< or >500 µg. Neither of these were significant (>400 µg p=0.274 and >500 µg p=0.288).

Table 17 Colonisation status according to steroid dose >400mcg and >500mcg

	Colonised (n=28)	Non-Colonised (n=22)
ICS >400 µg n (%) †	24(88.8%)	17(77.2%)
ICS <400 µg n (%) †	3 (11.1%)	5 (22.7%)
ICS >500 µg n (%) †	7 (25.9%)	3 (13.6%)
ICS <500 µg n (%) †	20 (74%)	19 (86.3%)

† Pearson's chi square p value for colonisation positive with steroid dose >400mcg = 0.274, and for steroid dose >500mcg =0.288

4.4.4 Body Mass Index (BMI)

A high BMI was associated with the likelihood of colonisation in our study. The mean [95% confidence interval, 95 CI] for the whole cohort was 25.5 [23.5-26.9] with colonised participants having a higher BMI 26.9 [24.7-29.1] vs 23.0 [20.6-25.5] in non-colonised p=0.019 Table 14.

4.5 Experimental Colonisation rates, duration and density in asthma compared to healthy volunteers

In our study the rate of experimental colonisation and the density were not significantly different to age matched healthy control participants Table 18. For comparison I used age-matched healthy controls from an influenza vaccine study (Sep 2015- Mar 2017), with identical methods and sampling, although participants received intramuscular tetravalent inactivated influenza vaccine either 3 days before or after inoculation with pneumococcus (209).

The median (IQR) dose of inoculum was 83417 (78333-85292) in asthma vs 78834 (76333-84167) p=<0.001 in healthy controls. There were 82 (54.3%) females in the healthy controls, with a median age of 20 years; this was significantly different compared to participants with asthma Table 18. The number of colonised (nasal wash positive for bacterial culture at any time point) was 28 (56%) in asthma vs 68 (45%) in healthy controls. This was not statistically significant p=0.178 Pearson's chi square. Colonisation at any time point was not significantly higher in asthma Table 18.

The density as calculated using the area under time cure (AUC) was similar in asthma compared to healthy controls (median [IQR] asthma 63.49 [14.04-116.3] healthy controls 81.18 [48.15-104.5] p=0.060). The density was not significantly different at any time point Figure 12 A-C. The area under the density time curve was not affected when participants with negative time points were excluded Figure 12D, E. The duration of colonisation was significantly shorter in asthma compared to healthy controls (median [IQR] 14 [7-29] vs 29[14-29]) p=0.034 Mann Whitney U test Figure 12F. The area under the time curve for density were similar in participants with ICS dose =<400µg or >400µg Figure 13A, B. and BMI < or >25 Figure 13C, D.

Table 18 Colonisation rates in Asthma compared to healthy Controls

	Asthma (N=50)	Healthy Controls (N=151)	P value
Demographics			
Median age (range) – year*	22 (20-26)	20 (19-22)	<.0005
Female – no. (%) †	38 (76)	82 (54.3)	<0.007
Median dose (range) – CFU/nostril ‡	83417 (78333-85292)	78834 (76333-84167)	<0.001
Colonisation Status positive any timepoint– no (%) +	28 (56)	68 (45)	0.178
Colonisation Status positive – day 2 (%) +	19 (38)	61 (40)	0.764
Colonisation Status positive – day 7 (%) +	22 (44)	62(41)	0.715
Colonisation Status positive – day 9 (%) +	17 (34)	60 (40)	0.470
Colonisation Status positive – day 14 (%) +	19 (38)	50 (33)	0.528
Colonisation Status positive – day 22 (%) +	12 (24)	43 (28)	0.538
Colonisation Status positive – day 29 (%) +	11 (22)	34 (23)	0.939
Duration days median (range) *	14 (7-29)	29 (14-29)	0.034
Colonisation Density log transformed AUC (cfu/ml) median (range) *	63.49 (14.04-116.3)	81.18 (48.15-104.5)	0.060

*MW Test, †Pearson chi square, ‡ Independent Samples T test

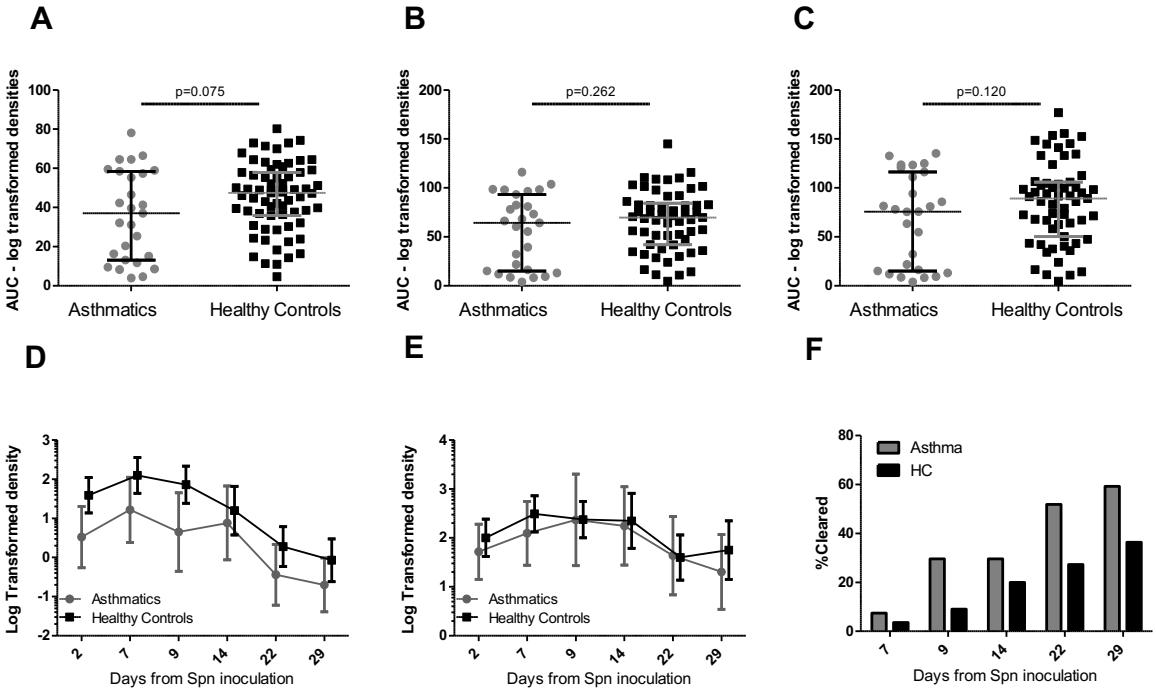


Figure 12: Pneumococcal densities in participants with asthma and healthy controls

A-C. Area under log-transformed bacterial colonisation density curve for participants colonised (positive bacterial culture from nasal wash) with pneumococcus at any time point who attended all visits up to A) day 14 (n=asthma 27, healthy controls (HC) 67), B) up to day 22 (n= 27 asthma, 57 HC) and C) up to day 29 (n=27 asthma, 59 HC). Individual volunteers are shown, and the lines represent median and inter-quartile range p values for Mann Whitney U Test are shown. D-E: Bacterial colonisation density at each time point; D) all positive participants (positive bacterial culture from nasal wash at any time point) with negative time points included (participants attended for the visit and the nasal wash sample was negative for bacterial culture) (n=28 asthma, 68 HC), E) all positive participants at any timepoint with negative values removed (asthma n=19, 22, 17,19 ,12 ,11 at days 2, 7, 9, 14, 22,29 respectively, HC n=61, 62, 60, 50, 43, 35 at days 2, 7, 9, 14, 22 and 29. Values are mean and 95% confidence interval for log transformed densities at each time point + 0.01 added to all values to allow log transformation). F) Cumulative clearance of colonisation (time point when nasal wash was negative for bacterial culture following a positive result at an earlier time point) in asthma vs HC at each time point. Clearance rates at each time point for asthma and HC; % clearance at days 7, 9, 14, 22, 29 – people with asthma 7, 30, 30, 52, 59 and HC 4, 9, 20, 27, 36 respectively).

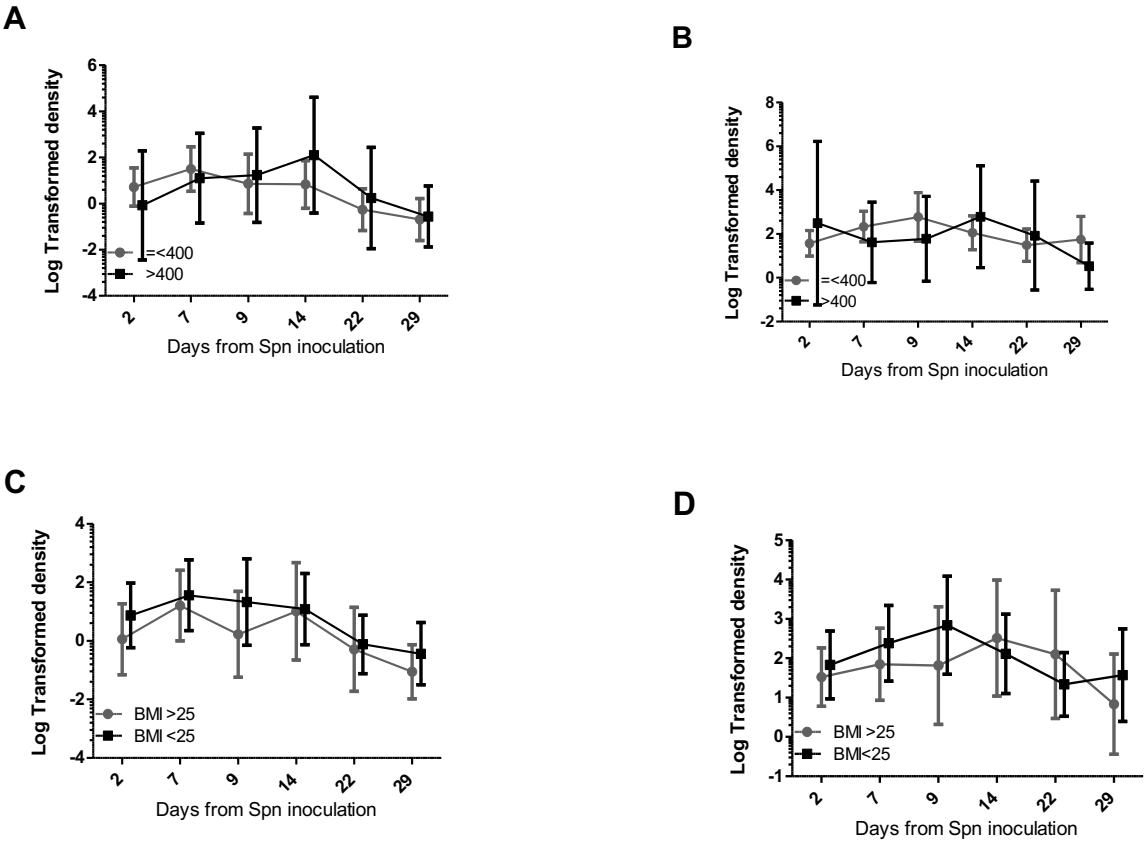


Figure 13: Bacterial colonisation density for asthma

A) dose of inhaled corticosteroid (ICS) >400µg n=7 or =<400µg n=21,all colonised (nasal wash positive for bacterial culture at any time point) participants with negative time points (nasal wash negative for bacterial culture) included B) ICS dose 400µg or <400µg all colonised participants with negative time points removed C) stratification by body mass index (BMI) >25 n=12, <25 n=16 all colonised participants with negative time points included D) BMI >25 or <25 all colonised participants with negative time points removed . Values are log transformed, bars represent mean and 95% confidence interval.

4.6 Antibodies to *S.pneumoniae* 6B in response to experimental colonisation in asthma compared to healthy controls

4.6.1 Asthma Anti 6B CPS IgG

The demographics of asthma participants are described in Table 19; data are available for 20 asthma participants (9 colonised and 11 non-colonised; the remaining 30 samples have not been processed using ELISA). These were performed in July 2016 on all the samples completed until then. Results for the remaining 30 participants who subsequently completed the study would be available following processing of these samples. Overall, there were more females in the asthma cohort 14 (70%) and the colonised and non-colonised asthma participants were similar in age (median [IQR] 22 (18 - 35) in positives vs 21 (19 - 22) in negatives p=0.539).

Anti 6B CPS IgG was measured in asthma participants at baseline (screen visit at day -5, as explained in Methods section Subjects and timelines) at day 7 and at day 29. ELISA was performed as described in the Methods 3.5.3. Data are presented for samples collected at day-5 (baseline) and at day 29 after experimental challenge. The samples collected at day 7 were not included in the analysis as similar data for healthy controls was not available for comparison and antibody responses are usually reported after 4 weeks post vaccination (123). The values were all log transformed before performing statistical analysis to normalise the data and allow parametric analysis.

4.6.1.1 Asthma

The baseline anti 6B IgG levels were similar in the colonised and non-colonised asthma participants; mean (standard deviation, SD) positive 6.65 ng/ml (0.83) vs negative 7.23 ng/ml (0.70) log transformed values, p= 0.06 independent samples t test). A similar pattern was seen in the values post colonisation, mean (SD) positives 7.66 ng/ml (1.05) vs 7.08ng/ml (0.66), p=0.16, Figure 14A, C. The fold changes (calculated by dividing the post values by the baseline followed by log transformation) were statistically different in the

colonised and non-colonised participants. Mean (SD) colonised 1.03 ng/ml (0.69) vs non-colonised -0.15ng/ml (0.25) p=0.0001 Figure 14D.

Table 19: Asthma Anti 6B CPS IgG

	Whole Group (N=20)	**Colonised** (N=9)	**Non-Colonised** (N=11)	**P value**
Demographics				
Age Median (IQR) – years*	21.5 (20 – 23.5)	22 (18 - 35)	21 (19 - 22)	0.539
Female – no (%) †	14 (70)	7 (77.7)	7 (63.6)	0.786
Inoculation dose CFU/nostril median (IQR) *	81833 (78000 - 85000)	83833(79500-87500)	78000 (78000-85000)	0.079
Anti 6B anti CPS IgG (ng/ml) ^				
Baseline Mean (SD) ‡	6.97 (0.79)	6.65 (0.83)	7.23 (0.70)	0.068
Post Inoculation (range) ‡	7.33 (0.87)	7.66 (1.05)	7.08 (0.66)	0.164
[1]Change from baseline in titre‡	0.34 (0.76)	1.03 (0.69)	-0.15 (0.25)	0.0001

*Mann Whitney U Test, †Pearson's chi square, ‡Independent samples t test

^Log transformed values – normally distributed, [1]Calculated by diving the post value by baseline

Table 20: Demographics Asthma and Healthy Control for Anti 6B CPS IgG

	Asthma (N=20)	**Healthy Controls (N=48)**	**P value**
Demographics			
Median age (IQR) – years*	21.5 (20 – 23.5)	21 (19.0 – 22.75)	0.45
Female – no. (%) †	14 (70)	31 (64.5)	0.67
Median dose (range) – CFU/nostril ‡	81833 (78000 - 85000)	81499(77833 – 88500)	0.59
Colonisation Status positive – no (%) †	9 (45)	24(50)	0.71
Sample availability – no. (%) *			
Baseline	20 (100)	48 (100)	

*MWT, †Pearson's chi square, ‡Independent samples t test

^Post inoculation sample availability 48 (100%) for healthy controls and 19 (95%) for asthma.

