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University of Southampton
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Airway bacteria and their relevance to disease severity in asthma

by
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Abstract

Asthma is a heterogeneous condition resulting from a complex interaction of genetic and environmental factors leading to airway hyperreactivity, reversible bronchoconstriction and chronic airway inflammation. Little is known about the role of chronic bacterial colonization in the underlying inflammation and how it can alter disease phenotype. In COPD, airway colonization with potentially pathogenic microorganisms (PPMs) leads to neutrophilic airways inflammation, increased frequency of exacerbation, worsening symptom scores and an accelerated decline in lung function. The Gram positive coccus *Staphylococcus aureus* is known to produce enterotoxins (SAEs) which can act as superantigens leading to an exaggerated T lymphocyte response and has been linked to steroid resistance in asthma.

Hypotheses tested were: (1) Bacterial colonization patterns differ between the asthmatic and healthy lower airway. (2) Airway colonization with potentially pathogenic bacterial species contributes to asthma severity and alters phenotype. (3) *S. aureus* colonizes the lower airway in asthma and contributes to treatment resistance through the action of superantigens.

The non-culture based, molecular microbiological technique of terminal restriction fragment length polymorphism profiling (T-RFLP) was used to demonstrate the patterns of airway bacterial colonization in severe asthmatics, mild asthmatics and healthy controls.

We have demonstrated that T-RFLP profiling of respiratory specimens is a more sensitive tool than standard respiratory culture in detecting potentially pathogenic bacterial species and is able to give a relative abundance of each species compared to the total bacterial load within the specimen.

Bronchoalveolar lavage (BAL) was used to sample the distal lower airways of volunteers and demonstrated a more diverse colonizing flora in severe asthmatics than mild asthmatics ($p=0.001$) and healthy controls ($p=0.029$). PPMs such as *Haemophilus sp.* and *Streptococcal sp*. were more frequently detected in severe asthma than in mild asthma ($p<0.001$) and healthy controls ($p=0.004$). The total abundance of PPMs as a percentage of total bacterial load was significantly higher in severe asthmatic BAL than mild asthmatic ($p<0.001$) and healthy subjects ($p=0.004$). Within the severe asthma group total BAL PPM abundance correlated with neutrophil counts, IL-8 and worsening FEV_1.

Induced sputum was used to sample the central, proximal, lower airways and demonstrated lower species diversity in severe asthma than in mild asthma

(p=0.012) and healthy controls (p=0.005). Within the severe asthma group, when the most abundant species within induced sputum was *Haemophilus sp.*, *Streptococcal sp*. or *Moraxella catarrhalis*, significantly worse FEV_1 (p=0.025), higher sputum neutrophil counts (p=0.001) and longer disease duration were seen. Dominance of these species within induced sputum was associated with a neutrophilic asthma phenotype (p=0.014).

Evidence of the effect of *S. aureus* superantigens was investigated with BAL T cell receptor (TCR) Vβ profiling. BAL *S. aureus* colonization was demonstrated in 38% of severe asthmatic subjects and was associated with higher Vβ 5.3 (p=0.001), Vβ 5.1 p=0.01), Vβ 13.1 (p<0.001) and Vβ 2 (p=0.022) positive CD4 T cells. This skewing of TCR Vβ populations provides a surrogate marker for the action of SAEs which may be relevant in the development of steroid resistant disease.

The findings of clinically relevant PPM airway colonization in severe asthma provide an opportunity for new targeted therapies in a group of patients where there are currently few therapeutic options.

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1 Introduction

1.1 The Global Burden of Asthma

It is estimated that 300 million people worldwide are affected by asthma. In Western Europe, where the prevalence of atopic disease is highest, we saw increasing incidence of asthma over the last decades of the 20th century[1]. Asthma UK currently estimates there are 5.2 million children and adults in the UK with physician diagnosed asthma[2]. The 2001 Health Survey for England estimated 15% of adults and 21% of children have, at some point, been given a diagnosis of asthma by their doctor [3].

1.2 Severe Asthma

The vast majority of asthmatics have mild or moderate disease. They tend to have a strong atopic basis to their disease with T Helper 2 (TH-2) lymphocyte driven inflammation of the airways, bronchial hyperresponsiveness and variable airflow obstruction which is sensitive to inhaled corticosteroid treatment.

The term severe or corticosteroid-refractory asthma however, can be used to describe patients whose symptoms remain difficult to control despite extensive re-evaluation of their diagnosis and management, followed by a six month period of observation by an asthma specialist[4].

Severe asthma represents 5-10% of asthma patients but is associated with a disproportionately large healthcare utilisation and around 40% of total costs[5;6].

The causes of steroid resistance in persistent asthma are poorly understood. It is recognised that this group of patients represents a heterogeneous group. Distinct subphenotypes can be defined according to clinical, aetiological, physiological, or pathophysiological characteristics[7].

1.3 The Role of Bacterial Colonization in Persistent Asthma

Up until the mid 20th century, asthma was believed to be causally related to bacterial infection[8;9]. Interest in the infectious aetiology of asthma diminished with greater understanding of non-infectious airway inflammation, until the emergence of the role of viral infection in precipitating exacerbations of the disease in the early 1990s [10;11].

Since then, there has been a resurgence of interest in bacterial infection, particularly with atypical organisms such as *Chlamydophilia pneumoniae* and *Mycoplasma pneumoniae.*

Martin *et al.* performed bronchoscopies on 55 chronic stable asthmatics and 11 normal control subjects using PCR to detect atypical organisms. They reported significantly higher incidence of PCR positivity for *M. pneumoniae* (n=25) and *C. pneumoniae* (n=6) in bronchial biopsies, lavage fluid, nasal biopsy or blood in the asthma group than in the normal controls (n=1)[12].

Multiple serological studies have shown an association with these organisms, particularly *C. pneumoniae*, and asthma[13;14]. Increasing *C. pneumoniae* heat shock protein 60 IgA antibodies are associated with decline in FEV_1 suggesting a dose response relationship[15]. IgG antibodies to *C. pneumoniae* were also found to be associated with worsening airways obstruction by Black *et al.* In the same study IgA antibodies to *C. pneumoniae* was associated with higher daytime asthma symptom scores. Both IgG and IgA *C. pneumoniae* antibodies were associated with the need for high dose inhaled corticosteroid therapy.[16].

The cytokine response to infection with atypical respiratory pathogens certainly adds biological plausibility to the hypothesis that these organisms are involved in asthma pathogenesis. However no studies have proved causation and the specificity of the tests for these organisms has prevented detection of other potentially pathogenic organisms that may co-colonize the asthmatic airway. Therefore, more recently, research into the relevance of bacterial colonization in stable, persistent asthma has failed to move beyond the search for a link to infection with these atypical organisms.

1.4 Potentially Pathogenic Bacteria

The advent of molecular microbiological techniques to identify bacterial DNA in respiratory specimens has led to a greater understanding of the diversity of species to be found within the lower airways of patients with respiratory disease [17].

The identification of bacteria in the lower airways will lead to the question of whether a species represents harmless colonization or has the potential for infection or immunological effect. For this purpose species can be divided into potentially pathogenic micro-organisms (PPM) or non-potentially pathogenic micro-organisms based on the bacterial species' intrinsic pathogenicity within the human airway[18]. Therefore PPMs include *S. pneumoniae*, *M. catarrhalis*, *H.*

influenzae, *H. parainfluenzae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae.*

Non-PPMs such as *Streptococcus viridans, Neisseria spp., Candida spp., Corynebacterium spp., Enterococcus spp.*, and coagulase-negative *Staphylococcus* are the normal flora of the oropharynx or gastrointestinal tract which, in the immunocompetent host do not cause respiratory infection.

1.5 The role of bacterial colonization in stable COPD

Colonization with potentially pathogenic micro-organisms (PPMs) (*H. influenzae*, *S. pneumoniae*, *M. catarrhalis*, *H. parainfluenzae*, *S. aureus*, *P. aeruginosa* and *K. pneumoniae*) in stable COPD is already recognised as a significant contributor to disease pathogenesis. Bacterial colonization has been linked to increased airway inflammation , accelerated lung function decline and increased frequency of exacerbation[19-21].

In a group of patients with moderate-severe COPD, *H. influenzae, M. catarrhalis* and *S. pneumoniae* colonization of induced sputum was associated with higher levels of inflammatory markers (Interleukin-8, leukotriene B4, Tumour Necrosis Factor α and neutrophil elastase), a trend towards higher sputum neutrophil differential counts, significantly higher plasma fibrinogen levels and worse quality of life (as assessed by the St George's Respiratory Questionnaire), when compared to patients colonized with non-pathogenic bacteria[19].

Similar results were seen when bronchoalveolar lavage was performed on ex-smokers with stable COPD, ex-smokers without COPD and healthy non-smokers [22]. Colonization with potentially pathogenic bacteria was seen in 34% of the COPD group, 0% ex-smokers and 6.7% of non-smokers. PPM colonization in COPD was associated with significantly higher absolute neutrophil counts, IL-8, active matrix metalloproteinase-9 and endotoxin (a Gram-negative cell membrane protein) than in non-colonized COPD subjects.

1.6 Studying Bacterial Colonization in the Lower Airways

Studies investigating the association between bacterial infection and asthma are hampered firstly by the difficulties of accessing the lower airways, and secondly, by the difficulties in detection of organisms.

Standardised protocols for the induction of sputum in severe asthma have provided a safe, non-invasive alternative to brochoscopic lavage for sampling the lower airways[23]. Sputum induction inevitably leads to contamination of samples by

squamous epithelial cells and oropharyngeal bacteria during expectoration[24]. Attempts can be made to minimise salivary contamination either by using protocols which separate saliva and sputum[25] or by separating viscid expectorated sputum from saliva during processing which has been shown to significantly lower squamous cell contamination[26].