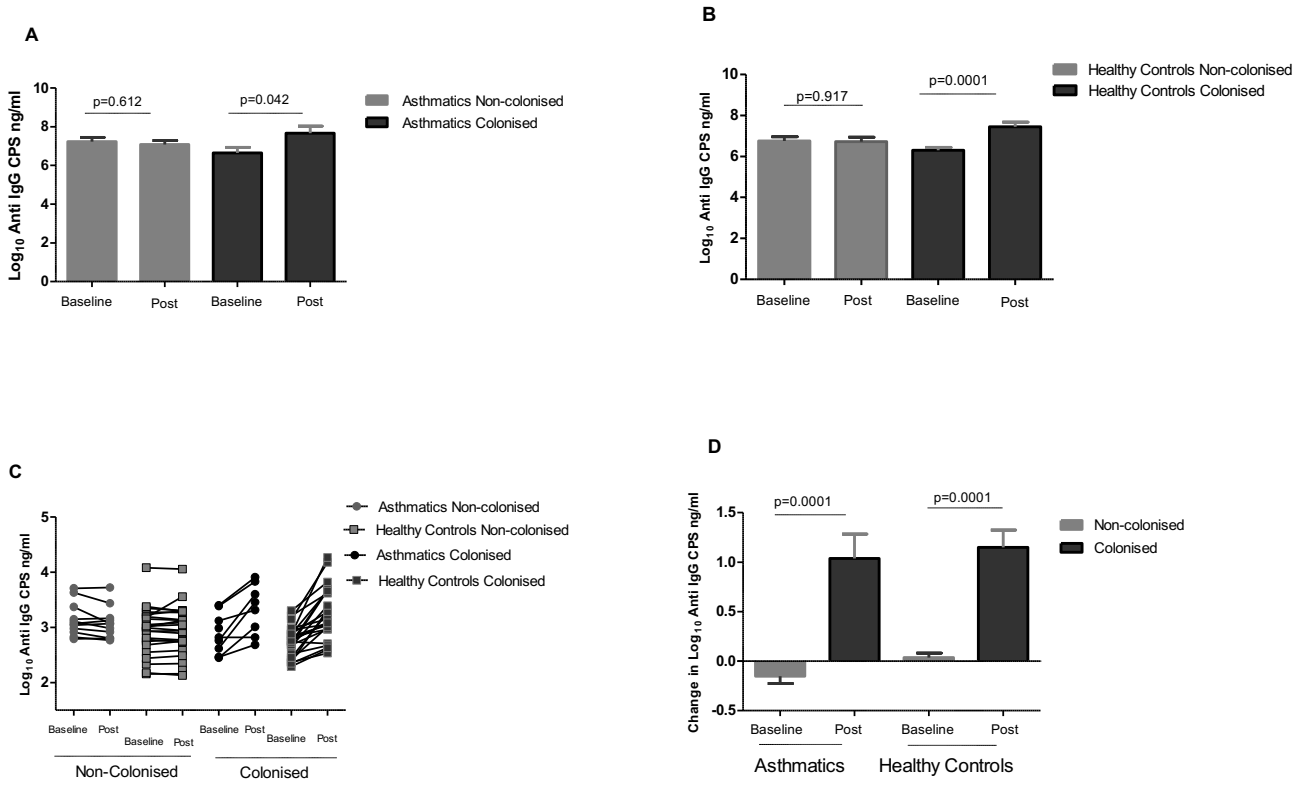


Figure 14: Log transformed Anti CPS IgG in people with asthma compared to healthy controls (HC): before and after experimental pneumococcal challenge

A) Asthma baseline and post levels in non- colonised (n=11) and colonised participants (n=9); p values for independent samples t test. B) HC baseline and post levels in non-colonised (n=24) and colonised participants (n=24); p values for independent samples t test C) Comparison of baseline and post levels in non-colonised and colonised asthma participants and HC D) Fold change from baseline (calculated by dividing the post value by baseline and log transformed after) in people with asthma and HC in non-colonised and colonised participants; p value for independent samples t-test

4.6.1.2 Anti CPS IgG in Asthma compared to Healthy Controls

Anti 6B IgG CPS were measured in healthy control group in a similar manner at baseline (day -5) and after inoculation at day 21. The samples were stored and analysed according to the WHO standard ELISA protocol. The healthy controls were all inoculated with *Streptococcus pneumoniae* 6B, as part of a previous EHPC study as described earlier (152). The two groups were comparable in age and gender distribution and received a similar dose of inoculum, as described in Table 20. The number of experimentally colonised participants was 9 (45%) in asthma and 24 (50%) in the healthy controls (Pearson's chi square p=0.70).

There were no significant differences in antibody titres at baseline and post inoculation in colonised and non-colonised healthy control participants Table 21. A similar pattern was seen in asthma compared to healthy controls Table 19. The difference in fold change of antibody titres was statistically significant between the colonised and non-colonised healthy controls (Mean (SD) colonised 1.15 (0.86) vs non-colonised 0.03 (0.21) Mann Whitney U test p=0.0001.

A comparison of the two groups is shown in Table 21 and Figure 14C, D. The colonised and non-colonised participants in both cohorts show a similar pattern.

Table 21: Baseline and post-challenge anti 6B capsular polysaccharide antibody concentrations in plasma: comparison of asthma and healthy control cohorts

	Colonised			**Non – Colonised**		
	Asthma	Healthy Controls	P value	Asthma	Healthy Controls	P value
Demographics						
Median Age (range) – year*	22 (18 - 35)	21 (19.0 – 26.5)		21 (19 - 22)	20 (19 - 22)	
Female – no (%) †	7 (77.7)	18 (75)		7 (63.6)	13(54.1)	
Baseline Mean ng/ml (SD)‡ ng/ml	6.65 (0.83)	6.30 (0.63)	0.20	7.23 (0.70)	6.75 (1.00)	0.16
Post Inoculation Mean ng/ml (range)‡	7.66 (1.05)	7.45 (1.09)	0.63	7.08 (0.66)	6.72 (1.05)	0.30
[1]Change from baseline in titre Mean (SD) ng/ml	1.038 (0.69)	1.15 (0.86)	0.74‡	-0.15 (0.25)	0.03 (0.21)	0.06*

‡Independent samples t test, *Mann Whitney U test

[1]Calculated by diving the post value by baseline

4.6.2 Anti-pneumococcal protein IgG

This was a pilot study to determine if people with asthma show an increase in anti-pneumococcal protein antibody responses as these are of interest in vaccine development. Therefore, only a subset of samples were analysed. Data are available for 20 asthma participants (9 colonised and 11 non-colonised; processed using Meso Scale Discovery (MSD)). These were performed in July 2016 on all the samples completed until then.

4.6.2.1 Demographics

The demographics of asthma participants and healthy controls are described in Table 22. The comparative data for healthy controls came from a dose ranging study as described above. The healthy control participants were inoculated with increasing doses of 6B. The inoculation dose was statistically different between the colonised and non-colonised healthy control participants (median [IQR] 82000 cfu [51250-128666] vs 21000 cfu [11166-29666] respectively p=<0.05). This was also seen on comparison of the asthma and healthy control groups (median dose [IQR] for asthma was 81833 cfu [78000 – 85000] vs healthy controls 49500 cfu [15916- 82000] p=0.001 Mann-Whitney U test.

The two groups were similar in age and gender distribution, with no difference in the rates of colonisation. There were more females in the asthma cohort 14 (70%) and the colonised and non-colonised asthma participants were similar in age (median [IQR] 22 years (18 - 35); colonised vs 21 years (19 - 22) vs negatives p=0.539).

4.6.2.1.1 Antibody responses to pneumococcal proteins in people with asthma responses

Anti-pneumococcal protein IgG for 27 proteins were measured in the asthma cohort at baseline (screen visit at day -5, day 7 and day 29 as explained in Methods chapter. MSD was performed as described in the Methods section 3.5.4, in the WHO reference laboratory at the Institute of Child Health, University College London. Data are presented for baseline (collected at day-5) and post (collected at day 29). The samples collected at day 7 were not included in the analysis as similar data for healthy controls was not available for comparison and it was very early post inoculation to see an immune response. Antibody responses are

usually reported and measured at least 4 weeks post vaccination (123). The values were log transformed to normalise data before statistical analysis.

4.6.2.1.1.1 Baseline

Of the 27 proteins, the baseline anti-pneumococcal protein IgG titres were similar for 23 proteins in colonised and non-colonised asthma participants. Statistically significant difference was seen in the values of anti protein IgG against LytC, SP0609, Spr0057 and Spr1 p=<0.05 independent samples t-test in the colonised vs non-colonised asthma participants Figure 15A.

4.6.2.1.1.2 Fold Change

The fold change (FC) was calculated by dividing the value at day 29 by the baseline, and then log transformed to normalise. The change in titres of anti protein IgG from baseline was statistically significant for 13 out of the 27 measured proteins in colonised vs non-colonised asthma participants (PspC, NanA, PcpA, PcsB, PhtD, PhtDD, PhtE, PiaA, PiuA, PsaA, PspAUAB099, Rrg23F, RygA) p<0.05 Figure 16A.

4.6.2.1.2 Healthy Controls

The baseline titre for anti protein IgG was significantly different in healthy controls in colonised vs non-colonised participants for two proteins: PcsB and Spr0057 p=0.02 Figure 15B.

4.6.2.1.2.1 Fold Change

The fold change (calculated by dividing the value at day 29 by the baseline, and log transformed for normalisation) was significant in colonised and non-colonised healthy controls for 3 anti protein IgG (PspA UAB055, PiuA, and SP2194 p<0.05 independent samples t test) Figure 16B.

4.6.2.1.3 Healthy Control vs Asthma

4.6.2.1.3.1 Baseline

Of the 27 proteins, anti protein IgG titre for only one protein Spr0057 was significantly different in colonised vs non-colonised participants in both people with asthma and healthy controls.

4.6.2.1.3.2 Fold Change

A similar pattern for fold change was seen for anti protein IgG against PiuA in both people with asthma and healthy controls. The change was significantly different in colonised vs non-colonised participants within each cohort.

Comparing anti-pneumococcal protein IgG in fold change in between the asthma and healthy control cohorts in - titres were significantly different for 14 proteins in non-colonised and for 8 proteins in the colonised participants between the two cohorts Figure 17 A and B.

Table 22 Demographics for Asthma and Healthy Control for anti-pneumococcal protein IgG

	Asthma (N=20)	Healthy Controls (N=41)	P value
Demographics			
Median age (range) – year*	21.5 (20 – 23.5)	22 (20-24)	0.361
Female – no. (%) †	14 (70)	22 (53.6)	0.223
Median dose (range) – CFU/nostril*	81833 (78000 - 85000)	49500 (15916-82000)	0.001
Colonisation Status positive – no (%) †	9 (45)	20 (48)	0.781
Sample availability – no. (%)			
Baseline	20 (100)	41 (100)	

*Mann Whitney U test, †Pearson's chi square

Post inoculation sample availability 48 (100%) for healthy controls and 19 (95%) for asthma.

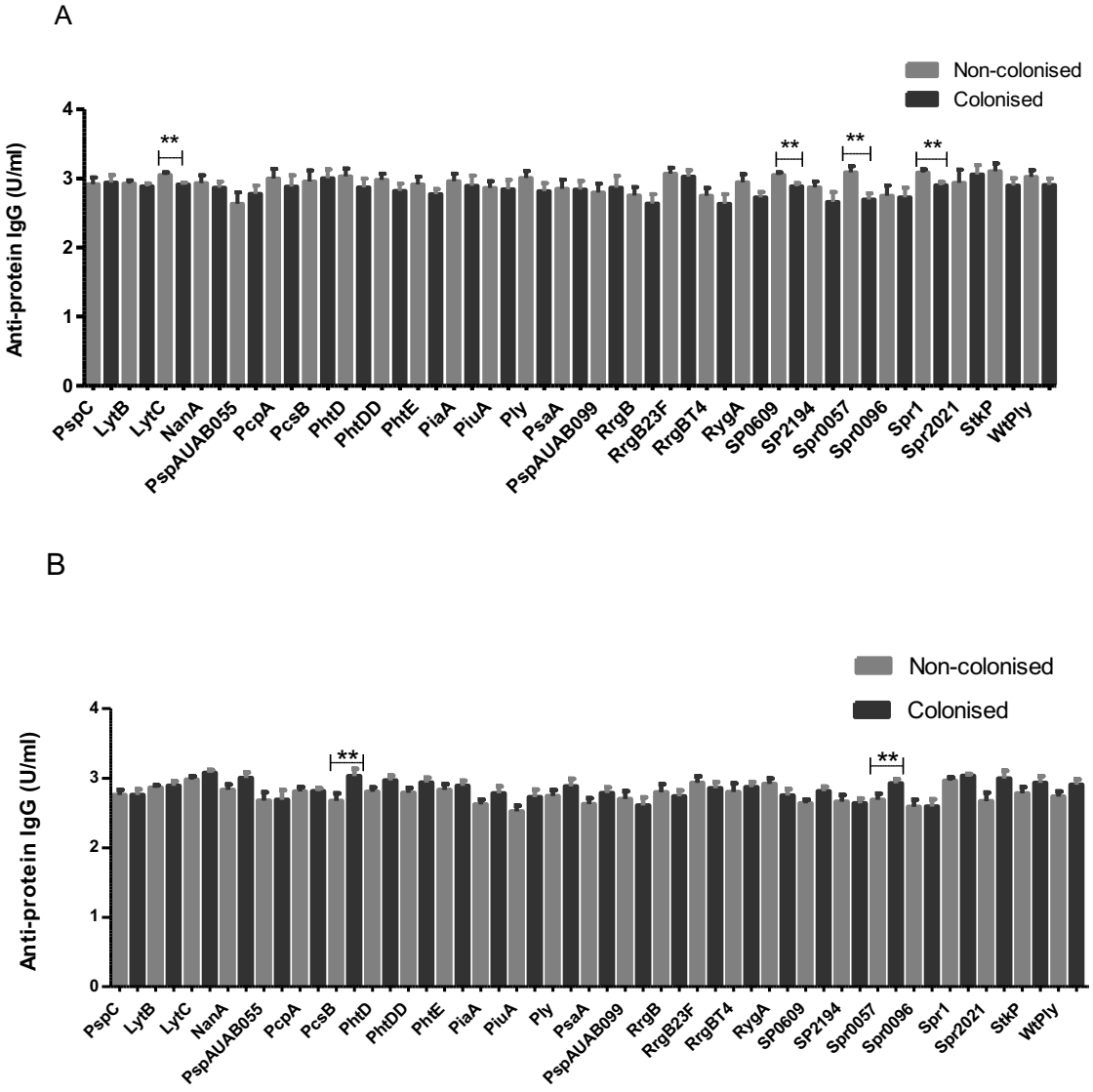


Figure 15: Serum IgG responses to 27 pneumococcal proteins at baseline measured using Meso Scale Discovery (MSD)

*A) Asthma n=20 (11 non-colonised and 9 colonised) B) Healthy Controls n=41 (20 non-colonised and 21 colonised). All values log transformed. Statistically significant difference in colonisation positive and negative log transformed values for IgG at baseline **p<0.05 – Independent samples t test. Bars represent standard error of mean. The p values were not corrected to take into account multiple testing.*

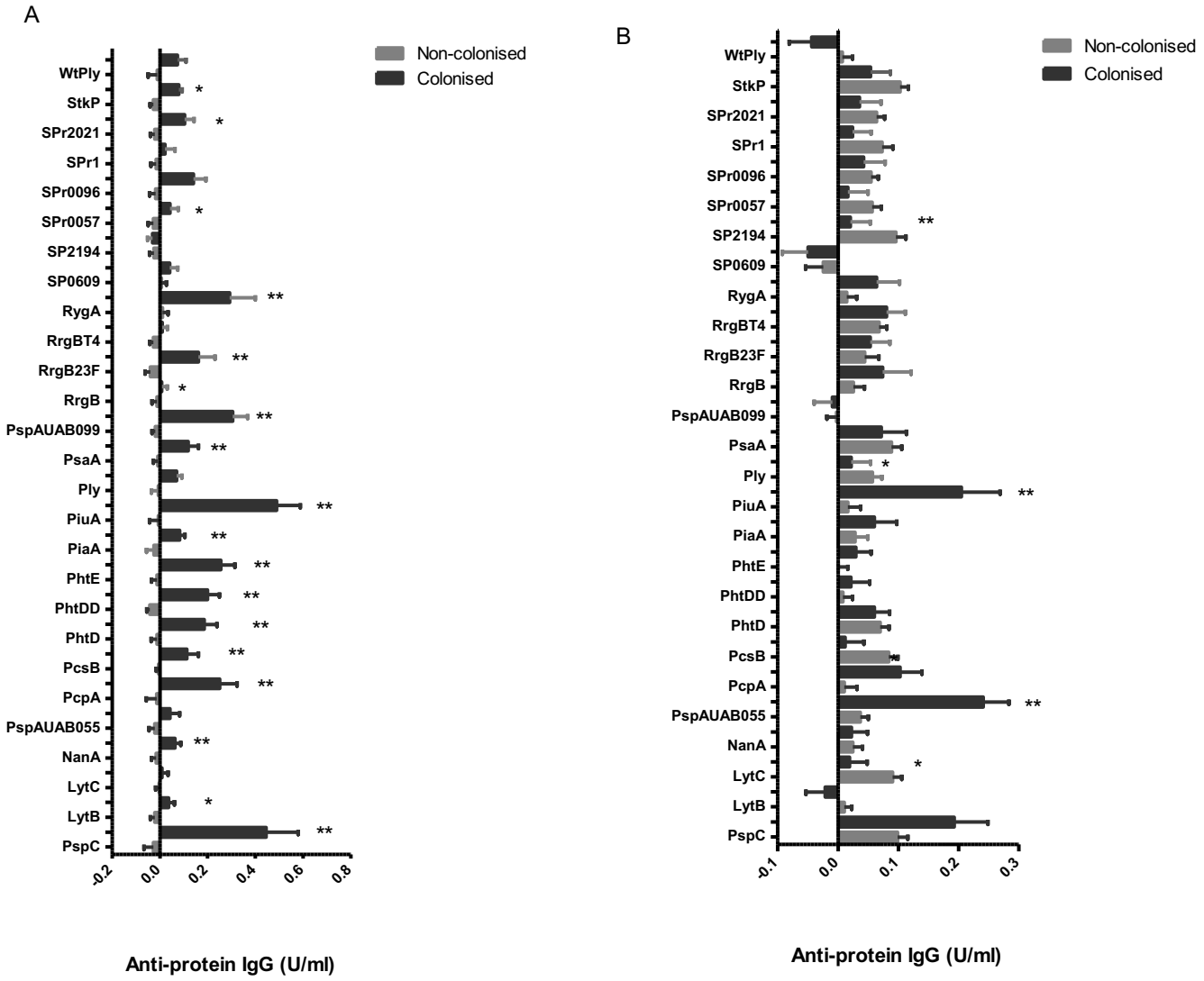


Figure 16: Serum IgG responses to 27 pneumococcal proteins measured with Meso Scale Discovery (MSD) – fold change (FC) values post inoculation divided by the baseline values and then log transformed.

*A) Asthma n=20 (11 non-colonised and 9 colonised) B) Healthy Controls n=41 (20 non-colonised and 21 colonised). All values log transformed. Statistically significant difference in colonisation positive and negative log transformed values for IgG at baseline **p<0.05, borderline significant*<0.1 p >0.05 – Independent samples t test. Bars represent standard error of mean. The p values were not corrected for multiple testing.*

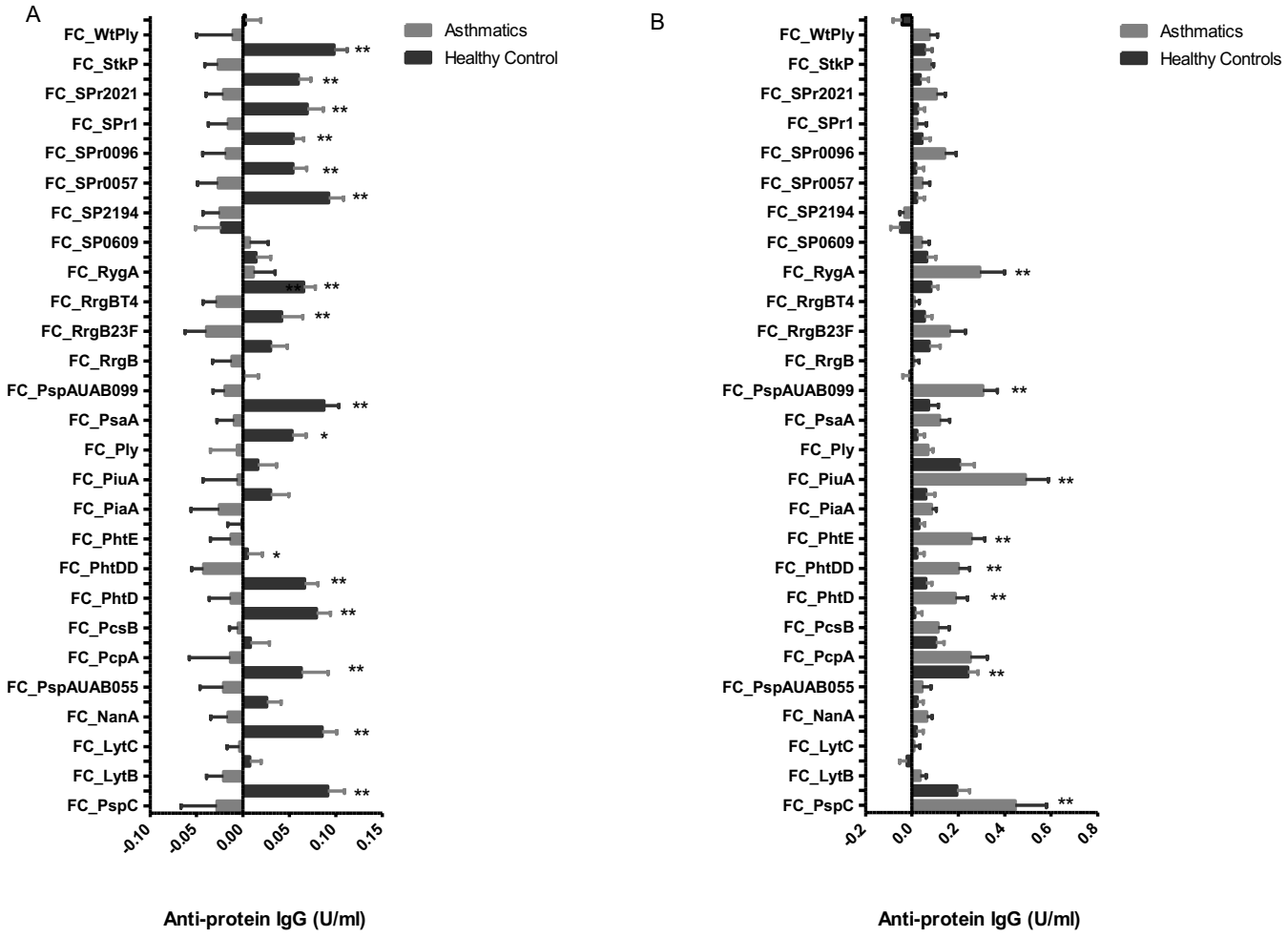


Figure 17: Serum IgG responses to 27 pneumococcal proteins measured with Meso Scale Discovery (MSD) – fold change (FC) values post inoculation divided by the baseline values and then log transformed; in non-colonised and colonised participants.

*A) Non-colonised participants with asthmatics n=11 vs Healthy Controls n=20 B) Colonised participants with asthma n=9 vs Healthy Controls n= 21). All values log transformed. Statistically significant difference in colonisation positive and negative log transformed values for IgG at baseline **p<0.05, borderline significant*<0.1 p >0.05 – Independent samples t test. Bars represent standard error of mean. P values were not corrected for multiple testing.*

4.7 Discussion

This is the first study to examine controlled challenge of an infectious bacterium in people with well controlled asthma, providing a unique opportunity to study experimental nasopharyngeal colonisation and its relation to clinical characteristics. The colonisation rates and density were not different in people with asthma compared to healthy controls. High BMI increased the likelihood of colonisation and it was unaffected by clinical characteristics such as FeNO and eosinophils.

4.7.1 Experimental Colonisation Rates, Density and Duration

4.7.1.1 Asthma

The experimental colonisation rates and density were not significantly different in asthma compared to healthy controls. This contrasts with cross-sectional observational studies which report high colonisation rates in asthma (132). However, these were conducted in general population with self-reported asthma and limited background information on medication, control, clinical characteristics and smoking history. These studies do not report objective measures such as FeNO and FEV_1 which may indicate severity and control. The discordance with previous observations may be secondary to other factors such as a high dose of ICS therapy, poor control, more severe disease, or the limitations of data recorded from a single time-point.

High colonisation rates amongst people with asthma with more severe disease could be seen secondary to higher doses of ICS, due to recurrent infections, or related more directly to asthma-related immune phenomena in the context of uncontrolled airway inflammation.

Experimental Colonisation density was similar in asthma compared to healthy controls. High ICS dose (>400μg) and BMI (>25) were not associated with a difference in area under the density time cure. This was a small study of participants with moderate asthma on a maximum ICS dose of up to 800μg. A dose of more than 400μg did not increase or reduce the density of colonisation. Pre-existing inflammation is seen to increase the colonisation density, and this may be seen in participants with uncontrolled airway inflammation as a result of severe, refractory disease or poor compliance. Our participants on a moderate

dose of ICS, had well controlled asthma with good compliance, which may explain a lack of observed difference.

BMI was associated with increased likelihood of colonisation. I looked at density in participants with a high BMI (defined as >25). There was no difference in the area under the density time curves for those with a BMI of more or less than 25. As obesity is associated with low level inflammation – a higher BMI may lead to increased colonisation density, however I have small numbers in my study with a median BMI in the normal range and this may explain a lack of difference.

The duration of experimental colonisation is significantly reduced in asthma. This may be due to underlying airway inflammation, which could be increased further and helps clear the bacteria in a timely manner. A short duration of colonisation may lead to lower colonisation rates in asthma in observational studies – as an episode may be cleared before sampling. However, observational studies report an increased rate of colonisation which may be secondary to underlying disease related factors as described above, such as severe uncontrolled disease with increased airway inflammation – facilitating colonisation by providing for example increased bacterial attachment sites such as PAFRs.

Nasopharyngeal colonisation is cleared by inflammatory cells recruited to the nasal mucosa (177). Faster clearance in a pre-existing inflammatory environment as in asthma may lead to a diminished mucosal and systemic immune response, as we have previously shown that colonisation is immunogenic (175).

4.7.1.2 Asthma vs Healthy Controls

A number of compositional differences in the asthma and control cohorts could have contributed to our results independent of airways disease. The number of female participants and the inoculation dose were significantly different in healthy controls compared to people with asthma. The median inoculation dose was lower in healthy controls and this may have reduced experimental colonisation (175). The difference in gender distribution and small numbers may impact on our result. Previously male gender has been associated with increased colonisation (131) and our cohorts had more females. An increased experimental

colonisation likelihood from a high BMI may also be independent of asthma as I do not have data in the healthy controls. In addition, the healthy control studies were carried out at different times of the year and seasonal variation in colonisation may affect the results.

4.7.2 Clinical Characteristics

4.7.2.1 FeNO and blood eosinophils

Levels of FeNO and blood eosinophils were not significantly raised in our cohort of colonised asthma participants, who all had well controlled asthma as recorded by objective (PEFR, spirometry) and subjective measures (ACT score). A blood eosinophil count of >0.3 or a FeNO of >40 did not affect colonisation outcome. A high FeNO and blood eosinophil count is seen in poorly controlled disease either refractory to therapy or inadequate compliance.

FeNO is a marker of airway inflammation used for diagnosis and management of asthma in current guidelines (222). A raised FeNO and blood eosinophil level despite adequate therapy prompts a discussion for change in treatment such as increasing dose of inhaled corticosteroids or if requiring repeated courses of oral corticosteroids consideration for monoclonal antibodies. The colonisation rates may be higher in participants with severe asthma, with a reduced duration and an increased density.

Blood and sputum eosinophil levels correlate and levels in blood are routinely used to guide therapy with steroids and specialised treatments such as monoclonal antibodies targeted against interleukin 5 (IL) (118, 231). A blood eosinophil level of >0.3 is accepted as raised for biological treatment in asthma (118). A study of participants with severe asthma may address this but would be challenging to perform.

4.7.2.2 Inhaled Corticosteroids

Dose of ICS was not associated with an increase in colonisation rate and density in our cohort of participants with well controlled asthma. Therapy with ICS is aimed at reducing airway inflammation (93) and FeNO readings are used for monitoring in clinical practice, with high levels suggesting poor control, inadequate dose or lack of compliance of ICS (50, 232, 233). Adequate dose of ICS with good control may

explain low levels of FeNO and blood eosinophils in our study. Poor asthma control secondary to uncontrolled airway inflammation may increase colonisation in severe asthma.

4.7.2.3 Asthma Severity and control

All our participants had well controlled asthma with a normal FEV_1 and no significant reversibility and good peak flow rates >80% predicted at baseline. These did not alter during study follow up, and one participant who had reduced PEFR was excluded due to an increase in the dose of ICS. PEFR variability of >12% did not influence colonisation outcome. The subjective control as measured by the ACT score was good.

Spirometry measurements and peak expiratory flow rate variability are used to diagnose and monitor asthma in clinical practice (223). They are both easy to record and interpret.