Samples containing less than 25% squamous cells culture significantly fewer bacterial species, and more closely resemble transtracheal aspirate findings[27], suggesting induced sputa with low levels of salivary contamination can accurately represent the colonising flora of the lower airway.

However, sputum induction samples the central airways[28]. In order to sample the more peripheral, small airways, more invasive bronchoscopic methods are required either by bronchoalveolar lavage or protected brush specimen.

Once obtained, the method of detection of bacterial colonization in clinical samples may be crucial. In the clinical setting bacterial pathogens are characterised through the prior culture of species on selective media[29]. However, the use of selective growth conditions requires assumptions about the pathogens expected to be present within a sample. Cultivable bacteria represent only 0.1 - 20% of species within microbial communities[30].

Therefore traditional methods of cultivation on selective media lead to a “culture bias” where quantification of bacterial load is difficult and the detection of unculturable or more fastidious species is impossible. Furthermore the cultivation of only viable colonies on selective media cannot give a true representation of the complex bacterial communities present within the airways.

An alternative approach to identification of species within clinical samples is by assessing extracted nucleic acids. Polymerase chain reaction assays are able to detect specific bacterial pathogens, however, more recently, terminal restriction fragment length polymorphism (T-RFLP) profiling has been developed to characterise complex microbial communities[31;32].

This method exploits the 16S ribosomal RNA gene sequence present in all bacteria but unique to each bacterial species. Nucleic acids can be extracted from clinical samples and 16S ribosomal sequences amplified. Sequences can then be digested using a restriction endonuclease, producing rRNA gene fragments lengths differing for each species. The ribosomal fragments are then separated by

gel electrophoresis to form a T-RFLP profile of the diversity of bacterial species within the specimen.

This technique has previously been shown to be both reproducible and effective at characterising microbial diversity in sputum and bronchoalveolar lavage from patients with cystic fibrosis[33] and COPD[17], but has never been applied to the study of bacterial colonization in chronic persistent asthma.

1.7 The role of the innate immune system in asthma

Asthma is predominately a T Hepler 2 (TH-2) lymphocyte driven disease, with TH-2 cytokines mediating eosinophilic airway inflammation[34]. However the innate immune system may also have a role to play in the ongoing airway inflammation of a subset of severe asthmatics with neutrophilic airway inflammation.

Phagocytic cells within the airways are able to recognize highly conserved bacterial motifs known as pathogen-associated molecular patterns (PAMPS) leading to cell activation and a pro-inflammatory response. Examples of PAMPs are lipopolysaccharides (LPS) present in the outer cell membrane of Gram-negative bacteria, and lipoteichoic acid (LTA), a major surface associated adhesion molecule of Gram-positive bacterial cell walls.

Pattern recognition receptors such as Toll-like receptors (TLRs) can be activated by bacterial lipopolysaccharides (LPS) leading to a pro-inflammatory cytokine release of tumour necrosis factor α, IL-1β and IL-8[35;36].

Simpson *et al.* induced sputum from stable asthmatics and divided them by the results of differential cell counts into subjects with neutrophilic asthma (> 61% sputum neutrophils), eosinophilic asthma (eosinophils > 1%, neutrophils<61%) or paucigranulocytic asthma (eosinophils<1%, neutrophils <61%). The neutrophilic asthma group demonstrated increased expression of TLR2, TLR4 and CD14 receptors as well as the cytokines IL-8 and IL-1β. This would suggest activation of the innate immune system. 3 of the 7 neutrophilic asthma group grew *Haemophilus influenzae* on culture of induced sputum and this group showed significantly higher levels of sputum endotoxin (LPS), suggesting bacterial colonization may be the underlying driver for their airway inflammation[37].

LPS challenge to the nasal mucosa of atopic individuals leads to an increase in the eosinophils in nasal lavage. An effect not seen in non-atopic subjects[38].

It may be the case that exposure to endotoxins can both prevent and cause asthma depending on timing of exposure, dose and genetic predisposition [39]. The hygiene hypothesis suggests endotoxin exposure in early life may reduce chance of developing asthma in later life[40;41]. Exposure to airborne endotoxin is associated with an enhanced TH-1 response and tolerance to allergen. However, exposure in later life when asthma is established may lead to increased severity of symptoms, increased susceptibility to viral infection and worsening of airways obstruction[35].

Studies linking endotoxin exposure with a reduced risk of allergic sensitization or atopic asthma[42;43] do not attempt to identify the bacterial source of the endotoxin. Endotoxin from different Gram-negative species differentially affect CD14 and TLR expression as well as secondary LPS-associated IL-6 and TNF-α responses[44].

This would suggest certain species have a more immunogenic or inflammatory effect through this innate immune activation.

The effect of Gram-positive organism cell membrane proteins such as LTA have not been as well studied as the effect of LPS. However LTA is known to activate macrophages stimulating release of IL-1β, TNF-α, IL-12, IL-6, IL-8 and inducible nitric oxide synthetase[45]. Bronchoscopic instillation of LTA into healthy volunteers leads to an increased, localized inflammatory response with increased TNFα and influx of neutrophils[46].

Figure 1 Innate immune response to bacterial PAMPs

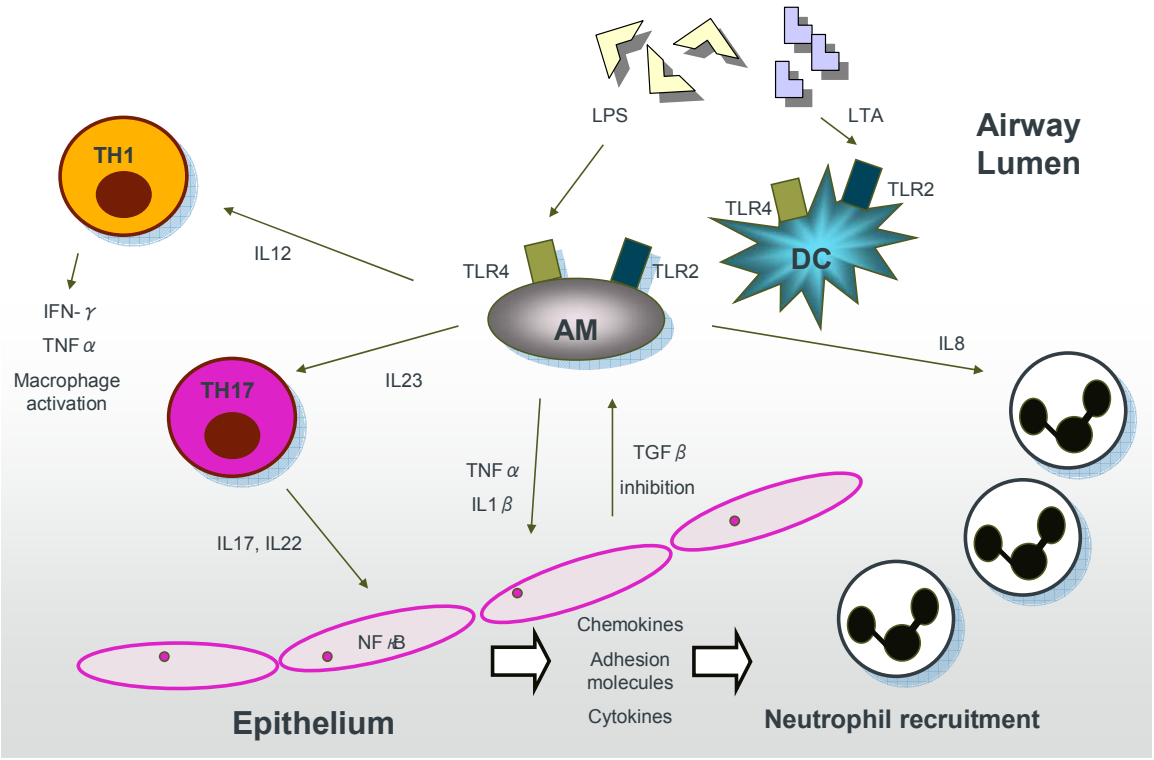

Figure 1 shows chemokine responses of airway dendritic cells (DCs) and alveolar macrophages (AMs) to the bacterial PAMPs LPS and LTA. The result is neutrophil recruitment through the direct action of IL-8 and the epithelial response to stimulation from TNFα, IL-17 and IL-22.

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1.8 Neonatal Airway Colonization and Asthma

Asymptomatic neonates born to asthmatic mothers with hypopharyngeal colonization with *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* or a combination of these organisms, are significantly more likely to have a diagnosis of asthma at 5 years of age than uncolonized neonates (33% vs 10%)[47]. Although this observation does not prove causation it does suggest that bacterial colonization of the upper airway may be linked to the development in later life of recurrent wheeze and asthma.

Given the evidence for the role of *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis* in the disease phenotype in COPD later on in life and this evidence for a role in early life in asthma, it would be valuable to look for the effect of airway colonization of these species in adult asthma.

Bisgaard *et al.* also looked at the rate of hypopharyngeal colonization with *Staphylococcus aureus*. Despite finding high rates of carriage (61% at 4 weeks and 13% at one year) they could not prove an association between colonization with this organism and development of recurrent wheeze or asthma.
Although this result would suggest that *S.aureus* may not be relevant in the initiation of asthma there is a growing body of evidence to suggest a role for *S. aureus* and their associated superantigenic, enterotoxin protein products in driving inflammation in asthma.