Asthma treatment is targeted to achieve adequate control defined as normal spirometry, symptom free and PEFR >80% predicted (223). To ensure safety, I included only these participants. The PEFRs did not alter after inoculation – which may have induced inflammation as a result of colonisation, thereby leading to increased symptoms.

Experimental colonisation may be altered in participants with low FEV_1 and low PEFRs. They may have a high rate of colonisation with increased density and reduced duration.

The guidelines recommend increasing or reducing ICS dose according to symptoms and disease severity, with the aim to achieve a dose with no symptoms and minimal side effects (31, 223). Our participants were on moderate and adequate dose of ICS and had well controlled asthma.

4.7.2.4 BMI

Our findings show that a high BMI is associated with colonisation, however our participants were not obese – defined as a BMI > 30. Asthma phenotyping has led to an appreciation of obesity asthma, often severe and therapy resistant and these patients require a larger proportion of resources (37, 234). In such cases obesity might be an iatrogenic component, perhaps secondary to repeated courses or maintenance use of

oral steroids, or may be an independent driver of disease (55). There is evidence to suggest obesity alters eosinophil recruitment, with an increase in sputum IL5 and submucosal eosinophils, but does not affect sputum eosinophils (62, 235). These findings may be independent of asthma, as I do not have similar data in healthy controls for comparison and there are no data to link obesity to nasopharyngeal colonisation in epidemiological studies.

4.7.2.5 Anti 6B CPS IgG

Amongst those with asthma, colonisation was associated with larger fold-increases in anti-CPS antibody titre than when individuals were not colonised. The levels at baseline were not significantly different in colonised and non-colonised participants.

These results are similar to healthy controls as previously published (175) and no significant differences are seen between the two groups, despite a shorter duration of colonisation in asthma. The values in healthy controls were from samples taken at day 21 and those from asthma participants at day 29. Given the patterns of IgG production after inoculation, it would be expected to continue to rise until at least 28 days, and a difference may be seen between the two groups if samples were collected at day 29 in healthy controls. The healthy controls all received hepatitis A vaccine which may have affected the antibody response in this cohort. However, this did not reduce experimental pneumococcal colonisation compared to the PCV arm in the study. This is a weakness of our comparative data and I speculate that as it did not affect the primary outcome of pneumococcal colonisation, it is unlikely to have lead to an increase in pneumocccal polysaccharide antibody levels.

In future, I intend to assess the anti-CPS function in asthma and control populations in order to complement the absolute concentration data. This will use opsonophagocytic assays to test the independent effect of the antibody while controlling for other factors, but results are not available yet. Our future re-challenge study arm will address this; colonised participants will be re-challenged with the same strain and with anti CPS IgG measurements taken at baseline and post inoculation. In our previous work we

have shown that anti CPS antibody levels protect against future carriage acquisition in healthy controls (175). These results may also be affected by disease severity specially in those participants who require multiple courses of oral corticosteroids for recurrent exacerbations.

4.7.2.6 Anti-pneumococcal protein IgG

An overall trend of increase in fold change was seen in antibody titres against pneumococcal proteins in colonised asthma participants. This was a pilot study and only a subset of randomly sampled participants were included in the MSD analysis. The pattern is similar as seen in healthy controls.

The titres for anti protein Ig antibodies fall in non-colonised participants in asthma and in some healthy controls. The reason for this is not clear and could be due depletion of antibodies from clearance of colonisation or an error in sample processing. This requires a larger cohort to study the trend and perhaps determine opsonophagocytic assays in non colonised and colonised participants to understand the functional ability of these antibodies.

However, the comparative data for healthy controls is from a dose ranging study, with a lower dose of inoculum and the samples were collected 2 weeks after the challenge. This may have affected the results. The asthma study participants were incoulated with a dose seen to achieve experimental colonisation rates of 50% in healthy controls based on earlier EHPC studies. The lower dose particpants in the healthy control study did not experimentally colonise and these results may have been different if all the healthy control participants received a similar inoculum dose. These data need to be interpreted with caution considering the inoculation dose was different in the healthy control arm, and samples were collected at differing time points.

There is variation in the number and type of proteins between the two groups. This may be due to small numbers in the both cohorts, specially asthma, and therefore results need to be interpreted with caution.

Overall titres for 13 pneumococcal protein IgG increased in asthma cohort compared to only 3 healthy controls. This analysis has been performed with a view to generating hypotheses for future research. I have therefore, not taken into account multiple testing, which may lead to these results by chance. Correction for multiple testing, on the other hand, is associated with type 2 statistical error which may not be appropriate in exploratory analysis (236).

A general trend of increase in titres from baseline in colonised asthma participants supports the use of these proteins as targets for novel vaccines (124). Pneumococcal choline-binding protein A (PcpA), genetically detoxified pneumolysin (PlyD1, PdA, PdB), chemically detoxified pneumolysin (dPly), and Pneumococcal histidine triad D (PhtD) and E (PhtE) (229, 237-239) are already widely studied antigens. PcpA is an important protein seen in clinically identified strains of *S.pneumoniae* (124). Further investigation in a larger asthma cohort across the disease spectrum is required to address the question whether a protein antigen-based vaccine would be protective in people with asthma.

4.7.3 Summary

To summarise, I have demonstrated that experimental colonisation rates and density are similar in people with well controlled asthma compared to healthy controls. The duration of colonisation is significantly reduced in participants with asthma and the systemic immune response to pneumococcal polysaccharide (anti CPS IgG) and protein antigens (anti-pneumococcal protein IgG) is not significantly different in comparison to healthy control participants.

Overall, the data suggest some differences which may be related to treatment or the underlying disease. However, no profound difference in immune responses was found, suggesting that a vaccine in asthma should not perform significantly differently than healthy population. It is as yet undetermined if people with asthma have a substantially increased specific risk of disease which would support targeted vaccination outside of usual prevention programmes.

5 Single-use and Conventional Bronchoscopes for Broncho alveolar Lavage in Research: A comparative study (NCT 02515591)

This is a published manuscript from BioMed Central (BMC) Pulmonary Medicine from May 2017 (https://www.ncbi.nlm.nih.gov/pubmed/28476111)(240).

5.1 Background

Bronchoalveolar lavage (BAL) samples can provide valuable information for diagnosis and ongoing management of asthma. Studying lower airway immune cells and microbiome in asthma may inform future therapies and allow development of new diagnostic tests. Research is ongoing to find new modalities for improving diagnostic methods, as currently available tests have sensitivity in adults in the region of 50%, with false positives and negatives well recognised. There is interest in studying volatile organic compounds and particles in exhaled air, multiple breath washout (used to study ventilatory heterogeneity) and impulse oscillometry (to study peripheral airway resistance) to predict a diagnosis of asthma (241-243). BAL can aid in investigating and validating findings to develop these non-invasive methods further by providing information on inflammatory and immune processes within the lungs. All the information together from blood, breath, sputum and BAL can guide phenotyping and targeted therapy development.

For the established therapies such as monoclonal antibodies, BAL can be useful to help understand the changes in for example airway remodelling in patients established on such treatment and provide a comparison of before and after (244, 245).

Many asthma research studies now include BAL samples as standard in a subset of patients and developing a safe and efficient technique is very relevant. I have demonstrated in this study that single use bronchoscopes can be used safely to obtain an adequate BAL sample and would extend this to future asthma studies involving EHPC.

Flexible bronchoscopy is widely performed in adults and children for investigation of pulmonary pathology (246, 247). Broncho alveolar lavage (BAL) sampling is used to study innate, cellular and humoral immune responses, determining the cell population profiles that can facilitate the diagnosis of various diffuse lung diseases (180, 197-200). It is used in early phase drug development studies and has a well-proven safety record in both research and in clinical applications. BAL is easily performed and well-tolerated with rare complications(248-250).

Typically, conventional flexible bronchoscopes are used but they are associated with significant costs related to initial purchase, ongoing maintenance, and sterilisation (251, 252). Single use bronchoscopes offer an alternative (251) and are currently used in many UK NHS trusts for both emergency and elective airway intubations (253). Single use scopes are more portable, and might also improve working efficiency (252). Their efficacy for research studies has not yet been demonstrated, notably in research BAL the cell number and viability, and the returned volume of epithelial lining fluid is critical. Maximising the volume of BAL fluid returned has potential advantages to both researchers and participants: procedures that return less than 100 mL are more frequently associated with side effects such as cough, pleuritic chest pain and fever. Larger total instilled volumes of a minimum of 100 mL and a recommended standard 240 mL using standard 4 x 60 mL aliquots have therefore been recommended by the European Respiratory Society (ERS) to improve standardization when more efficient alveolar sampling and accurate quantitative measurements are required (254). For cellular studies, function and viability are important, and may be maximised by the use of manual suction which minimises cellular shear forces (248). Rapid processing by designated laboratory staff highly trained in handling of BALF (BAL fluid) samples is ideal. However, it should be noted that there is no

strong relationship between the volumes returned and cell numbers obtained (unpublished data from our group).

This study presents a comparison of single use disposable bronchoscopes and conventional bronchoscopes with regards to BAL volumes, cell yields and viability using each method in healthy controls, to establish a safe and effective sampling method which can be used in people with asthma.

5.1.1 Methods

The aim of the study was to compare the BAL volume yield, total cell yield and viability between samples obtained using single use and conventional bronchoscopes and develop a safe sampling method using single use bronchoscopes to be extended to people with asthma post experimental pneumococcal challenge in future. The single use, flexible bronchoscopes were provided free of charge for use by Ambu®, with no input in the study design, analysis or manuscript drafting.

Table 23 compares different features of the single use and flexible bronchoscopes.

5.1.2 Recruitment

We enrolled healthy volunteers aged 18 - 55 years old, to undergo bronchoscopy using the Ambu® Scope™ Regular 5.0/2.2 single-use flexible intubation bronchoscope. The study was carried out in the Clinical Research Unit (CRU) at the Royal Liverpool University Hospital (RLUH). The primary aim was to compare the BAL volume yield (mL), cell yield (total cell number) and proportion of viable cells (alveolar macrophages [AM] and lymphocytes), with recent data from procedures using conventional bronchoscopes. Conventional procedures were performed on 50 healthy volunteers recruited at the same site with identical inclusion and exclusion criteria. The demographics are described in Table 24.

A physical examination including vital signs was performed. A detailed history of complications associated with other procedures or trauma was obtained, and risks for bleeding sought according to guidelines (200), specifically medications (e.g. clopidogrel, aspirin, Coumadin, heparin), and relevant medical conditions (e.g. uraemia). Exclusion criteria were: a history of allergic reaction to

benzodiazepines, or any anaesthetic agent; smoking history of >10 pack years; any tobacco smoking in the preceding 3 months; pregnancy; abnormalities of screening blood tests (haemoglobin, white cell count, platelets, liver transaminases, bilirubin, renal and clotting profile).

5.1.3 Bronchoscopy and Broncho alveolar lavage

Bronchoscopy was carried out as a day case according to previously published protocol by our group (248). Briefly, local anaesthesia was attained using topical lidocaine gel and spray, with further 4% lidocaine administered to the larynx and 2% lidocaine to the bronchial tree via the scope. Warmed 0.9% saline was instilled to the right middle lobe in sequential aliquots (60 mL, 50 mL, 50 mL and 40 mL), with aspiration into a sterile syringe using gentle manual suction, as in our previous BAL studies. BAL yields were recorded, and fluid transported immediately to the laboratory on melting ice (248). We used continuous monitoring of heart rate, blood pressure and oxygen saturations during the procedure, with supplemental oxygen given by nasal cannula.

All procedures were carried out by one of two senior bronchoscopists, experienced in obtaining BAL for research purposes. Hospital procedures required that conventional bronchoscopy was performed in the surgical theatres, whereas flexible bronchoscopy was performed in the research ward: this was the only difference between the groups.

5.1.4 Sample processing

BAL fluid (BALF) was filtered through double layered gauze to remove mucus plugs. Cells were pelleted by centrifugation (1500rpm for 10min at 4^{o}C) and washed with 50 mL cold RPMI medium (Gibco™ RPMI 1640 Medium) containing antibiotics (Penicillin, Neomycin and Streptomycin, Sigma-Aldrich, Sigma Chemical Co. St. Louis, MO, USA). The centrifugation step was repeated once, and the cell pellet was re-suspended in culture medium, with the addition of 10% FBS Gibco-Invitrogen (Life Technologies GmbH, Eggenstein, Germany). Cell suspensions were examined as 5 times diluted in trypan blue for counting and viability assessment using a haemocytometer.

5.1.5 Statistical analysis

Primary outcome measures were compared with values from the preceding 50 conventional procedures using the Mann Whitney U Test. Statistical analyses were performed with Graph Pad Prism version 5.0, Graph Pad Software, La Jolla, CA, USA). It was estimated that 8 volunteers in each group (16 total) will provide a 90% power to detect non-inferiority 1.5 times the standard deviation of the expected return BAL volume.

5.2 Results

Ten participants (6 male), mean age of 23.4 years (range 20-26 years) were enrolled. All participants were intubated nasally, and only one requested sedation (midazolam 3mg used). The median BAL volume yield from the single-use bronchoscopes was 152 mL (IQR 141-166 mL) as compared to conventional 124 mL (110-135 mL), $p<0.01$ Figure 18. The median total cell yield from single-use bronchoscopes was 7.33×10^6 ($5.13 \times 10^6 – 9.80 \times 10^6$) compared with 7.0×10^6 ($4.53 \times 10^6 – 1.64 \times 10^7$) for conventional procedures, $p=0.61$ Figure 19. The median cell viability for samples from single use bronchoscopes was 98.5% (93.8-100) compared to 98.2% (93.7-100%), $p=0.75$ Figure 20. The comparison for the demographics is described in Table 24.

Table 23: Features of Single-use and Multiple Use Bronchoscopes

		Multiple Use Bronchoscope	**Single-use Bronchoscope**
Optical Systems	Field of View	120°	85°
	Direction of View	Forward Viewing	Forward Viewing
	Depth of field	2-100mm	8-19mm
Insertion section	Distal end outer diameter	4.8 mm	5.4mm
	Insertion tube outer diameter	4.9 mm	5.0mm
	Working length	600 mm	600mm
Instrument Channel		2.0 mm	2.0mm
Risk of cross infection		Yes	No
Potential delay due to cleaning		Yes	No
Cost		Sterilisation Servicing Initial equipment cost	Repeated purchase cost
Portability		Depends on location of image processing unit	Can be hand held

Table 24: Demographics of participants for Single use and Conventional Bronchoscopes

	Single-use (n=10)	Conventional (n=50)
Age (yrs.) mean ±SD[#]	23.4 ±1.8	25.9 ±4.2
Males (%)	6 (60%)	19 (37.2%)

[#]Un-paired T-test, SD standard deviation

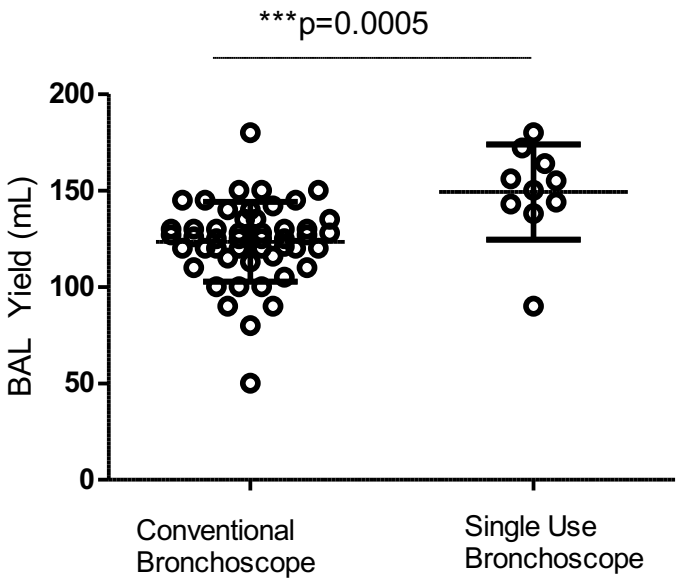


Figure 18: BAL Fluid Volume Yield (mL) from Conventional vs Single-use Bronchoscopes

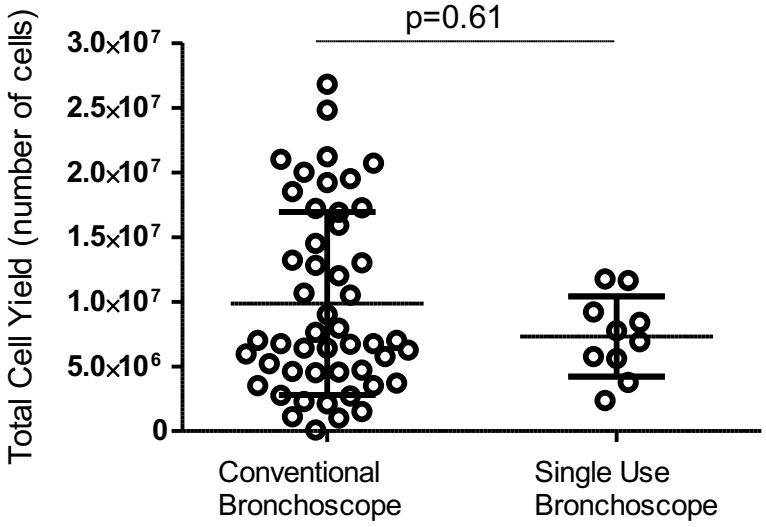


Figure 19: Total cell yield (no.) from Conventional vs Single-use Bronchoscopes

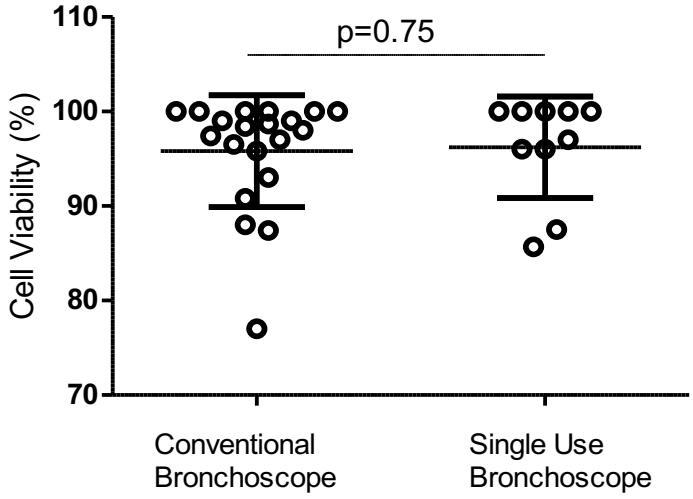


Figure 20: Cell Viability (%) from Conventional vs Single-use Bronchoscopes

5.3 Discussion

Broncho alveolar lavage with single-use flexible bronchoscope achieved greater BAL volume yields than with conventional bronchoscopes. There was no significant difference between the cell yield and viability between the methods.

Single-use bronchoscopes have been evaluated in the critical care setting with favourable evaluation for bronchoscopy, tracheostomy, intubation and suction (251). Our group has experience of over 1500 research BALs, and the procedures in this study were performed by senior bronchoscopists with extensive experience of BAL for research purposes; allowing good comparison with the use of conventional bronchoscopes. BAL volume yields were similar in male and female participants in our study, as seen in other studies(255).

BAL sampling has been used for investigating, diagnosing and profiling pulmonary conditions such as interstitial lung disease and in immune compromised hosts (197, 198, 200). It can provide

information on alveolar cell profiles and immune responses to guide therapy. BAL samples from participants colonised with pneumococcus and with pneumococcal pneumonia have been used to study mucosal immune responses (201, 202).

The greater BAL volume return achieved with single-use bronchoscopes could lead to reduced risk of post-procedural side effects such as cough, pleuritic chest pain and fever, which may improve tolerability and participant comfort. However, I have not systematically collected these data.

Single use flexible bronchoscopes have the potential for use in pharmaceutical preclinical and clinical studies for medicine development.

5.4 Bronchoscopy in Asthma

Samples from BAL are helpful in defining asthma phenotypes and targeting therapy. This has been used in asthma for some time mostly using conventional bronchoscopes. This technique using single use flexible bronchoscopes can be used in asthma. We have previously shown increased activity of alveolar macrophages in experimentally colonised healthy controls, and it would provide a helpful insight into the lung immune responses in both colonised and non-colonised asthma participants. This would help research into new medications to treat asthma according to phenotype and guide vaccine development. This can also be used to study the effect of new therapies on airway remodelling. Using EHPC we can conduct early phase studies with a small cohort of participants. This sampling method is safe in healthy controls and may be extended to include people with asthma post challenge in future.

5.5 Conclusion

Single-use flexible bronchoscopes can be used to obtain BAL for research purposes to study immune responses and in early phase drug development studies in healthy controls and can be implemented in asthma to obtain adequate BAL samples.

6 Discussion

In this thesis, I have described the first study investigating human pneumococcal challenge in people with asthma. Our literature review highlights an interesting overlap between the pathophysiology of asthma and pneumococcal colonisation, with airway inflammation being common to both.

Preventing nasopharyngeal colonisation is an important step in controlling invasive disease from the bacterium. Mucosal innate and adaptive immune responses form part of the frontline defence in preventing colonisation. This is complemented by systemic responses through secretion of cytokines by T cells such as interleukin (IL) 17 by Th17 cells. IL17 enhances inflammatory response by recruitment of neutrophils and macrophages to help with timely clearance of the bacterium from the nasopharynx and in asthma promotes fibroblast proliferation leading to airway remodelling. The balance of this inflammatory mechanism may play an important part in pneumococcal disease in asthma – increased exposure may lead to reduced antigen exposure and subsequent poor future protection whereas reduced secretion may result in delayed clearance and perhaps an increase in disease.

Nasopharyngeal pneumococcal colonisation in early life is associated with increased risk of developing asthma later (35). Asthma and an exacerbation in the preceding 12 months can affect the likelihood of becoming colonised by *S.pneumoniae (131).*

Invasive pneumococcal disease and pneumonia are increased in asthma, and the response to vaccination is unclear along with limited information on baseline immune responses. This may be due to the underlying disease mechanisms or related to treatment. These factors require investigation, to reduce burden of pneumococcal disease affecting people with asthma and to achieve better symptom control as bacterial infections may cause exacerbations leading to a deterioration of symptoms.

Using experimental pneumococcal challenge in a well characterised cohort of people with moderate asthma on ICS therapy, I have shown:

- The rate and density of experimental nasopharyngeal colonisation are similar compared to healthy controls
- The duration of colonisation is significantly shorter compared to healthy controls
- Clinical characteristics such as FeNO, blood eosinophils, spirometry and PEFR do not affect experimental colonisation
- A trend towrds an increased likelihood of experimental colonisation with increasing BMI
- The systemic immune response (anti CPS IgG and anti-pneumococcal protein IgG) is similar to healthy controls

I will discuss each one of these findings, potential reasons for the outcomes and their implications in details.

6.1 Colonisation Rates, Density and Duration

The colonisation rates and density were not significantly different in our cohort of well characterised asthma participants compared to healthy controls, suggesting that people with well controlled asthma are not at increased risk of pneumococcal colonisation.

Our findings are in contrast to those from observational studies, which report high colonisation rates in asthma. These were conducted in general population with self-reported diagnosis of asthma and lack of information on objective and subjective measures of disease, treatment and compliance. The participants in these studies may have had sub optimally controlled, severe disease with poor compliance.

All our participants were symptom free at the time of inoculation with markers of disease severity within normal range. This was due to careful participant selection and inclusion criteria which led to recruitment of asymptomatic volunteers on optimal treatment.

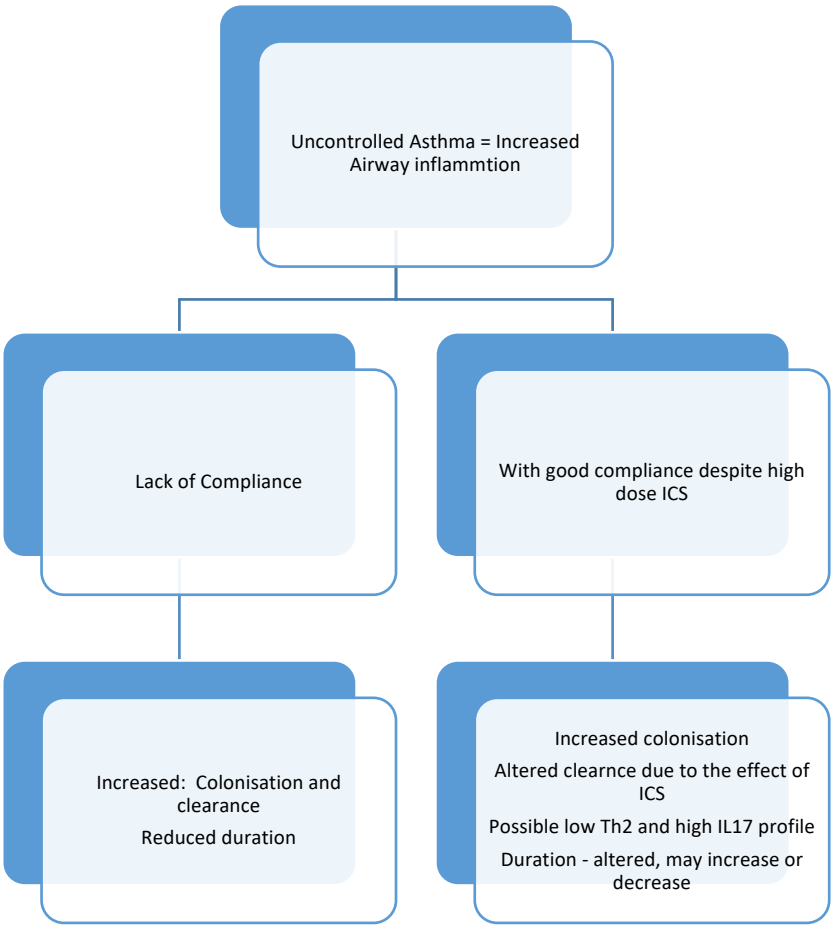
The colonisation rates and density were not associated to the dose of ICS in our study. All our participants were on moderate dose and a high dose of ICS may influence colonisation outcome – for example the anti-inflammatory effect may reduce mucosal immune response and lead to increased colonisation rates. Similarly, symptomatic asthma participants with uncontrolled airway inflammation despite therapy with high dose ICS may also have high bacterial colonisation rates and density secondary to for example increased bacterial attachment sites. Platelet activating factor receptor (PAFR) provides attachment sites for *S.pneumoniae* on the mucosal surface and in asthma leads to airway inflammation by activation immune cells, increased recruitment of inflammatory cells, vascular permeability and mucus production by goblet cells.

I hypothesise that asthma participants with uncontrolled airway inflammation would have increased nasopharyngeal colonisation rates and density secondary to increased bacterial attachment sites for

instance. Although, excessive airway inflammation may contribute to a brisk mucosal immune response leading to early clearance of the bacteria and therefore they may have a shorter colonisation period.

The duration of colonisation was significantly shorter in people with well controlled asthma compared to healthy controls. The shorter duration may be due to underlying airway inflammation in asthma, or an effect of immune profiles such as IL17 which facilitates clearance of colonisation. A shorter duration may be associated with a reduced systemic immune response. If colonising bacteria are killed, and antigen presentation takes place over a reduced time frame, I hypothesise that the absolute humoral responses would be lessened. The effect on T cell responses of a shortened window of antigen availability might also be important as both humoral and cell mediated immunity is required for protection from pneumonia and invasive disease (256). The concurrent use of steroids may alter the development of cell-based immunity by reducing the numbers of immune cells including macrophages and antigen presenting cells within the mucosa. These cells engulf the bacteria and present derived antigen via the major histocompatibility cell complex to T cells in the local lymphoid tissue. This subsequently leads to activation and differentiation of T cells and they stimulate B cells for antibody production and migrate from the lymphatic tissue to the infection site releasing cytokines to help clear the bacteria (257). There is interest in understanding the role of ICS in this mucosal immune pathway with BAL studies being undertaken in healthy volunteers on ICS (NCT02476825). There are no data available on duration of nasopharyngeal colonisation in asthma.

I have explained this further in a diagram below. Uncontrolled asthma leads to increased airway inflammation which may be due to lack of compliance or despite high dose ICS with compliance as seen in patients requiring further treated with monoclonal antibodies. In those with poor compliance I speculate higher colonisation rates and rapid clearance due to inflammation, and in those with good compliance on high dose ICS I hypothesise increased colonisation rates with altered clearance – could be enhanced or slow secondary to the effect of ICS.



My findings generate the following main questions:

1. Are colonisation rates and density higher in uncontrolled asthma with a shorter duration?
2. How does therapy with ICS affect colonisation outcome – rate, density and duration?

I can address the first question by a pilot epidemiological study of asthma participants on high dose ICS. This would include two cohorts - symptomatic participants despite therapy and well controlled on high dose ICS. The selected participants with appropriate disease markers – for example – poorly

controlled with more than 2 or 3 exacerbations in one year, ACT score less than 18; well controlled with none or 1 exacerbation per year and ACT score more than 20; would have nasopharyngeal samples collected at different time points over a 6 week period, to determine colonisation rates, density and duration. Participants for this may be recruited from a pre-existing research database or a national registry with predefined criteria.

The second question may be addressed by studying healthy control participants treated with ICS for a period of 8 weeks – 4 weeks before bacterial challenge and followed for 4 weeks after in a similar manner using EHPC. This will require ethical approval and the exposure to ICS justified. This would have three cohorts of healthy control participants – not on any ICS, on moderate dose and high dose ICS. The results can be compared to historic healthy control EHPC studies and my asthma cohort.

The results showing a shorter duration of colonisation with rates and density comparable to healthy controls in the context of observational studies showing increased risk of invasive pneumococcal disease in asthma require further investigation. Studies described above may address these with factors such as ucontrolled airway inflammation and therapeutic effect of ICS contributing to the increased disease burden in this patient population. Contribution of phenotype Th2 and Th17 may play a part in this as well. All these factors may contribute to an increased risk of pneumococcal disease seen in patients with asthma.