1.9 Evidence for the role of *Staphylococcus aureus* in the asthma

Staphylococcus aureus is a Gram-positive bacterial species which has the ability to release enterotoxins which can act as conventional antigens resulting in the production of specific S*taphylococcus aureus* enterotoxin (SAE) IgE. However these enterotoxins can also act as superantigens by their ability to bind receptor structures found on T cells, B cells and major histocompatability II (MHC II) proteins outside the antigen binding groove, bypassing the need for processing by antigen presenting cells. This enables *S. aureus* to effectively modify the immune response to impair host defence and enable colonization.
In the case of the T cell superantigens, the enterotoxin binds the variable β-chain (Vβ) of the T cell receptor (TCR) and the class II MHC molecule outside the peptide binding grove, on the surface of the antigen presenting cell (Figure 2). This

leads to an exaggerated immune activation with nonspecific stimulation of up to 20 % of naïve T cells compared with <0.01% when specific antigens are presented within the antigen binding groove of the MHC class II molecule[48].

T cell superantigens produced by *S. aureus* species include staphylococcal enterotoxin A (SEA), SEB, SEC1, SEC2, SEC3, SED through to SEU and TSST-1 (toxic shock syndrome toxin 1).

Unlike the antigen binding groove, the Vβ region of the TCR has limited variation. There are around 60 Vβ subsets currently described. Activation of T cells via this route leads to proliferation of the specific enterotoxin binding subset.

S. aureus species are able to produce more than one enterotoxin simultaneously and each toxin may recognise more than one Vβ segment[49].

For example, in atopic dermatitis, the most commonly found SAE detected on the skin, SEB, binds human T cells expressing TCRVβ3, 12, 14, 15, 17, or 20[50;51]. This leads to excessive T cell activation and clonal expansion of T cells expressing these specific Vβ subclasses producing a specific TCR Vβ signature.

Table 1 shows the human TCR Vβ specificity and rate of detection in nasal isolates, of *S. aureus* enterotoxins.

SAEs have been implicated in the initiation or maintenance of the inflammatory response in a wide range of diseases, including ; toxic shock syndrome, allergic rhinitis, nasal polyposis, Kawasaki Disease, atopic dermatitis, and, more recently severe asthma.

Table 1 Human TCR specificity for *S. aureus* superantigens

Superantigen	Detection Nasal Isolates(%) [52]	Human TCR Specificity
TSST-1	12	Vβ 2.1; Vβ 2[53]
SEA	10	Vβ1.1; 5.3; 6.3; 6.4; 6.9; 7.3; 7.4; 9.1; 16; 18; 21.3; 22
SEB	18	Vβ 3.2; 6.4; 12; 13.2; 14; 15; 17; 20; 22
SEC	7	Vβ 3; 6.4; 6.9; 12; 15.1; 17; 20
SED	9	Vβ 1.1; 5.3; 6.9; 7.4; 8.1; 12.1
SEE	0	Vβ 5.1; 6.3; 6.4; 8.1; 18; 21.3
SEG	53	Vβ 3; 12; 13.1; 13.2; 13.6; 14; 15
SEH	5	Vα 10
SEI	49	Vβ 1.1; 5.1; 5.2; 5.3; 6B; 23.1
SEJ	10	ND
SEK	9	Vβ 1; 5.1; 5.2; 5.3; 6.7; 21.3; 23
SEL	7	Vβ 5.1; 5.2; 5.3; 6.7; 7; 9; 16; 22; 23
SEM	44	Vβ 6a; 6b; 7.1; 8; 9; 18; 21.3
SEN	52	Vβ 5.1; 5.3; 9; 20
SEO	43	Vβ 5.1; 7; 21.3
SEP	27	Vβ 5.1; 8; 16; 18; 21.3
SEQ	7	Vβ 2.1; 5.1; 6.7; 21.3
SER	9	Vβ 3; 5.1; 8; 11; 12; 13.2; 14
SEU	ND	Vβ 12; 13.2; 14

Table adapted from Thomas et al.[54]

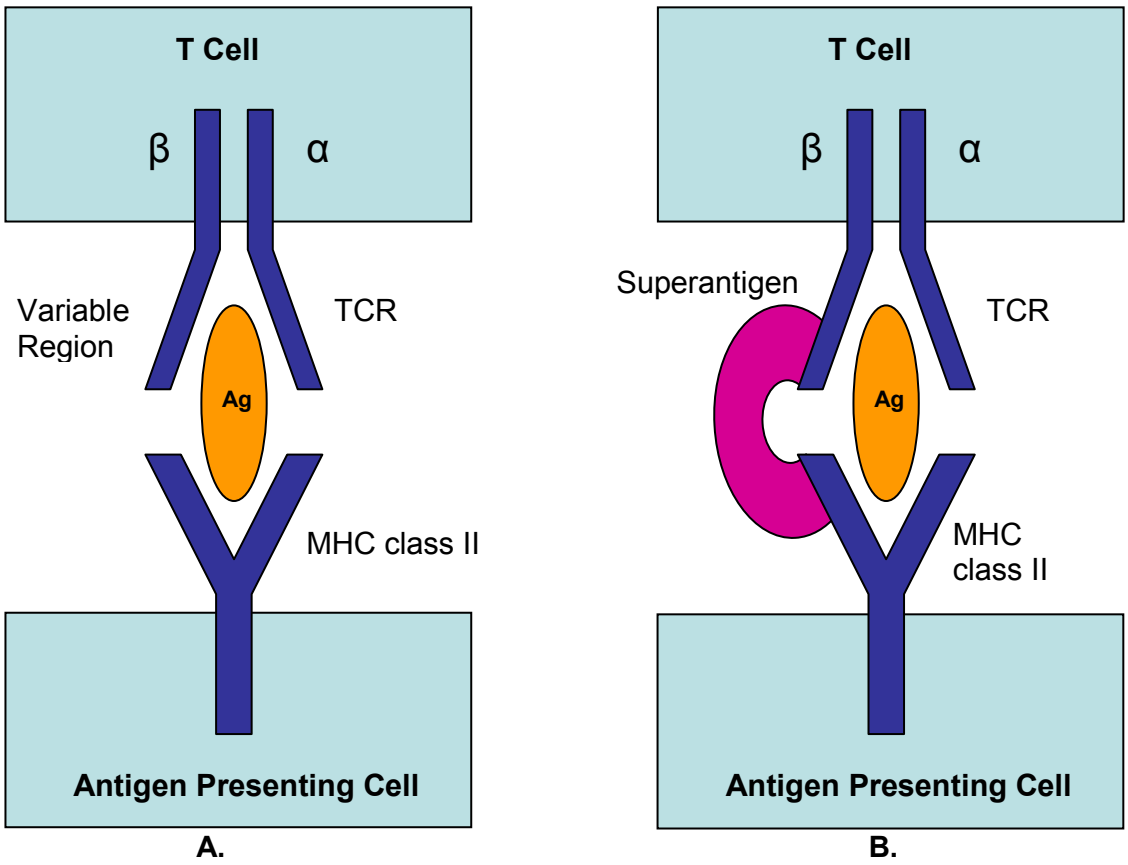


Figure 2 A. Conventional antigen presentation. B. Superantigen binding

1.10 The role of *Staph. aureus* in Allergic Rhinitis

S. aureus is frequently found as part of the normal bacterial flora of the upper respiratory tract. Perennial allergic rhinitis (PAR) is associated with a significantly higher nasal carriage rate of *S. aureus* when compared to normal controls (44 vs 20 %) and the presence of *S. aureus* is associated with significantly higher symptom scores[55].

In patients with PAR, a positive serum specific IgE to staphylococcal enterotoxins A,B,C,D or TSST 1 is associated with higher eosinophil cationic protein (ECP) and higher total IgE, suggesting more severe disease[56].

Patients with PAR and allergy to house dust mite, who have nasal *S. aureus* colonization, show a trend toward higher symptom scores and have significantly raised ECP, elastase and total nasal IgE when compared to uncolonized subjects[57].

Okano *et al.* used a mouse model of AR to investigate the effect of staphylococcal enterotoxin B (SEB). BALB/c mice were sensitised by intranasal exposure to Schistosoma mansoni egg antigen (SmEA) with and without SEB exposure. Control mice were exposed to SEB alone without prior SmEA sensitisation. SEB enhanced the development or AR in SmEA sensitised mice as measured by higher SmEA-specific IgE, nasal eosinophilia, and higher IL-4 and IL-5 in SmEA-stimulated nasal mononuclear cells. SmEA sensitized mice treated with SEB produced SEB specific IgE, whereas control mice treated with SEB alone did not[58].

These studies would suggest, not only that *S. aureus* carriage is more frequent in patients with PAR, but that the bacteria modifies the disease, producing a more severe phenotype with increased IgE production and eosinophilic inflammation. However, in mouse models SEB alone cannot induce AR but in previously sensitised mice will increase susceptibility and amplify the response to allergen challenge.

1.11 Staph. aureus Colonization and Nasal Polyposis

Nasal polyposis (NP) is associated with an increased rate of nasal staph aureus carriage. The condition is strongly linked to aspirin sensitivity and asthma. In one study colonization rates from middle nasal meatus swabs was 33.3% in healthy controls and 63.6% in subjects with NP. Within the NP group, carriage rates

increased to 66.7% with coexisting asthma and 87.5% if the subject was aspirin sensitive[59]. Specific IgE to staphylococcus enterotoxin A, C and toxic shock syndrome toxin 1 (SAE mix) was measured in nasal polyp tissue homogenates. Rates of specific IgE tissue positivity to the SAE mix mirrored staph aureus carriage rates (27.8% in NP and 58.8% in subjects with NP plus asthma). Specific IgE to SAE mix in nasal tissue was associated with significantly increased ECP and total IgE providing further evidence of the proinflammatory effect of staphylococcal superantigens.

1.12 The role of *Staph. aureus* in Atopic Dermatitis

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin. Like asthma, the causes are multifactorial, with interactions between susceptibility genes, environmental triggers, allergens, defective barrier function and abnormal immune responses.

Patients with AD have markedly increased rates of *Staph. aureus* colonization and infection[60;61]. 5% of healthy skin is colonized with staph aureus compared to between 75 and 100% in patients with AD.

Allergic skin inflammation exposes extracellular matrix (ECM) adhesins to the *S. aureus* cell membrane leading to increased bacterial adhesion to lesional skin. One such ECM adhesin is fibronectin. Fibronectin synthesis is stimulated by IL-4, a TH-2 cytokine which has leant weight to the hypothesis that TH-2, rather than TH-1 responses increase Staph aureus colonization in AD[62].

More than 70 % of *S. aureus* isolated from AD lesions are enterotoxin producing strains of the species. *S. aureus* enteroxins, particularly SEB which is most commonly found on the skin of AD patients can penetrate the skin barrier and exacerbate the inflammatory response either through superantigenic activity leading to clonal expansion of T cells bearing particular Vβ regions, or by acting as conventional antigens leading to the production of SAE specific IgE and an IgE mediated inflammatory response[63].

Skin colonization with SAE producing strains is associated with significantly higher symptom scores and disease severity[64;65].

1.13 *Staph. aureus* enterotoxins and corticosteroid insensitivity

Atopic dermatitis is more effectively treated with antibiotics in combination with corticosteroids than steroid treatment alone[66]. This may suggest that bacterial products induce glucocorticosteroid (GC) insensitivity.

Many patients with chronic, severe asthma have poor control of symptoms despite high dose corticosteroid therapy.

Clinically these patients can be labelled as "steroid resistant", although this term is unhelpful as many of asthmatics, although poorly responsive to low dose GC therapy will show clinical improvement at higher steroid doses.

In vitro phytohaemagglutinin (PHA) proliferation experiments using PBMCs from severe, steroid insensitive asthmatics who showed a poor FEV_1 response to oral prednisolone, demonstrated a lower response to glucocorticoid inhibition than PBMCs from steroid sensitive asthmatics[67].

The effect of *S. aureus* enterotoxins on steroid sensitivity has been demonstrated using PBMCs from healthy subjects. Dexamethasone is able to inhibit 99% of PHA-induced cell proliferation in vivo, compared to 19% inhibition in SEB treated cells. In the same study SEB significantly increased the expression of glucocorticoid receptor β, when compared to PHA stimulated cells untreated with enterotoxin[68]. The glucocorticoid receptor β is an isoform of the glucocorticoid receptor α which acts as an inhibitor of glucocorticoid action and shows significantly increased expression in PBMCs from steroid insensitive asthma compared to steroid sensitive asthmatics[69].

This provides a possible mechanism by which bacterial superantigens may be able to induce a state of steroid insensitivity leading to difficulty in controlling the inflammatory process in the asthmatic airway.

1.14 Evidence in murine models of a link between *S. aureus* enterotoxins and lower airway disease

Nasal or bronchial administration of SEB to ovalbumin sensitised mice leads to an augmented eosinophilic inflammation, with increased mRNA expression of IL-5, IL-4, IFN-gamma, IL-12 p40, eotaxin-1 and TGF-beta in bronchi, and raised serum total and OVA-specific IgE [70].

As well as increasing the bronchial allergic inflammatory response in sensitised mice, the effect of airway exposure to SEB was investigated in a non-allergic murine model of asthma, where it was found to increase CD4+ T cell-dependent

airway inflammation with increased IL-4, TNF-α and airway hyperresponsiveness.[71]

1.15 Evidence in humans of a link between *Staph. aureus* enterotoxins and lower airway disease

Serum measurement of specific IgE to SAEs can be used as a marker of exposure. Bachert *et al.* characterised IgE responses specific to SAEs in nasal polyposis and used this to develop a highly sensitive and specific test for a mix consisting IgE to SEA, SEC and TSST-1. This was applied to serum from healthy controls, mild asthmatics treated with only short acting bronchodilators as needed and an FEV_1 >80% predicted, and severe asthmatics requiring high dose inhaled corticosteroid with FEV_1<80% predicted. Total IgE, IgE specific to a mix of inhaled allergens and eosinophil cationic protein (ECP) were also measured.

IgE antibodies to the SAE mix were found more often in asthmatics than healthy controls (62% severe asthmatics vs 13% healthy controls , p=0.01) and were linked to levels of total IgE, ECP and steroid dependency.[72]

This study was subsequently extended and positivity for SAE mix IgE was found to be significantly higher in severe asthmatics than in patients with more mild disease. Asthmatic subjects showed a significant association between total IgE and levels of IgE to SAE mix. Aspirin sensitive severe asthmatics were found to be significantly more likely to be SAE mix IgE positive than non-aspirin sensitive patients. Possibly linked to this, nasal polyposis in severe asthmatics was significantly associated with antibodies to SAE mix.

There was no difference in aeroallergen sensitisation between the severe asthmatic group and the healthy control group to confound these findings. This led to the hypothesis that bacterial enterotoxin superantigens may be the allergens driving what is thought of as non-atopic asthma [73].

1.16 TCR Vβ Profiling in Asthma

The clonal expansion of specific regions of the Vβ repertoire in the presence of superantigens has led to an interest in profiling the proportions of T cells expression Vβ regions of interest.

Wahlstrom *et al*. attempted to identify T cell subsets associated in allergic asthma. They used RT-PCR followed by southern blot to identify TCR repertoires and then quantify Vβ gene usage in T cells from blood and BAL, before and after repeated allergen challenge with either birch or grass pollen. Peripheral blood samples from healthy individuals were used as controls.

Vβ gene usage altered in individuals post allergen challenge, although this differed between subjects. In the asthmatic group there was no significant alteration in Vβ expression in BAL or blood pre and post allergen challenge.
However BAL and peripheral blood T cells from asthmatics showed increased Vβ3, Vβ5.2 and Vβ6.1-3 usage and decreased Vβ16, Vβ18 and Vβ19 usage when compared to PBLs of healthy controls. This difference was evident before and after allergen challenge, suggesting over and under expression of certain Vβ subsets in asthmatics compared to healthy controls.
TCR Vβ7 and Vβ9 usage was significantly higher in BAL than in peripheral blood lymphoctyes in asthmatics before as well as after challenge.[74]

Hauk *et al.* looked for evidence of superantigenic activation of T cells by looking at the TCR-Vβ repertoire of BAL fluid and PBMCs from subjects with poorly controlled asthma, well controlled asthma and healthy controls. Poor asthma control was defined as having an FEV_1<75% and good control if FEV_1>80% predicted.
BAL and PBMCs were stained with monoclonal antibodies to CD3, CD4, and CD8 for 9 possible TCR-Vβ chains (Vβ2, 3, 5.1, 6.7, 8, 12, 13.1, 13.2, and 17). Flow cytometry was used to determine the percentage of T cells expressing each TCR Vβ region[75].
Poorly controlled asthmatics had significantly higher TCR-Vβ8+ cells in BAL than the combined well controlled asthmatic and healthy volunteer groups (p=0.0001).
Expression of TCR Vβ8+ cells was significantly higher in BAL than PBMCs suggesting there may be a local airway stimulus.

Hauk *et al.* suggested that microbial superantigens may represent an ongoing stimulus to chronic airway inflammation resulting in a skewing of the TCR Vβ repertoire in poorly controlled asthma. They did not, however, attempt to identify the superantigen producing bacteria in their sample population [75].

1.17 Research Questions

The development of asthma and, in particular, steroid resistant severe asthma, remains poorly understood. Complex interactions between intrinsic and acquired factors are involved and the significance of bacterial colonization in disease pathogenesis or development of specific disease phenotypes needs further evaluation.

There is growing evidence for the role of potentially pathogenic micro-organisms in the ongoing maintenance of inflammation and decline in FEV1 in COPD. However few studies have examined PPM colonization of the lower airway in asthma.

Recent developments in molecular microbiological techniques such as T-RFLP profiling enable examination of induced sputa and bronchoalveolar lavage to give a comprehensive account of bacterial colonization in the airways of severe asthmatics.

The hypotheses to be tested through this work are :

1. Bacterial colonization patterns differ between the asthmatic and healthy lower airway.
2. Airway colonization with potentially pathogenic bacterial species contributes to asthma severity and the development of a steroid resistant phenotype.
3. *S. aureus* colonizes the lower airway in asthma and contributes to treatment resistance through the action of superantigens.

Airways distal to the trachea are usually considered sterile in healthy non-smoking subjects [76]. The techniques adopted to address the research question will allow comparisons to be made between the profile of bacterial colonization in the proximal airways (trachea and major bronchi) using induced sputum and the more distal small airways, using bronchoalveolar lavage, in severe asthmatics, mild asthmatics and healthy controls.

The presence and relative abundance of PPMs will be correlated with markers of inflammation and clinical measures of disease severity to gain an insight into the role of airway colonization in changing asthma phenotype.

The molecular microbiological methods adopted to detect airway bacterial species will include terminal restriction fragment length polymorphism (T-RFLP) profiling.

T-RFLP profile results and culture results will be compared from split samples to assess the reliability of culture as a method of determining bacterial colonization.