6.2 Clinical Characteristics

Clinical characteristics defining the condition and phenotypes of asthma do not influence experimental colonisation outcome in this study. All my participants had normal lung function with absence of reversibility. FeNO and blood eosinophils were raised (defined as FeNO>40 and blood eosinophils >0.3) in a small number of participants but did not affect colonisation outcome.

Factors such as blood eosinophils and fractional exhaled nitric oxide (FeNO) are simple measures of inflammation but are only raised in patients with poorly controlled disease. A small sample size of

well controlled participants may explain lack of these factors influencing colonisation. I do not have data on the effect of these factors from epidemiological studies.

I speculate that asthma at the severe spectrum of disease may increase the likelihood of pneumococcal colonisation. Severe disease is defined by low FEV_1, uncontrolled airway inflammation, recurrent exacerbations and increased use of healthcare resources. Suboptimal control may be secondary to inadequate therapy or compliance with ICS or sometimes despite maximal dose of ICS with good compliance. These patients then require treatment with new therapies such as monoclonal antibodies against IL5. The persistent eosinophilia seen in these patients leads to airway remodelling which may also affect immune responses and propensity to infection. Participants with severe and uncontrolled disease were excluded due to safety concerns.

The questions from this observation for further study are:

1. Is reduced lung function associated with nasopharyngeal colonisation outcome for example secondary to recurrent infections, persistent inflammation and airway remodelling?
2. Are high FeNO and blood eosinophil levels associated with nasopharyngeal colonisation rates, density and duration?

Both these questions could be answered by the epidemiological study described above of asthma participants on high dose ICS. In addition, to the inclusion criteria described, I would define parameters for FEV_1, FeNO and blood eosinophils along with the dose of ICS in each cohort. The values would be in the normal range for the well-controlled arm and deranged (high FeNO) for the poorly controlled arm. FeNO and blood eosinophil levels would be measured at baseline and at follow up visits along with ACT scores. These measurements are useful to include as they are easy and non-invasive to perform and simple to interpret and monitor over time, although measuring lung function may be more useful.

6.3 BMI

A trend of increased likelihood of experimental colonisation is seen with a higher BMI being in people with well controlled asthma. The asthma cohort had a median BMI within the normal range. BMI has not been studied in the context of nasopharyngeal colonisation in asthma or healthy controls. Obesity related asthma is a recognised phenotype, which can be secondary to a high body mass index (BMI) or a consequence of the condition and treatment (55). Several factors may contribute to asthma due to obesity such as structural changes due to reduced lung volumes, airway compliance, altered airway mucosa and ageing processes. It is often late onset, affects women commonly and may be associated with oxidative stress. This may also be iatrogenic from transformation of early onset, uncontrolled allergic asthma treated with multiple courses of OCS (258).

It is plausible that obesity affects nasopharyngeal colonisation independent of asthma. Obesity is associated with an abnormal persistent low level of inflammation and changes in adipocytes, macrophages and related cytokines are seen outside of asthma (259). Within adipose tissue macrophage subtypes both anti and pro inflammatory are important in mediation of obesity associated inflammation and are perhaps shaped by the characteristic presence of nutrients such as excess glucose and triglycerides (260, 261). Pro-inflammatory macrophages utilise the glycolytic pathway and the anti-inflammatory macrophages use fatty acids as substrates. Obesity associated changes in immune cells and cytokines favour pro-inflammatory macrophage phenotype (262, 263).

Both B and T cells are thought to play an important part in obesity related inflammation with T cells seen before macrophages in adipose tissue. T regulatory cells secreting Th2 profile cytokines (IL5, IL4, IL13) are found in lean tissue and decrease with increasing BMI in a manner like CD4 + T helper cells (Th2). In contrast CD8+ cells increase with obesity (264, 265).

Obesity may increase susceptibility to infections from impaired immune defences (266, 267). It was identified as an independent risk factor during the 2009 H1N1 influenza outbreak (268), and

impaired function of anti-inflammatory lung macrophages in obese mice infected with influenza was associated with reduced survival (269). An association of nasopharyngeal colonisation with high BMI should prompt consideration of the benefit from pneumococcal vaccination, and may inform choice of targeted therapies in asthma, as obesity alters Th2 driven inflammation (62) (235). Clinically, weight loss is associated with improvement in asthma severity, control, and quality of life (55).

This is an important finding as obesity is now a major public health concern, with increasing prevalence globally. Vaccination programmes are designed to reduce risk at both a population and individual level. If our findings are reproduced, the individual protection in this group of patients need consideration in guidelines and recommendations for pneumococcal vaccination.

The following questions now require investigation:

1. Does high BMI affect nasopharyngeal colonisation independent of asthma?
2. Do people with obesity related asthma have an increased likelihood of nasopharyngeal colonisation?

The first question may be addressed by setting a cohort study, defining groups based on BMI (<25, 25-30 and >30) and following them for a period of 6 weeks with nasopharyngeal samples. Or by retrospective analysis of historic EHPC studies using a case control design – defining cases with a BMI 25-30, >30 and controls with <25. BMI measurement has now been included in ongoing and future EHPC trials of healthy control participants and will assess colonisation outcomes prospectively in participants with a high BMI and compared to lean individuals.

The second question about obesity asthma may be addressed by a case control epidemiological study – cases defined as BMI >25 and controls with BMI <25. Participants from a national registry or from data set of a tertiary clinic may be included.

6.4 Systemic Responses

The systemic responses to capsular IgG were similar in my cohort of asthma participants and healthy controls, although it was measured in only a subset of participants. The samples were collected at different time points (earlier for healthy controls), and these may be significantly higher if measured at day 29 in healthy controls. Similar data is not available at day 14 in asthma for comparison, and this may have shown a reduced response. Results require a cautious interpretation due to small numbers in the asthma cohort and differences in collection times.

Despite an early clearance colonisation is immunogenic in people with well controlled asthma on ICS therapy. This may be attributed to a very well characterised and symptom free cohort of asthma participants, who had not received any oral corticosteroids (OCS) in the last 4 weeks and neither had more than one exacerbation in the preceding 12 months requiring OCS. This result may be different in severe asthma participants on high dose ICS and who often require multiple courses of OCS.

The IgG to anti-pneumococcal proteins increased in colonised people with asthma as in healthy controls. The titres were measured against 27 pneumococcal proteins in both the cohorts. The response in titres to each protein was different in the two groups. This is an exploratory analysis with small number of participants in both cohorts. Healthy controls were part of a dose ranging study with a significant difference in inoculation dose and different sample collection time points for the two cohorts. The titres may have been higher and increase in IgG against more proteins may have been seen if measured at 4 weeks in healthy controls as in asthma. They may also be affected by the dose of inoculated bacterium. It is reassuring to see a similar trend for anti-pneumococcal protein IgG in people with well controlled asthma as they are potential targets for novel vaccines, although due to multiple statistical testing, this hypothesis-generating analysis may be affected by false-positive (type 2) errors.

This generates the following questions:

1. Is colonisation immunogenic in severe asthma?
2. Could the titres for anti-pneumococcal antibodies be used to compare vaccine response in asthma in comparison to healthy controls?
3. Are the antibodies functionally comparable to healthy controls?

A pilot case-control study of people with severe asthma and age matched healthy controls may address this. Severe asthma participants can be recruited from a tertiary asthma clinic with well-defined inclusion criteria such as number of OCS courses in the last 12 months and no radiological evidence of structural lung disease within the last 2 years. Severe asthma participants for this study would require careful selection, as they often have co-existing conditions such as bronchiectasis which is recognised for immune deficiency. Age-matched healthy controls can be recruited from general population who have no underlying lung disease or any other medical condition which may compromise the immune system such as diabetes. Also, volunteers with past history of pneumococcal vaccination would have to be excluded. These antibodies are measured in clinical practice in severe asthma patient cohorts.

The second question may be addressed by a pilot study of cases of asthma vaccinated with PCV. Although this may be challenging as they often receive PPV and have other underlying conditions such as bronchiectasis. Also, healthy control volunteers are not usually offered a pneumonia vaccine in the UK. Another option would be to study the response using EHPC – defining participants with asthma and healthy controls and offering them PCV. Measurements of IgG would be taken at baseline and 4 weeks after vaccination and colonisation status determined with nasopharyngeal samples. This would include carefully selected participants with moderate and severe asthma and healthy control.

I aim to measure opsonophagocytic assays for these antibodies and also re-challenge some colonised participants to asses if these offer protection against future disease.

In Figure 2 in the introduction describes the possible mechanism of interactions during nasopharyngeal colonisation at the mucosa. I have adapted this and highlighted in bold the possible factors which may explain our findings.

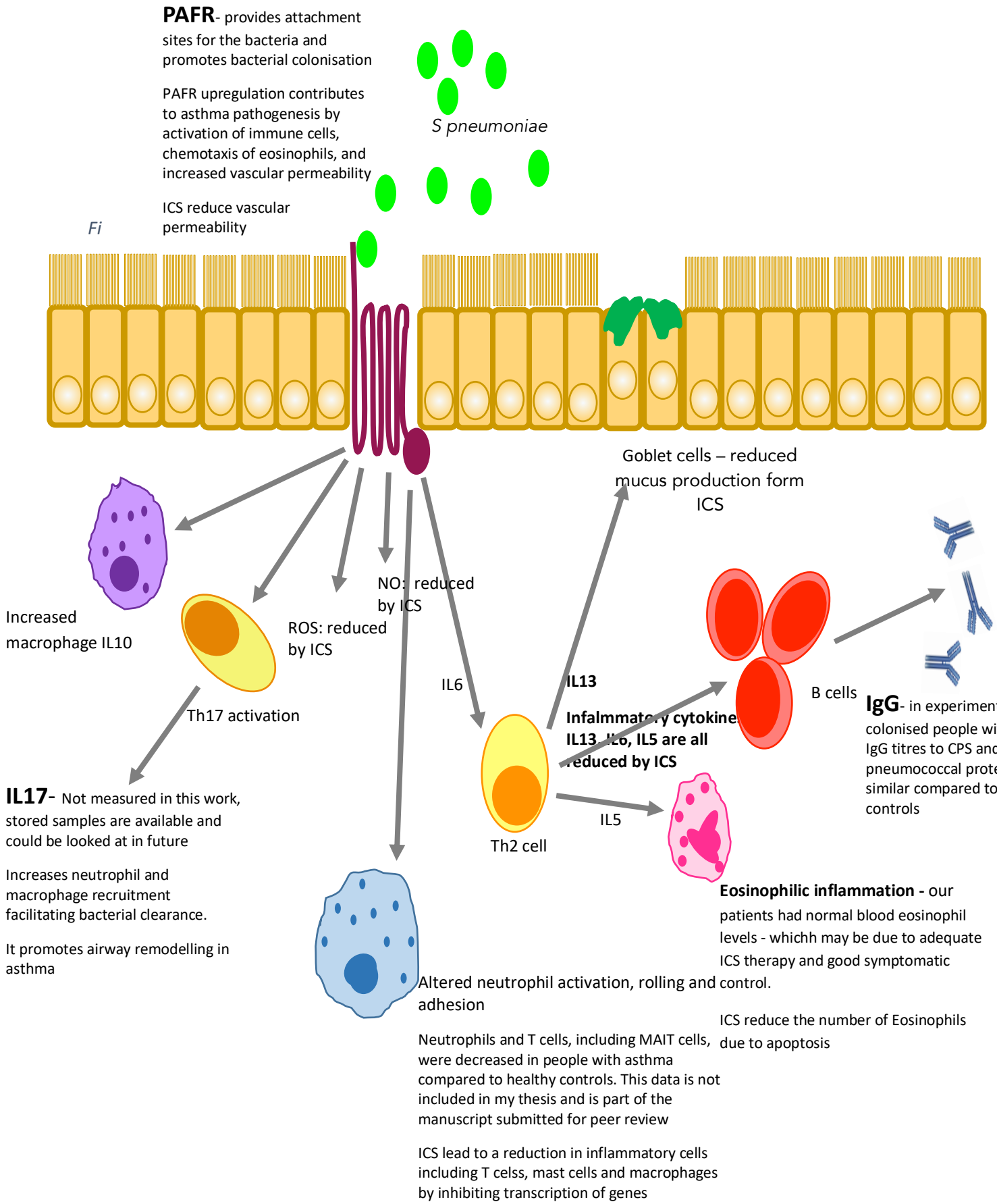

PAFR- provides attachment sites for the bacteria and promotes bacterial colonisation
PAFR upregulation contributes to asthma pathogenesis by activation of immune cells, chemotaxis of eosinophils, and increased vascular permeability
ICS reduce vascular permeability
S pneumoniae
Fi
Goblet cells – reduced mucus production form ICS
Increased macrophage IL10
ROS: reduced by ICS
NO: reduced by ICS
IL6
IL13
Infalmmatory cytokine IL13, IL6, IL5 are all reduced by ICS
B cells
IgG- in experimentally colonised people with as IgG titres to CPS and pneumococcal proteins is similar compared to heal controls
Th17 activation
IL17- Not measured in this work, stored samples are available and could be looked at in future
Increases neutrophil and macrophage recruitment facilitating bacterial clearance.
It promotes airway remodelling in asthma
Th2 cell
IL5
Eosinophilic inflammation - our patients had normal blood eosinophil levels - whichh may be due to adequate ICS therapy and good symptomatic control.
ICS reduce the number of Eosinophils due to apoptosis
Altered neutrophil activation, rolling and adhesion
Neutrophils and T cells, including MAIT cells, were decreased in people with asthma compared to healthy controls. This data is not included in my thesis and is part of the manuscript submitted for peer review
ICS lead to a reduction in inflammatory cells including T celss, mast cells and macrophages by inhibiting transcription of genes

Figure 21: Adapted from Figure 2 - Schematic diagram depicting the molecular interactions between S. pneumoniae and immune cells at the nasopharyngeal mucosa, with additions to include our findings

The Platelet activating factor receptor (PAFR) provides increased attachment sites for the bacteria, thereby facilitating colonisation, and upregulation leads to asthma pathophysiology – this may explain increase bacterial colonisation in people with asthma.

Interleukin (IL) 17 may be measured from stored samples later on – facilitates bacterial clearance following colonisation by recruitment of inflammatory cells and simultaneously contributes to asthma pathogenesis by promoting airway remodelling.

IL5 cause chemotaxis of inflammatory cells specially eosinophils to the submucosa and mucosa causing airway inflammation – eosinophils were suppressed in our cohort of participants with well controlled, moderate disease.

Serum IgG is important for protection against bacterial disease. When measured in a subset of our participants the response following colonisation was similar in people with asthma compared to healthy controls.

(NO – nitric oxide, ROS – reactive oxygen species)

6.5 BAL

BAL samples are helpful to study the mucosal immune responses in the lungs. These can aid research for developing diagnostic techniques and studying airway remodelling secondary to new therapies. It is important to have a simple method without the risk of cross infection. Our sampling method with single use bronchoscopes is safe and effective in healthy controls. I can now extend this to asthma participants from challenge studies. Bronchoscopy would be offered to participants with mild well controlled disease and then extended to those on the severe spectrum of disease. There are several ongoing industry sponsored trials using bronchoscopy to study changes in airway mucosa following therapy with monoclonal antibodies.

Possible areas that BAL sampling may address in asthma include:

1. Improve understanding of alveolar mucosal immune response - secondary to ICS therapy and as a result of the condition. This can be achieved by using BAL sampling in healthy controls on ICS as described above and performing BAL samples in asthma participants on high and low dose ICS.
2. Gain better knowledge of mucosal changes in asthma – such as airway remodelling, mucosal receptors, immune cells and antibodies present within the alveoli.
3. Study the effect of therapies aimed at reducing airway inflammation and remodelling. This may be carried out in patients suitable for therapy with monoclonal antibodies before initiation of treatment and after

6.6 Challenges and strengths

The main challenge I had to overcome was recruitment. Finding participants with objective evidence of asthma, on appropriate treatment and compliant with all our inclusion and exclusion criteria was a difficult task. To improve recruitment and complete the study I had to make several amendments as described in methods.

It is a strength of my study to investigate a well characterised cohort of asthma participants with detailed information on lung function, inflammation parameters, subjective and objective measures of control (ACT score and PEFR respectively), prescribed medication and smoking status, with follow up post challenge.

Using the challenge model to study experimental colonisation with information on the dose of exposure, time of onset and duration is also a strength of our work.

I have discussed the pathology of asthma a non-communicable disease and linked this to an infectious communicable disease, explaining how the two may be associated with each other.

Excluding severe asthma participants with low lung function on high dose of ICS may have altered colonisation outcome and is a weakness of my study. I was unable to include such individuals for safety reasons, due to an increased risk of exacerbation. However, I hypothesise for future studies that uncontrolled airway inflammation will lead to increased colonisation with rapid clearance. The effect of ICS at low, medium and high dose needs investigation in helathy volunteers with ethical approval. In addition measuring IL17 in participants with asthma may help address the part played by this cytokine in nasopharyngeal colonisation of pneumococcus.

Comparative data for the healthy controls was available from historic EHPC studies where participants were inoculated at different times of the year, and the serum blood samples were collected at different times. The seasonal variation may affect colonisation rates and density as reported in studies.

6.7 Future

This study demonstrates safe implementation of EHPC in people with mild to moderate asthma. This can be extended to people with more severe disease on high dose ICS, based on results of non-interventional study. The model can also inform future vaccination studies in healthy controls and people with asthma to study immune responses post vaccination. The BAL sampling method can be used in asthma to study alveolar immune responses, to develop new therapies, investigate vaccine

targets and asses the response of new therapies on airway remodelling. The effect of BMI in healthy population and asthma requires investigation.

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8 Appendices

Appendix A

Participant information sheet

Experimental Human Pneumococcal Challenge: Effect of asthma on immune response to pneumococcus

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 a bacterium 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 the 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 sepsis). These severe infections are very uncommon in healthy adults: about 50 cases in Liverpool per year. However, those with asthma 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 those with asthma. We have already studied this using more than 500 volunteers and found this type of study to be safe.

All of the volunteers we have studied so far have been “healthy volunteers”. In order to develop a vaccine that will protect those with asthma, we need to understand the immune responses in adults with asthma.

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 have:

- Mild, well controlled asthma
- Do not smoke
- Not had a life-threatening asthma attack
- Not in close contact with children under 5

Your participation will provide us with helpful information. We ensure that it is safe for you to take part and if we find any reason you may be at higher risk of infection, then we will not enrol you in the trial.

You will not be eligible if:

- You are aged more 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 disease, or lung disease due to smoking
- The study doctor thinks that a health condition, or medication means that you are at increased risk of infection
- If you are pregnant. (We would advise you to use contraception during the study)

What happens if I choose to take part?

1. Consent –we ask you to sign a consent form when you are sure you want to take part.

2. Taking samples – we take samples from the nose, throat and blood (see below). We also do breathing tests (described later in the leaflet). Again, this is to check that you are well enough to take part in the study and to confirm a diagnosis of asthma if not done previously.

3. Being given drops of pneumococcus in the nose - we put a few drops of liquid with a small number of bacteria in your nose.

4. Monitoring– we will ask you to contact us daily (by phone or text) to make sure you are well.

- We will ask you to complete a symptom questionnaire for seven days during the study. It asks simple questions such as do you have a cough, wheeze etc.
- We will also ask you to provide details of someone who can be contacted in an emergency, as mentioned in the consent form.

5. Monitoring visits – we take samples from your nose to see whether the bacteria are present and if your asthma is affected.

This study takes less than four weeks. After six to twelve months we will invite some participants to repeat this study.

A small number of participants may be allocated at random to receive drops of water in their nose rather than pneumococcus bacteria. This is called a placebo and will help us determine if people develop

symptoms (e.g. runny nose) because of the pneumococcus bacteria or simply as a reaction to our other study procedures. You will not know if you have received pneumococcus or a placebo until the end of the study and all participants will follow the same study protocol.

What kind of samples do you take?

- *Samples from the nose:*

1. ***Nasosorption****:* This collects cells from your nose we place a small piece of blotting paper inside your nostril for a few minutes.

2. ***Nasal probe***: We run a small plastic rod along the inside of each nostril.

3. ***Nasal wash****:* 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.

• ***Blood samples***: We take blood samples from a vein in your arm. We will never take more than 10 teaspoons (50 ml).

• ***Breathing tests***: These tests are part of asthma care and you may have had them done before:

1. ***Spirometry***: This is a basic breathing test which measures the amount of air that can be blown out of the lungs. This is done using a spirometer and you will be asked to take a deep breath in and blow into the spirometer as hard and fast as you can until your lungs are completely empty. This routine will be repeated to ensure the results are consistent. Depending on the results you may be given an inhaler (bronchodilator) and have the test repeated 15-20 minutes later to see if there is any improvement. This is called reversibility.

2. ***FeNO:*** This is a breathing test, done using a special machine. It involves blowing air out of the lungs into a machine at a steady rate. This test measures the inflammation in the lungs and is routinely used for diagnosis and monitoring of asthma in clinical practice.

3. ***Peak Expiratory Flow rate (PEFR):*** This is a measure of the fastest rate of air (airflow) that you can blow out of your lungs. It records airflow in litres per minute (L/min). It is measured using a peak flow meter which is a small device that you blow into. Participants will be given a peak flow meter and shown how to take a peak flow reading. It will be explained that the marker should be set to zero, take a deep breath and seal your lips around the mouthpiece and then blow as hard and as fast as you can into the device. They will be asked to note the reading on the chart provided. Each time we will ask you to check the reading three times and record the best of these three.

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. You may choose to gift your samples for future research. This may be used to study the DNA from your blood sample. This is mentioned in your consent form. If you choose not to donate your DNA you may still take part in the study.

What will happen at each study visit?

Initial Visit, Pre-screen and Screening appointments (spread over about two weeks)	We explain the study in detail, obtain your signed consent, ask some basic questions to ensure that you are eligible and do some breathing tests. We will also write to your GP to confirm some aspects of your medical history (e.g. which vaccinations you have had before) and inquire about asthma tests.

	At the next visit we will do some more test as detailed earlier, check your blood pressure and temperature, listen to your heart and lungs, breathing tests (FeNO) and blood tests. We will also give you a PEFR meter to measure and record your peak flow rates at home. If you are well enough to take part in the study, we do the throat swab, nasal samples and other blood tests. We then book your next appointments. If you cannot come in on a specific date, we can be flexible to accommodate you.

Between one to seven days after Visit 3:

Appointment for being given pneumococcus up the nose	We collect samples as described earlier. We 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 7 days a week 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. We advise following your personal asthma plan at all times and to seek medical help as necessary.***

Up to six visits over the next five weeks

Clinic Appointments on days 2, 7, 9 14, 22 and 29	At each visit, a number of samples will be taken which may include throat swab, nasosorption, nasal wash, nasal probe and blood tests

End of the first study	If our laboratory test finds that the pneumococcus bacteria stays in your nose, we will ask you to take a course of antibiotics to clear it. We may ask you to be in the re-challenge study.

What about the re-challenge?

We think that having small number of bacteria in your nose—even for a short time—might protect you against illness from this bacterium, 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 a few months have passed. Y*ou do not have to take part in the re-challenge if you do not want to*. These visits will take about 2 to 3 weeks.

What will happen at each visit of the re challenge??

Re-challenge study: pre-screen	We make sure you are still fit to take part in the study by repeating the questions and examination done at the start of the first study. We do the throat swab, nasal wash and blood test, and ask you to monitor PEFRs.

1-7 days later

Appointment for 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 for 7 days

Clinic appointments days 2, 7 and 14	Samples as explained earlier.
Visit 5: End of the study	At the end of the re-challenge, after a final throat swab and nasal wash, 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 monitor them closely. We have experience of using this model safely in more than 500 healthy volunteers with no serious side effects.

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. All other tests are standard for asthma. If these results are outside the normal values, we will inform your GP.

What if there is a problem?

You can contact the research team 7 days a week by phone to answer your questions and arrange to see you as necessary. We would recommend using and following your personal asthma action plan at all times.

What if I wish to complain?

If you wish to complain about any aspect of the study, you can contact the study doctor. You may also use the Royal Liverpool University Hospital's independent complaints department (contact number 01517064903). Making a complaint 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 wish 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, your 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 may be looked at by those who monitor the research.

Are there any benefits to 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 the medical care of people with asthma.

How much will I be paid?

The money you are paid is compensation for inconvenience, loss of income and your time. 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. If you receive a placebo instead of pneumococcus the payment is unchanged. Our payments are listed below:

First Study	**Visit length**	
Initial Visit	45 min	-
Pre-screen appointment	60 min	£40
Screening appointment	30 min	£30
Inoculation with pneumococcus appointment. 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
Clinic appointments for samples on days 2 and 7	30 min	£20
Clinic appointment for samples on day 9	20 min	£15
Clinic appointments for samples on days 14 and 22 (*not all participants will be called for day 22)*	15 min	£10
Clinic appointment for samples on day 29	25 min	£20

Re-challenge		
Re-challenge study pre-screen.	45 min	£30
Inoculation with pneumococcus appointment. 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 mins	£50
Re challenge study clinic visit day 2, 7 and 14	20 min	£15

After you have had time to read the information leaflet a member of our team will discuss the study 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

Contact details

General questions: please contact the research team on 0151 706 3381 during normal working hours. Web site: *******

Emergency contact details at any time 7 days a week:

Mobile: 07595463833

Royal Liverpool Hospital Switchboard: 0151 706 2000.

Please ask for the study team ("EHPC team" or "Pneumonia research team")

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 and The Bill and Melinda Gates Foundation. The research has been reviewed for scientific content by an external panel.

Royal Liverpool University Hospital Independent Complaints Department 0151 706 4903

The National Research Ethics Service Committee (XXXX) has reviewed the study and given approval for it to take place.

Appendix B

Invitation letter from Primary Care

To be printed on GP Letter Headed Paper>

<Insert Patient Title and Name>

<Insert Patient Address1>

<Insert Date>

Dear <Insert Patient Title and Name>

We want to invite you to be part of a research study.

A group of researchers from the Liverpool School of Tropical Medicine, is trying to find out why people with asthma are more likely to get pneumonia. The research is done at the Royal Liverpool University Hospital.

As your GP, we are not linked with the study. But we are helping to give details of the research to people who might be able to take part. We will not pass any of your details to the research team: your details are confidential.

The details are explained in the participant information sheet attached with this letter. This study has been reviewed and approved by the National Research Ethics Service Committee.

Whether you agree to take part of not is entirely up to you. It makes no difference to the care you receive at the GP or anywhere else.

If you do take part, you will be paid for your time and inconvenience.

To find out more about the study please:

- Call 0151 706 4856
- Text 2VOL to 8802

Email: 2volresearch@lstmed.ac.uk

Website: www.lstmed.ac.uk/pneumoniavaccine

Yours Sincerely,

Dr <Insert GP Title and Name>

Insert Name of Practice >

Appendix C

GP questionnaire

Dear Dr _____

Your patient _____,

DOB _____ has agreed to participate in pneumonia vaccine research : Experimental Human Pneumococcal Carriage: Effect of asthma on immune response to pneumococcus

This study involves inoculation with pneumococcal bacteria and monitoring over 4 weeks for experimental carriage of the bacteria.

We appreciate you are busy and would be grateful if, you would please complete, sign, date and stamp the attached forms or print a relevant summary of their records so that the participant may commence the study as early as next week if possible. This will ensure safety of the participant and provide the necessary information to check that your patient is eligible to participate. Please return the completed forms by fax to Catherine Lowe on (0151) 706 4856.

Thank you.

If you have any concerns or questions regarding this study, please contact us on 0151 706 3381

Sister Catherine Lowe

catherine.lowe@rlbuht.nhs.uk

Tel (0151) 706 3381

Fax (0151) 706 4856

Mobile: 07912053981

Medical Report to be completed by the General Practitioner

General Medical History	Please Circle	Please Provide Details
Is the above person registered with your practice and do you have their full medical record for the past 12 months?	YES NO	A minimum of 12 months history is required before participants can take part in this research. Please contact us if the notes are not available but request the records so the questionnaire can be completed as soon as they are received.
Does the patient have asthma?	YES NO	If YES, please provide a copy of any diagnostic tests done.

		Details
Does the patient have an immunosuppressive condition? (Including diabetes, active malignancy, immunosuppression secondary to medications etc)	YES NO	_____
Has the patient been treated for an asthma exacerbation in the last twelve months with oral corticosteroids?	YES NO	If YES, please state how many times in the last twelve months?
Is the patient allergic to penicillin?	YES NO	
Is the patient prescribed any regular medications?	YES NO	Details (or please attach copy of latest prescription)

		Details
Is there objective evidence that the patient has previously had pneumococcal disease? (Culture-proven or molecular diagnosis)	YES NO	_____
		Vaccine and date
Has the patient received a pneumococcal vaccine at any point? (Prevnar/ pneumovax)	YES NO	_____
		Why?
Are you aware of any reason why this patient might **not** be considered suitable for clinical trial studies?	YES NO	_____

Appendix D

Asthma Action Plan

MY ASTHMA TRIGGERS

Taking my asthma medicine each day will help reduce my tion to these triggers. Avoiding them where possible will also help.

MY ASTHMA REVIEW

I should have at least one routine asthma review every year.

I bring:

y action plan to see if it needs updating

y inhaler and spacer to check I'm using them in the best way

y questions about my asthma and how to cope with it.

asthma review date:

__ / __ __ / __ __ __ __

asthma nurse contact

ne:

ne number:

-of-hours contact number

your GP surgery who to call when they are closed)

ne:

ne number:

t more advice & support from Asthma UK:

Speak to a specialist ma nurse about aging your asthma on: 00 222 5800

Get news, advice and download information packs at: www.asthma.org.uk

Health & care information you can trust

80216 © 2016 Asthma UK registered charity number in England and s 802364 and in Scotland SC039322.

reviewed and updated 2016; next review 2019.

ns et al; Factors associated with hospital admissions and repeat emergency rtment visits for adults with asthma; Thorax 2000;55:566–573

Use it, don't lose it!