The role of *S. aureus enterotoxins* in persistent asthma has not previously been established although there is a growing body of circumstantial evidence for their action. In addition, diseases sharing similar immunological pathology with asthma have been linked to SAEs.
T cell receptor Vβ repertoire profiling in BAL and blood will be undertaken in asthmatic subjects and healthy controls to look for surrogate evidence of superantigenic induced T cell clonal expansion within the lower airway. TCR Vβ profiles will be compared between subjects colonized, within the lower airway, with the superantigen generating species, *Staphylococcus aureus* and *Streptococcal sp.*

Evidence for a role of bacterial colonization contributing to disease persistence and steroid resistance in asthma would have clear therapeutic implications, leading on to the question of whether prolonged antibiotic treatment or vaccination, with the aim of eradicating specific species from the lower airway, could improve symptoms in a group of patients where there are currently few effective therapeutic options.

2 Materials and Methods

2.1 Subjects

Three patient cohorts were recruited; healthy controls, mild asthmatics and severe asthmatics. The study was approved by the Southampton and South West Hampshire Ethics Committee and all subjects provided written, informed consent. Severe asthmatic subjects were recruited from the Southampton University Hospitals NHS Trust severe asthma service. These subjects had a clinical diagnosis of asthma with historical evidence of variable airflow obstruction. Severe asthma was defined according to the American Thoracic Society workshop on refractory asthma criteria[77]. Exacerbation of asthma or antibiotic therapy within six weeks of a scheduled study visit, either led to subject exclusion, or rescheduling of the visit.

Healthy volunteers and mild asthmatic subjects were recruited from the University of Southampton, Allergy and Inflammation Research volunteer database. Healthy volunteers had no history of respiratory disease and a PC_{20} > 8mg/ml at methacholine challenge testing. Mild asthmatics had a clinical history of asthma treated at the British Thoracic Society asthma management guideline treatment step one or two[78], and evidence of bronchial hyperreactivity (PC_{20} < 8mg/ml). Current smoking was an exclusion criteria for all subject groups.

2.2 Study Protocol

All subjects were given a participation information sheet prior to giving informed consent. Subjects were asked to attend for three study visits. At visit 1 a full history was taken, physical examination performed and asthma control questionnaire[79] completed, followed by venesection, spirometry with reversibility testing, skin prick testing and sputum induction.

At visit 2 exhaled nitric oxide and methacholine challenge were performed for healthy control and mild asthmatic subjects. Severe asthmatic subjects were excluded from methacholine challenge testing for safety reasons.

At visit 3 bronchoscopy was performed.

2.3 Spirometry

Subjects were asked to abstain from caffeine for 6 hours, long acting β agonists (LABA) for 12 hours, short acting β agonists (SABA) for 6 hours and theophylline preparations for 48 hours prior to testing.

Forced expiratory volume in 1 second (FEV_1) and forced vital capacity were measured using a spirometer (Vitalograph Medical Instrumentation Ltd). The maximum of 3 attempts was recorded. Percentage predicted values were calculated from standardised reference values[80].

2.4 Bronchodilator Reversibility

In order to assess FEV_1 bronchodilator reversibility, baseline spirometry was performed then salbutamol 200µg was administered via aerochamber (Trundel Medical International Ltd). Post bronchodilator spirometry was performed at 15 minutes and reversibility calculated as: FEV_1post – FEV_1baseline / FEV_1baseline x 100.

2.5 Peak Flow Monitoring and Variability

Asthmatic subjects were issued with a mini-Wright peak flow meter (Airmed, Clement Clarke International Ltd) to record the best of 3 prebrochodilator morning and bedtime peak flow measurements over a two week period. PEFR variability (%) was calculated as: Maximum PEFR – Minimum PEFR / Maximum PEFR x 100

2.6 Allergen Skin Prick Testing

Subjects were asked to refrain from taking antihistamines for 4 days prior to testing. Skin prick testing to the following aeroallergens was performed: Aspergillus Fumigatis, Alternaria Tenius, Grass Mix Pollen, Birch Pollen, Weed Mix, Rape Pollen, Dermatophagoides Pteronyssinus, Dermatophagoides Farinae, Cat Fur and Dog Fur (Merck Skin Testing Solution). A histamine positive control and saline negative control were used. Wheal size over 3mmx3mm larger than the negative control with evidence of a histamine wheal was considered positive. Subjects were described as atopic if skin prick positivity to one or more aeroallergen was demonstrated.

2.7 Methacholine Challenge Testing

A modified protocol developed from the standardised bronchial inhalation challenge described by Chai *et al.* was used[81]. Subjects were asked to refrain from using SABA use for 6 hours and LABAs for 24 hours before testing. Histamine solutions (0.03–8mg/ml in normal saline) were sequentially administered via Spira Electro Dosimeter. FEV_1 was recorded at 1 minute and 3 minutes after inhalations. The lower of the best FEV_1 readings taken at 1 minute and 3 minutes was used to calculate the percentage fall from a post saline baseline FEV_1. Doubling concentrations of histamine were given approximately every 5 minutes until a maximum concentration of 8mg/ml or a drop in FEV_1 >20% post saline baseline was seen. Post procedure salbutamol 200 µg was administered. Spirometry was repeated at 15 minutes post bronchodilator to ensure FEV_1 had returned to >90% baseline.

2.8 Exhaled Nitric Oxide Measurement

Exhaled nitric oxide (ENO) was measured prior to any other respiratory function tests. Subjects were asked to abstain from caffeine and bronchodilators as previously described. The Logan LR2500 (Logan Research, UK) trace gas and nitric oxide analyser system was used. Subjects were asked wear a noseclip, exhale fully, then inhale through the mouth to total lung capacity, then exhale slowly for 30 seconds at a constant rate aided by the biofeedback LED indicator. A minimum of 3 adequate tests were performed and the mean ENO calculated.

2.9 Sputum induction

Sputum induction was performed using hypertonic saline according to European Respiratory Society guidelines[82]. Baseline FEV_1 and PEFR were measured. Asthmatic subjects were then given salbutamol 200mcg via aerochamber and spirometry repeated at 15 minutes. Subjects were seated in an environmental chamber and given written instructions. They were asked to blow their nose and rinse their mouth before induction. Subjects were instructed in how to expectorate into a sterile petri dish for the procedure and were encouraged to swallow saliva before coughing. 4.5% hypertonic saline was administered by ultrasonic nebuliser (DeVilbiss Ultraneb 3000) for a total of 20 minutes. Nebulisation was stopped every 5 minutes to check FEV_1 or PEFR. If FEV_1 or PEFR dropped by >20% from the post bronchodilator baseline, the procedure was discontinued.

Asthmatic subjects felt to be at high risk of bronchospasm were asked to give induced sputum using a modified protocol were 0.9% saline was initially nebulised with FEV_1 checks after 30 seconds, 1 minute and 5 minutes. The saline concentration was then increased to 3% with spirometry after nebulisation periods of 30 seconds, 1 minute and 5 minutes. If an adequate sample had not been obtained the saline concentration was increased to 4.5% with nebulisation periods of 30seconds, 1, 2, 4 and 8 minutes between check FEV_1 measurement. If at any stage FEV_1 dropped by >20% of the post bronchodilator FEV_1 the procedure was discontinued.

2.10 Sputum Processing

Sputum plugs were selected from expectorate and divided for differential cell counting and the remainder stored at -80^{0}C for subsequent molecular microbiological testing.

Sputa for differential cell counts were immediately processed with dithioerythritol to separate cells from the fluid phase of sputum. The homogenised sample was then filtered using a 100µm cell strainer (BD Falcon) to remove mucus. Samples were centrifuged at 1500rpm for 10 minutes (Haraeus Labofuge 400R), the fluid phase supernatant was then removed and frozen at -80^{0}C, and the cell pellet resuspended in 1000µl phosphate buffered saline. A total cell count was made using a haemocytometer and cell viability determined using tryphan blue exclusion.

Cytospins were prepared using 70 x10^3 cells and centrifuged at 450rpm for 6 minutes (Shannon Cytospin 2). Cytospins were fixed with and stained using Rapi-Diff I, II and III (DiaCheM Int. Ltd, UK) and differential cell counts were obtained from 400 non-squamous cells.

2.11 Flexible Fibreoptic Bronchoscopic Examination and Sampling

Severe asthmatics, mild asthmatics and healthy control subjects were recruited for bronchoscopic sampling of the lower airways.

2.11.1 Bronchoscopic procedure.

Informed consent was obtained at a prior visit. Subjects were asked to attend The Wellcome Trust Clinical Research Facility having been nil by mouth for a minimum of 6 hours. The procedure was fully explained to the subject and then a full physical examination was performed. History was reviewed to ensure the subject

had not had an exacerbation of asthma or antibiotic therapy in the preceding 6 weeks.

Subjects were cannulated in the dorsum of the right hand and blood taken. Baseline spirometry was performed and then salbutamol 2.5mg was administered via a compressed air driven nebuliser (Respironics Sidestream). 15 minutes post nebulisation repeat spirometry was performed.

Immediately pre-procedure, subjects were given intravenous atropine (600mcg), midazolam (up to 2mg) and fentanyl (up to 50mcg).

Local anaesthesia was administered to the nose and oropharynx using up to 3ml of lignocaine gel (Instillagel) nasally and lidocaine hydrochloride (Xylocaine) orally.

Bronchoscopy was performed nasally wherever possible (Olympus BF Type P60 Bronchoscope, Olympus Evis CLV U20 Light Source Video System) and the vocal cords anaesthetised with up to 6mls 2% lignocaine. Oxygen saturations were monitored throughout. Following scope intubation, anaesthesia of the bronchial tree was achieved using up to 12mls 1% lignocaine. Further sedation could be used during the procedure (up to 5mg midazolam total) if required.