Your action plan is a personal guide to help you stay on top of your asthma. Once you have created one with your GP or asthma nurse, it can help you stay as well as possible.

People who use their action plans are four times less likely to end up in hospital because of their asthma.

Your action plan will only work at its best to help keep you healthy if you:

1. **Put it somewhere easy for you and your family to find** – you could try your fridge door, the back of your front door, or your bedside table. Try taking a photo and keeping it on your mobile phone or tablet.
2. **Check in with it regularly** – put a note on your calendar, or a reminder on your mobile to read it through once a month. How are you getting along with your day-to-day asthma medicines? Are you having any asthma symptoms? Are you clear about what to do?
3. **Keep a copy near you** – save a photo on your phone or as your screensaver. Or keep a leaflet in your bag, desk or car glove box.
4. **Give a copy of your action plan or share a photo of it with a key family member or friend** – ask them to read it. Talk to them about your usual asthma symptoms so they can help you notice if they start. Help them know what to do in an emergency.
5. **Take it to every healthcare appointment – including A&E/consultant.** Ask your GP or asthma nurse to update it if any of their advice for you changes. Ask them for tips if you're finding it hard to take your medicines as prescribed.

THE STEP-BY-STEP GUIDE THAT HELPS YOU STAY ON TOP OF YOUR ASTHMA

Your asthma action plan

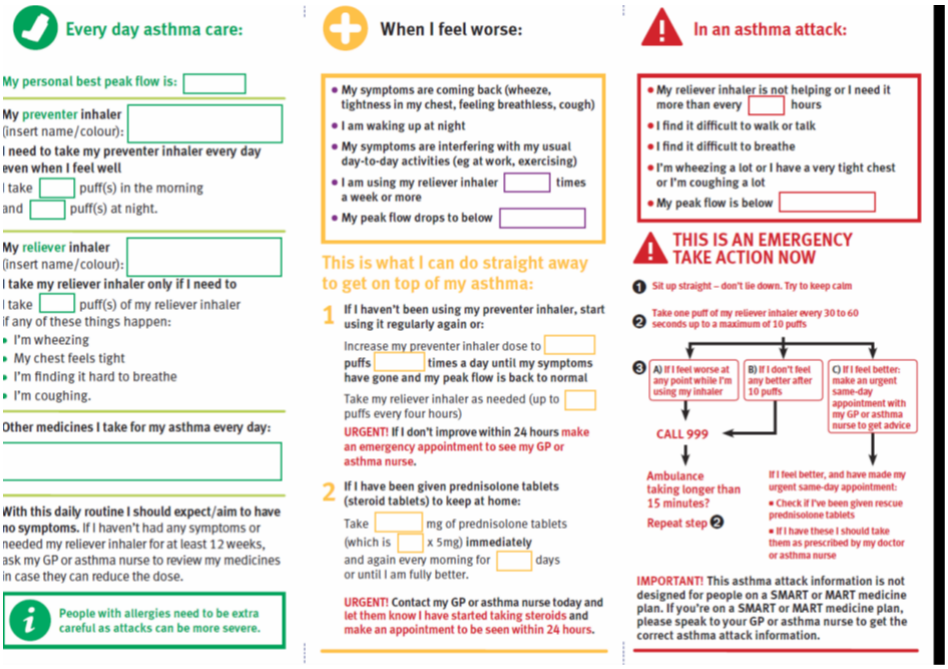
FILL THIS IN WITH YOUR GP OR ASTHMA NURSE

If you use a written asthma action plan you are **four times less likely** to be admitted to hospital for your asthma.*

Name and date:

asthma UK

Any asthma questions? Call our friendly helpline nurses 0300 222 5800 (9am – 5pm; Mon – Fri) www.asthma.org.uk



Every day asthma care:

My personal best peak flow is:

My preventer inhaler (insert name/colour):

I need to take my preventer inhaler every day even when I feel well

I take ___ puff(s) in the morning and ___ puff(s) at night.

My reliever inhaler (insert name/colour):

I take my reliever inhaler only if I need to

I take ___ puff(s) of my reliever inhaler if any of these things happen:

- I'm wheezing
- My chest feels tight
- I'm finding it hard to breathe
- I'm coughing.

Other medicines I take for my asthma every day:

With this daily routine I should expect/aim to have no symptoms. If I haven't had any symptoms or needed my reliever inhaler for at least 12 weeks, ask my GP or asthma nurse to review my medicines in case they can reduce the dose.

People with allergies need to be extra careful as attacks can be more severe.

When I feel worse:

- My symptoms are coming back (wheeze, tightness in my chest, feeling breathless, cough)
- I am waking up at night
- My symptoms are interfering with my usual day-to-day activities (eg at work, exercising)
- I am using my reliever inhaler ___ times a week or more
- My peak flow drops to below ___

This is what I can do straight away to get on top of my asthma:

1. **If I haven't been using my preventer inhaler, start using it regularly again or:**

   Increase my preventer inhaler dose to ___ puffs ___ times a day until my symptoms have gone and my peak flow is back to normal

   Take my reliever inhaler as needed (up to ___ puffs every four hours)

   URGENT! **If I don't improve within 24 hours** make an emergency appointment to see my GP or asthma nurse.

2. **If I have been given prednisolone tablets (steroid tablets) to keep at home:**

   Take ___ mg of prednisolone tablets (which is ___ x 5mg) **immediately** and again every morning for ___ days or until I am fully better.

   URGENT! Contact my GP or asthma nurse today and let them know I have started taking steroids and make an appointment to be seen within 24 hours.

In an asthma attack:

- My reliever inhaler is not helping or I need it more than every ___ hours
- I find it difficult to walk or talk
- I find it difficult to breathe
- I'm wheezing a lot or I have a very tight chest or I'm coughing a lot
- My peak flow is below ___

THIS IS AN EMERGENCY TAKE ACTION NOW

1. Sit up straight – don't lie down. Try to keep calm
2. Take one puff of my reliever inhaler every 30 to 60 seconds up to a maximum of 10 puffs
3. A) If I feel worse at any point while I'm using my inhaler → CALL 999 → Ambulance taking longer than 15 minutes? Repeat step 2
   B) If I don't feel any better after 10 puffs → CALL 999 → Ambulance taking longer than 15 minutes? Repeat step 2
   C) If I feel better: make an urgent same-day appointment with my GP or asthma nurse to get advice

   If I feel better, and have made my urgent same-day appointment:
   - Check if I've been given rescue prednisolone tablets
   - If I have these I should take them as prescribed by my doctor or asthma nurse

IMPORTANT! This asthma attack information is not designed for people on a SMART or MART medicine plan. If you're on a SMART or MART medicine plan, please speak to your GP or asthma nurse to get the correct asthma attack information.

Appendix E

PEFR Diary

Week	Date	Time	1	2	3
WEEK ONE		AM			
		PM			
		AM			
		PM			
		AM			
		PM			
		AM			
		PM			
		AM			
		PM			
		AM			
		PM			
		AM			
		PM			
		PM			

Appendix F

PEFR variability calculator

Two-Week Peak Flow Variability Calculator

INITIALS:											Reviewed by:				
PATIENT ID:											Signed:				
DATE STARTED:											Date:				
	Day														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
BEST of 3 AM PEF															
BEST of 3 PM PEF															
PEF [Mean]	#DIV/0!														
PEF [Highest]	0	This is the highest PEF in all of the 28 readings above													
PEF [Lowest]	0	This is the lowest PEF in all of the 28 readings above													
															PEF Variability
Mean diurnal peak expiratory flow (PEF) variability calculated by ((PEF[highest]-PEF[lowest]) / (PEF[mean]))*100															#DIV/0! %

Four Week Peak Flow Variability Calculator

Initials: _____

Patient ID:

Date Started:

PEF [MEAN]
PEF [Highest]
PEF [Lowest]
PEF Variability

Day	Best of 3 AM	Best of 3 PM
1		
2		
3		
4		
5		
6		
7		
8		
9		
10		
11		
12		
13		
14		
15		
16		
17		
18		
19		
20		
21		
22		
23		
24		
25		

Appendix G

Asthma Control Test

Asthma UK is the only charity dedicated to the health and well-being of the 5.2 million people in the UK with asthma. By taking control of their asthma, most people's day-to-day lives should be free from disruption such as troubled sleep or not being able to exercise.

Asthma Control Test™

Why take the Asthma Control Test™?

The Asthma Control Test™ will provide you with a snapshot of how well your asthma has been controlled over the last four weeks, giving you a simple score out of 25. Asthma symptoms can vary from month to month, so it is worth keeping the test handy to see if your score changes. You can also share your results with your doctor or asthma nurse to help explain just how your asthma affects you.

Are you in control of your asthma? Or is your asthma in control of you? Here's how to find out

Step 1: Read each question below carefully, circle your score and write it in the box.
Step 2: Add up each of your five scores to get your total Asthma Control Test™ score.
Step 3: Use the score guide to learn how well you are controlling your asthma.

Q1 During the **past 4 weeks,** how often did your asthma prevent you from getting as much done at work, school or home? — Score:

All of the time	Most of the time	Some of the time	A little of the time	None of the time
1	2	3	4	5

Q2 During the **past 4 weeks,** how often have you had shortness of breath? — Score:

More than once a day	Once a day	3-6 times a week	1-2 times a week	Not at all
1	2	3	4	5

Q3 During the **past 4 weeks,** how often did your asthma symptoms (wheezing, coughing, chest tightness, shortness of breath) wake you up at night or earlier than usual in the morning? — Score:

4 or more times a week	2-3 nights a week	Once a week	Once or twice	Not at all
1	2	3	4	5

Q4 During the **past 4 weeks,** how often have you used your reliever inhaler (usually blue)? — Score:

3 or more times a day	1-2 times a day	2-3 times a week	Once a week or less	Not at all
1	2	3	4	5

Q5 How would you rate your **asthma control** during the **past 4 weeks?** — Score:

Not controlled	Poorly controlled	Somewhat controlled	Well controlled	Completely controlled
1	2	3	4	5

Total Score

What does your score mean?

Score: 25 – WELL DONE
- Your asthma appears to have been UNDER CONTROL over the last 4 weeks.
- However, if you are experiencing any problems with your asthma, you should see your doctor or nurse.

Score: 20 to 24 – ON TARGET
- Your asthma appears to have been REASONABLY WELL CONTROLLED during the past 4 weeks.
- However, if you are experiencing symptoms your doctor or nurse may be able to help you.

Score: less than 20 – OFF TARGET
- Your asthma may NOT HAVE BEEN CONTROLLED during the past 4 weeks.
- Your doctor or nurse can recommend an asthma action plan to help improve your asthma control.

What can you do now?

Like many other people in the UK, it is possible that your asthma could have less impact on your everyday life. You can get a free pack full of information about how to take control of your asthma, including an action plan to fill in with your doctor or asthma nurse, from Asthma UK.

"US English version modified for use in UK". The production of this leaflet has been supported by GlaxoSmithKline

Registered charity number 802364

Appendix H

Participant Inoculation Leaflet

EXPERIMENTAL HUMAN PNEUMOCCAL CARRIAGE:

THE EFFECT OF ASTHMA ON IMMUNE RESPONSE TO PNEUMOCOCCUS

Information Sheet

EMERGENCY RESEARCH TEAM

7 days a week – phone number

OR CALL

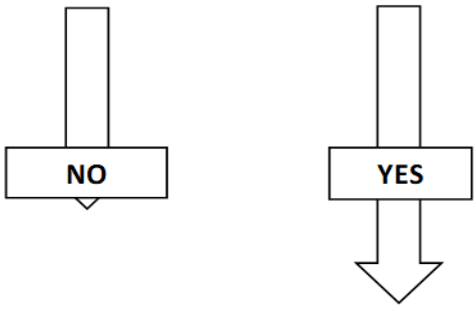
0151 706 2000

Hospital Switchboard

Ask for XXXXXXXXXXXX

Do you have any of the following?

- Fever (>37.5 ˚C)
- Headache
- Rash
- Drowsiness
- Cough



Contact Research Team by 12noon every day for

Call the Emergency Research Team on XXXXX XXX XXX 7 days a week

Or Call 0151 706 2000 and Ask for XXXXXXXXXXX

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

- New rash
- Drowsiness
- Cough
- Earache
- Wheeze
- Shortness of breath

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 I

Daily Symptom Log

In the following table, please mark one circle in each row according how much your symptoms have bothered you in the last 24 hours. ***We expect that the majority of our patients will not experience any of these symptoms.***

In the past 24 hours, how much have you been bothered by?	Did not have symptom	Had symptom, but not bothered	A little bothered	Moderately bothered	Quite a bit bothered	Ext bo
Sneezing	○	○	○	○	○	
Runny nose	○	○	○	○	○	
Congestion/stuffiness	○	○	○	○	○	
Itchy nose	○	○	○	○	○	
Post-nasal drip (nasal secretions running down back of throat)	○	○	○	○	○	
Total nasal symptoms	○	○	○	○	○	
Coughing	○	○	○	○	○	
Chest pains	○	○	○	○	○	
Shortness of breath	○	○	○	○	○	
Coughing up phlegm	○	○	○	○	○	
Sweating	○	○	○	○	○	
Chills	○	○	○	○	○	
Headache	○	○	○	○	○	

Appendix J

Participant trial Business card

CLINICAL TRIAL PARTICIPANT

Trial No. __5173_____ Participant No. _____

Study Title: Experimental Human Pneumococcal Carriage: Effect of asthma on immune response to pneumococcus

Participant Name _____

Date of Birth _____

This volunteer is part of a study and has been inoculated with live bacteria in the nose.

For information about the study in case of an emergency or accident, please contact:

Name _ Respiratory Research Team_____

Telephone _____

Keep this card with you for the duration of the study

Appendix K

Reminder Letter to participants from GP

<To be printed on GP Letter Headed Paper>

<Insert Patient Title and Name>

<Insert Patient Address4> <Insert Date>

Dear <Insert Patient Title and Name>

We want to invite you to be part of a research study. Please accept our apologies if you have already responded to the initial letter sent out by the practice for this study.

A group of researchers from the Liverpool School of Tropical Medicine, is trying to find out why people with asthma are more likely to get pneumonia. The research is done at the Royal Liverpool University Hospital.

As your GP, we are not linked with the study. But we are helping to give details of the research to people who might be able to take part. We will not pass any of your details to the research team: your details are confidential.

The details are explained in the participant information sheet attached with this letter. This study has been reviewed and approved by the National Research Ethics Service Committee.

Whether you agree to take part of not is entirely up to you. It makes no difference to the care you receive at the GP or anywhere else.

If you do take part, you will be paid for your time and inconvenience. You do not have to reply or respond to this letter if you did so the first time.

To find out more about the study please:

- Call 0151 706 3381
- Text 2VOL to 8802

Email: 2volresearch@lstmed.ac.uk

Website: www.lstmed.ac.uk/pneumoniavaccine

Yours Sincerely,

<Insert GP Signature>

Dr <Insert GP Title and Name>

< Insert Name of Practice >