2.11.2 Bronchoalveolar lavage (BAL) procedure and processing

BAL was performed by wedging the bronchoscope in the posterior segment of the right upper lobe. Up to 120ml of pre-warmed (37°C) 0.9% saline was instilled in 20ml aliquots with aspiration into a sterile trap between each controlled instillation.

5mls of BAL was retained pre-processing and frozen at -80°C for T-RFLP. The remaining BAL was processed immediately.

The BAL was filtered using 100µm cell strainer (BD Falcon). The filtrate was centrifuged at 2000rpm for 10 minutes at 4°C. BAL supernatant was removed and frozen at -80°C. The remaining cell pellet was resuspended in 2ml of phosphate buffered saline. 10µl of cell suspension was mixed with 90µl tryphan blue and 10µl of the mix used for live, dead and squamous cell counting using a haemocytometer. The cell suspension was diluted using the cell count to a concentration of $0.5x10^6$ cells/ml and 70µl aliquots were used to make cytospins.

2.11.3 Post procedure

Post procedure subjects were moved to a recovery suite for an hour before being allowed to eat and drink. Post procedure spirometry was performed to ensure there had been no significant drop in FEV_1 (>10% baseline). Subjects were then

discharged home under the supervision of a friend or relative with a post procedure advice sheet.

2.12 Supernatant cytokine measures

Cytokine measurements including interleukin-8 (IL-8), interleukin-6 (IL-6), interleukin-1β (IL-1β), epithelial-derived neutrophil attractant-78 (ENA-78), growth related oncogene-α (GRO-α), eosinophilic cationic protein (ECP) and α2 macroglobulin were obtained from selected induced sputum and BAL supernatant.

All cytokine measures were performed by The University of Southampton School of Medicine Division of Infection, Inflammation and Immunity using enzyme-linked immunosorbent assays (ELISAs). All ELISAs were performed in accordance with manufacturers' instructions using the CXCL8/IL-8 DuoSet kit (R&D Systems Cat. No DY208), CXCL1/GRO alpha DuoSet kit (R&D Systems Cat. No DY275), IL-1β DuoSet Kit (R&D Systems Cat. No DY201), IL-6 DuoSet kit (R&D Systems Cat. No DY206) and the CXCL5/ENA-78 DuoSet ELISA kit (R&D Systems Cat. No DY254). α2 macroglobulin (Sigma), and eosinophilic cationic protein (Mesacup ECP) were also measured by ELISA.

2.13 Culture independent analysis of respiratory samples

The molecular microbiological approach adopted to indentify bacterial species within clinical samples was Terminal Restriction Fragment Length Polymorphism (T-RFLP) profiling.

T-RFLP profiling was performed at King's College London. Respiratory specimens were frozen within 30 minutes of collection and batch shipped to King's for analysis.

Proir to analysis sputum samples are washed using phosphate buffered saline to remove any adherent saliva.

During the T-RFLP profiling process bacterial DNA is extracted from the clinical specimen (induced sputum or BAL)[33]. The extracted nucleic acid undergoes PCR amplification using specific primers for the phylogenetically informative 16S RNA gene[32].

An IR dye primer is added and PCR amplicons are then cleaved using restriction endonuclease digestion. The ribosomal gene fragments are then resolved on a DNA sequencer to form a T-RFLP profile (Figure 3) [83]. T-RFLP profiles are analysed using Phoretix 1D Advanced software v.5.10 (Nonlinear Dynamics,

Newcastle upon Tyne, UK), with band sizes determined by comparison to size markers (Microzone, Lewes, UK) to identify the presence of specific bacterial species within the samples electrophoretic strip. Band volume (the product of the band area and signal intensity) is determined and expressed as a percentage of the total volume of bands in a given electrophoretic profile to give species abundance as a percentage of total bacteria load within the sample. A threshold band volume of 0.1% of the total profile signal for the specimen was predetermined as the detection threshold. The species with the highest percentage abundance within the sample is designated the dominant species.

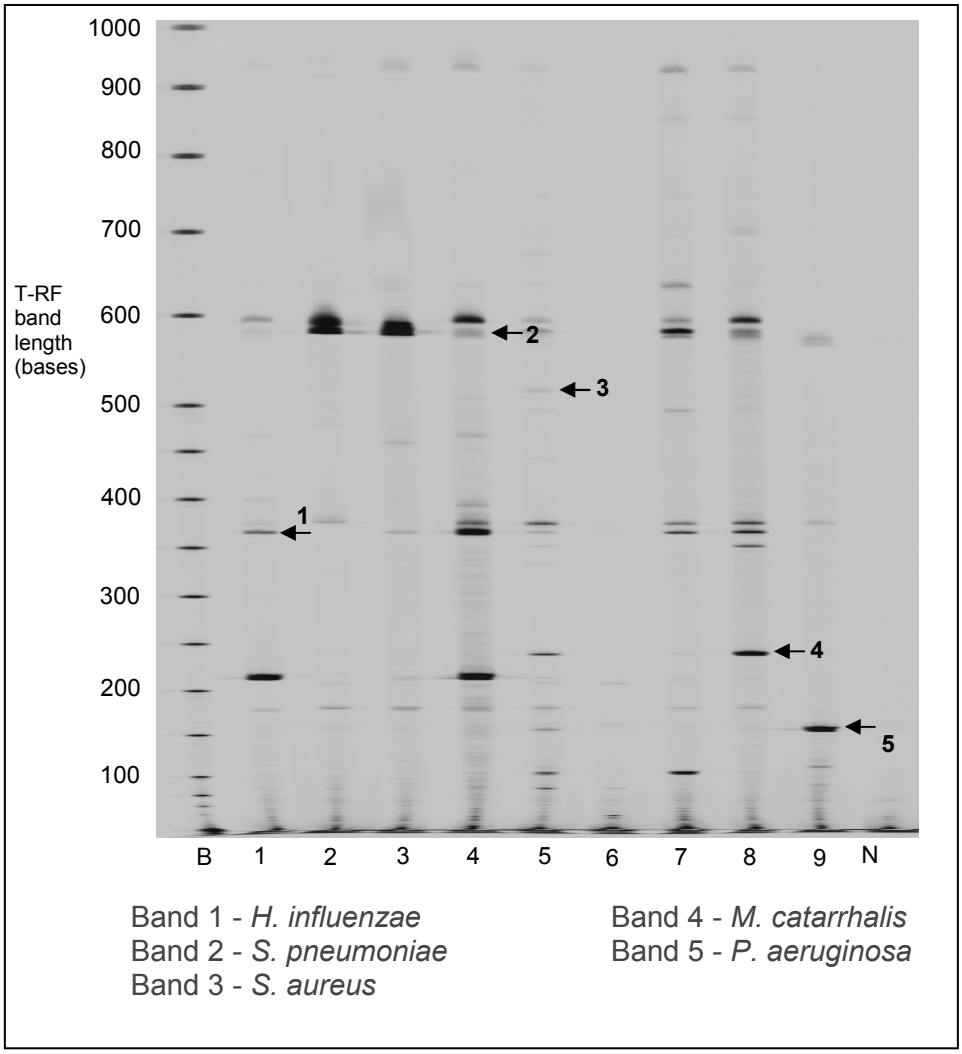


Figure 3 An example of a T-RFLP profile gel (B- band size marker, specimens 1-9, negative control N)

2.14 Statistics

Data were analysed using SPSS (Version 15; SPSS,Inc, Chicago, USA). Continuous parametric data were compared using the independent samples t test. Correlations between normally distributed data were performed using Pearson's correlation coefficient. Paired data sets were compared using the paired samples t test.

Non-normally distributed data were compared using the nonparametric Mann-Whitney U-test. Correlations between nonparametric data sets were undertaken using Spearman's rank correlation.

Pearson Chi-squared test was used to compare categorical data unless expected cell counts were less than 5 when Fischer's exact test was applied. Two-tailed tests were used and the level of significance was taken as $p=0.05$.

3 Assessing Bacterial Airway Colonization in Severe Asthma through Induced Sputum

3.1 Aim

We aimed to describe the normal flora of the lower respiratory tract in subjects with stable severe asthma using conventional culture based species identification and with terminal restriction fragment length polymorphism (T-RFLP) profiling.

Standardised protocols for the induction of sputum in severe asthma provide safe, non-invasive sampling the lower airways[84-86]. Despite passage through the oropharynx, if there is low salivary contamination of the sputum sample with fewer than 25% squamous cells on differential cell counting, cultured bacterial species resemble transtracheal aspirate findings[24;27;87;88].

However bacterial species identified by culture represent, at best, only 20% of total species within complex microbial communities[30]. The culture bias introduced by the use of selective media and the cultivation of only viable colonies cannot give a true representation of the colonising bacterial flora present within the airways.

Terminal restriction fragment length polymorphism (T-RFLP) profiling is a molecular microbiological technique developed to characterise complex microbial communities[31;32].

This method exploits the 16S ribosomal RNA sequence present in all bacteria but unique to each bacterial species and provides a semi-quantitative description of diversity and abundance of species within a specimen.

This technique has previously been shown to be both reproducible and effective at characterising bacterial species diversity and dominance in sputum from patients with cystic fibrosis[33].

3.2 Methods

Induced sputum was collected from 21 subjects with severe asthma as defined by American Thoracic Society workshop on refractory asthma[77]. All subjects were a minimum of 6 weeks since last exacerbation or antibiotic treatment. Expectorated sputum plugs were selected from the fluid phase and homogenised. The separated sample was then divided for T-RFLP profiling, standard culture and differential cell counting.

Culture of sputa was performed according to Health Protection Agency (HPA) National Standard Methods (BSOP 57)[89] at Southampton General Hospital. Standard respiratory culture media, chocolate agar and blood agar were used with incubation at 35-37^{o}C, 5-10%CO_2 for 40-48 hours.

The sample for T-RFLP profiling was frozen at -80^{0}C within 30 minutes and the sample for culture was delivered to the HPA within 2-3 hours.

3.3 Results

T-RFLP profile results can be expressed as a list of species contained within the clinical sample and the percentage abundance of that species in relation to the total bacterial load within the specimen.

Mean number of bacterial species detected in the 21 severe asthma specimens was 7.7 (SD+/- 4.05) with a range of 1-15 species. The clinical significance of species that make up small proportions of the total bacterial load is unknown therefore T-RFLP results can be reported as the three dominant species in the specimen (ie. three species of highest percentage abundance).

Mean squamous cell contamination of samples (measured as a percentage of 400 non squamous cells counted for the cell differential) was 9.7% (SD+/-14.0).

Table 2 below shows the T-RFLP (3 most dominant species) and culture results for 21 induced sputum samples from severe asthmatics.

	1st Dominant species (% abundance)	2nd Dominant species (% abundance)	3rd Dominant species (% abundance)	Culture result
1	*Moraxella catarrhalis (98.0)*	*Veillonella sp. (1.2)*	*Unassigned sp. (0.8)*	*Streptococcus pneumoniae, Oral flora*
2	*Veillonella sp. (100)*			*Oral Flora*
3	*Veillonella sp. (49.9)*	*Prevotella (15.0)*	*Neisseria subflava, N, elongate (8.4)*	*Oral Flora*
4	*Haemophilus sp. (55.0)*	*Neisseria subflava, N, elongate (25.9)*	*Neisseria sp. (6.7)*	*Oral Flora*
5	*Haemophilus sp. (46.0)*	*Streptococcus sp. – S. pseudopneumoniae, S. pneumoniae, S. mitis (26.2)*	*Porphyromonas sp. , Prevotella sp. – P. loescheii, P. denticola, P. veroralis, P. oris (8.5)*	*Oral Flora*
6	*P. aeruginosa (48.8)*	*Moraxella catarrhalis (26.7)*	*Haemophilus sp. (12.8)*	*Oral Flora*
7	*Moraxella catarrhalis (95.8)*	*P.aeruginosa (4.2)*		*Haemophilus Influenzae, Oral flora*
8	*Veillonella sp. (80.2)*	*P.aeruginosa (5.6)*	*Moraxella catarrhalis (4.95)*	*Oral Flora*
9	*Haemophilus sp. (87.5)*	*Fusobacterium periodonticum (7.13)*	*Unassigned sp. (1.13)*	*Haemophilus Influenzae, Oral flora*
10	*Moraxella catarrhalis (53.0)*	*Streptococcus sp. – S. pseudopneumoniae, S. pneumoniae, S. mitis (26.6)*	*Unassigned sp. (5.0)*	*Oral Flora*
11	*Moraxella catarrhalis (54.0)*	*P.aeruginosa (41.6)*	*Haemophilus sp. (2.15)*	*Oral Flora*
12	*Moraxella catarrhalis (41.6)*	*P.aeruginosa (35.5)*	*Haemophilus sp. (17.3)*	*Oral Flora*
13	*Streptococcus sp. – S. pseudopneumoniae, S. pneumoniae, S. mitis (25.6)*	*Prevotella sp. – P. melaninogenica, P. intermedia (22.9)*	*Haemophilus sp. (14.3)*	*Oral Flora*
14	*Prevotella sp. – P. melaninogenica, P. intermedia (37.1)*	*Porphyromonas sp. , Prevotella sp. – P. loescheii, P. denticola, P. veroralis, P. oris (15.7)*	*Haemophilus sp. (14.8)*	*Oral Flora*
15	*Prevotella sp. – P. melaninogenica, P. intermedia (33.4)*	*Porphyromonas sp. , Prevotella sp. – P. loescheii, P. denticola, P. veroralis, P. oris (20.5)*	*Haemophilus sp. (12.2)*	*Oral Flora*
16	*Veillonella atypica, V. parvular, V.criceti, V.ratti (28.9)*	*Unassigned species (28.1)*	*Moraxella catarrhalis (13.5)*	*Oral Flora*
17	*Prevotella sp. – P. melaninogenica, P. intermedia (23.8)*	*Megasphaera sp. (21)*	*Unassigned sp. (18.1)*	*Oral Flora*
18	*Streptococcus sp. – S. pseudopneumoniae, S. pneumoniae, S. mitis (40.3)*	*Prevotella sp. – P. melaninogenica, P. intermedia (20.8)*	*N. flava (7.4)*	*Oral Flora*
19	*Haemophilus sp. (95.8)*	*Veillonella sp. (2.4)*	*Fusobacterium periodonticum (1.4)*	*Haemophilus Influenzae, Oral flora*
20	*Unassigned species (36.5)*	*Haemophilus sp. (15.3)*	*Streptococcus sp. - S. pseudopneumoniae, S.pneumoniae, S.mitis (15.5)*	*Oral Flora*
21	*Streptococcus sp. – S. pseudopneumoniae, S. pneumoniae, S. mitis (70.8)*	*Unassigned species (12.7)*	*Veillonella sp.(10.5)*	*Oral Flora*

Haemophilus sp. = Haemophilus influenzae, segnis, parainfluenzae, Terrhaemophilus aromaticivorans

Table 2 Induced sputum paired T-RFLP profile and culture results in severe asthma

		Potentially pathogenic microorganism identified by T-RFLP		Total
		No	Yes	
PPM correctly identified by culture	No	4	15	19
	Yes	0	2	2
Total		4	17	21

Table 3 T-RFLP and culture PPM identification in severe asthmatic induced sputum

Potentially pathogenic micro-organisms (PPMs) defined in this study as the respiratory pathogens *Moraxella catarrhalis*, *Haemophilus sp.*, *Streptococcus sp., Pseudomonas aeruginosa* and *Staphylococcus aureus* were identified by T-RFLP in the induced sputum in 17/21 (81.0%) of subjects. PPMs were identified using culture in 4/21 (19.0%) (Table 3). There was no significant association between the identification of pathogenic species using T-RFLP and culture in paired samples (Ficher's Exact Test, p= 1.0).

3.4 Discussion

A molecular microbiological technique and standard respiratory specimen culture were used to describe the colonizing flora of the lower respiratory tract in severe asthma. All subjects were clinically stable with a minimum of 6 weeks since last exacerbation or antibiotic therapy and subjects did not have symptoms of acute infection. No subjects had a clinical history of significant bronchiectasis. Therefore the lower airway flora described can be considered a stable colonized state. However, the word colonizing is likely to be inappropriate as the presence airway bacteria exerts a pro-inflammatory effect without the need for invasive infection. Bacteria continually shed a variety of outer cell wall membrane fragments into the airway which act as pathogen associated molecular patterns (PAMPs) and are recognized by the pattern recognition receptors of the innate immune system. Soluble fragments such as lipopolysaccharide (LPS), a Gram-negative outer cell membrane product, can act as a conventional antigen through the polysaccharide portion of the molecule, however, the lipid portion, which is not antigenically specific, triggers a pro-inflammatory innate immune response through Toll-like

receptor 2 (TLR-2) activation. Potentially pathogenic species such as *Haemophilus influenzae* have been linked with this response in asthma[37].

The healthy non-smoking adult airway is traditionally thought of as sterile[90;91]. However the studies providing the origins of this theory were based on the examination of lower airway specimens by culture. Growth of bacteria on selective media requires assumptions about the pathogens expected to be present within a sample and the presence of sufficient viable bacteria. Cultivable bacteria represent only 0.1 - 20% of species within microbial communities[30]. Using the more sensitive molecular microbiological techniques such as T-RFLP profiling, it is possible to demonstrate a normal flora in the airway of the healthy adult, although information regarding bacterial load is more difficult to infer.

Investigation of bacterial colonization in asthma has previously focused on the detection of atypical respiratory pathogens. Serological evidence suggests an association between previous *C. pneumonia* infection and severe chronic asthma [13;14;92].

In one bronchoscopic study of 55 chronic stable asthmatics and 11 normal control subjects using PCR to detect atypical organisms, significantly higher incidence of PCR positivity for *Mycoplasma pneumoniae* (n=25) and *Chlamydophilia pneumoniae* (n=6) in bronchial biopsies and lavage fluid was reported in the asthma group than in the normal controls (n=1)[12]. However in our study of induced sputum we have not identified any evidence of the presence of either *Mycoplasma pneumoniae* or *Chlamydophilia pneumoniae*. This may truly reflect an absence of these pathogens. However, induced sputum preferentially samples the trachea and major bronchi rather than the more distal sampling of bronchoalveolar lavage[93-95] which could be the more relevant site of colonization for some species.

Sputum induction risks contamination of samples by the bacterial flora of the oropharynx during expectoration. In a previous study of cystic fibrosis patients, T-RFLP profiles of spontaneously expectorated sputum and paired mouthwash samples did not show significant evidence of contamination from oropharyngeal species[96], suggesting examination of sputum can give an accurate account of lower airway colonization.

Attempts were made to minimise salivary contamination using protocols to separate sputum plugs from saliva during processing which has been shown to significantly lower squamous cell contamination[25;82]. Low squamous cell

contamination (Mean 9.7%) in the samples was seen, suggesting bacterial flora demonstrated are likely to represent the flora of the lower airway.

There was no significant correlation between sputum culture results and results from T-RFLP profiling. Potential respiratory pathogens were identified on culture in 4 of 21 specimens. In two of these specimens the pathogen was reported as one of the three most dominant species within the specimen on T-RFLP profiling. In the two other culture positive specimens the pathogen identified was not detected in the T-RFLP profile. In 15 of 21 specimens a potential respiratory pathogen was identified in the three most dominant species but was not detected by sputum culture.

The clinical relevance of this finding in clinically stable severe asthmatics is not yet clear. It is possible to detect potential respiratory pathogens, which appear to be the dominant species within the lower airways of asthmatics with molecular microbiological techniques which are not detected by standard culture for respiratory pathogens on standard culture media.

If molecular detection of bacterial species is considered the gold standard, in our severe asthmatic induced sputa samples, standard HPA respiratory specimen culture could not be considered either sensitive or specific for identification of potentially pathogenic colonising bacteria. This would suggest any investigation of the relevance of bacterial colonization in airways disease should avoid culture based identification of species.

4 Diversity of Colonising Bacterial Flora in Severe Asthma, Mild Asthma and Healthy Control Subjects

4.1 Aim

To document the diversity of flora in the lower airway of subjects with severe asthma, mild asthma and healthy control subjects.

Molecular microbiological techniques demonstrate the presence of bacterial species in the lower airways which have previously been considered sterile. It is necessary to demonstrate the "normal flora" of the airway in health in order to understand the relevance of lower airway bacteria in asthma.

Colonising flora diversity within a respiratory specimen can be estimated by the number of species identified within the sample. Induced sputa, sampling the trachea and major bronchi, and bronchoalveolar lavage, sampling distal to the third generation airways, were collected from severe asthmatics, mild asthmatics and healthy controls. Species diversity was compared between subject groups and comparison was made between proximal and distal airway species diversity by comparing species number in induced sputum and BAL within subject groups and using paired, same subject samples.

4.2 Methods

Three subject groups were recruited. Severe asthmatic patients were identified and defined as previously described. Mild asthmatic subjects were defined as patients with a previous physician diagnosis of asthma with control of symptoms at British Thoracic Society treatment step 1 or 2. Mild asthmatic subjects were recruited from the University of Southampton subject database and all had evidence of bronchial hyperreactivity (demonstrated at metacholine challenge testing with PC_{20}<8mg/ml) or evidence of reversible airways obstruction. Healthy volunteers were recruited from local advertising within the University of Southampton and from the University volunteer database. They had no history of respiratory disease and a negative bronchial provocation test (PC_{20}>8mg/ml). No current smokers were recruited into the study.

Both induced sputum and bronchoalveolar lavage were collected as previously described and specimens used for T-RFLP profiling. When paired IS and BAL

specimens were obtained from the same subject (asthmatic subjects only), sampling was performed within a six week window.

Airway bacterial diversity was estimated by the number of bands observed on the T-RFLP profile, with the number of bands representing the number of species detected.

The mean number of detected species was compared between subject groups and between induced sputum and BAL sampling using an independent sample t test. Paired t tests were used to compare species diversity in induced sputum and BAL collected from the same subject.

4.3 Results

4.3.1 BAL species diversity

Table 4 shows the clinical characteristics of the three subject groups from whom BAL was obtained. BAL from severe asthmatics contained significantly higher number of T-RFLP profile bands than from mild asthmatic, $p<0.001$ and healthy control subjects, $p=0.029$ [Mean band number (SD) 6.70 (2.93) vs 3.65 (2.35) vs 3.86 (2.91) respectively]. There was no significant difference between the number of species identified in mild asthmatic and healthy control distal airways, $p=0.848$ (see Table 5 and Figure 4).

Table 6 demonstrates the species (T-RFLP band corresponding to species genus) and rates of detection in the three subject groups in bronchoalveolar lavage.

4.3.2 Induced Sputum species diversity

Table 7 shows the clinical characteristics of subjects who provided induced sputa. Induced sputa from severe asthmatics demonstrated significantly fewer T-RFLP profile bands than from mild asthmatic ($p=0.012$) and healthy control subjects with no history of airways disease ($p=0.005$) [Mean (SD) T-RFLP bands 7.78 (3.59) vs 10.91 (2.95) vs 11.78 (3.87) respectively]. The number of species detected in mild asthma and healthy control induced sputum was not significantly different (see Table 8 and Figure 5).

Table 9 demonstrates the species (T-RFLP band corresponding to species genus) and rates of detection in the three subject groups in induced sputa.

Table 4 Subject clinical characteristics (BAL sampling)

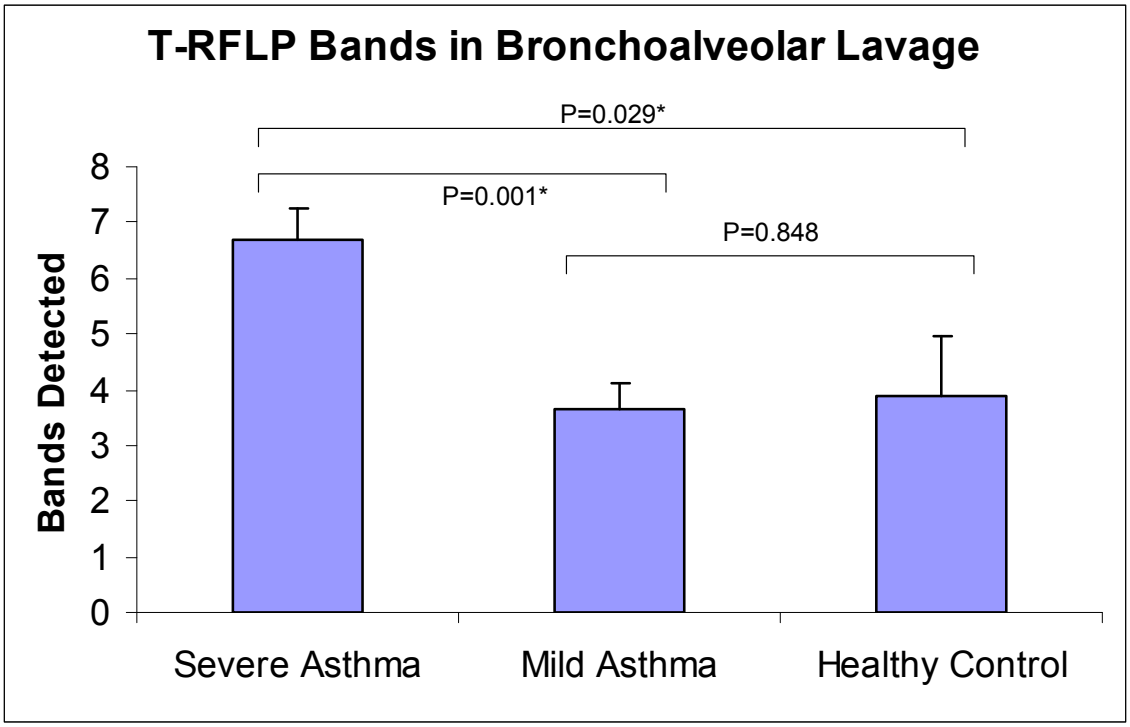
BAL Subjects	Severe Asthma (N=27)	Mild Asthma (N=26)	Healthy Control (N=7)	
Age^	43.7 (12.7)	27.5 (7.1)	27.9 (7.3)	p<0.001 (ANOVA)
Sex M/F	20/7	18/8	4/3	p=0.680 (Chi-square)
Ex-Smokers	12	2	0	p=0.02 (Chi-square)
Pack years^^	0 (3.0)	0 (0)	0 (0)	p=0.001 (Kruskal-Wallis)
Asthma duration(years)^	24.8 (16.0)	15.6 (10.0)	N/A	p=0.016 (t test)
FEV_1 Post BD (% Predicted)^^	81.1 (33.9)	104.7 (16.7)	108.5 (11.3	p=0.001 (Kruskal-Wallis)
FEV_1 Reversibility (%)^	9.5 (8.7)	4.1 (4.3)	N/A	p=0.06 (t test)
PEFR Variability (%)^	29.3 (15.4)	14.6 (8.5)	N/A	p=0.01 (t test)
ACQ^	2.88 (1.03)	0.81 (0.57)	N/A	p<0.001 (t test)
ENO^ (ppb)	12.6 (10.4)	22.5 (26.5)	13.3 (5.7)	p=0.206 (ANOVA)
BDP mg eq/day^	2202 (1376)	15 (78)	N/A	p<0.001 (t test)
Maintenance Prednisolone^^ mg/day	N=15/27 10 (19)	N/A	N/A	

^ Mean (SD) ^^ Median (IQR)

Table 5 Mean T-RFLP band identification in BAL

	Number	Mean T-RFLP Bands in BAL	Std. Deviation	Std. Error Mean
Severe Asthma	27	6.70	2.93	0.56
Mild Asthma	26	3.65	2.35	0.46
Healthy Control	7	3.86	2.91	1.10

Figure 4 Mean T-RFLP band identification in BAL



BAL T-RF Bands Detected	Severe Asthma (N=27)	Mild Asthma (N=26)	Healthy Control (N=7)	Pearson Chi-squared
Haemophilus sp.(1)	22	5	2	**p<0.001**
Streptococcus sp.(2)	16	4	2	**p=0.004**
Moraxella catarrhalis	4	0	0	p=0.073
Pseudomonas aeruginosa	2	6	1	p=0.279
Staphylococcus aureus	8	7	2	p=0.976
Veillonella sp.(3)	15	3	2	**p=0.003**
Veillonella sp.	1	0	0	p=0.537
Prevotella sp.(4)	12	6	1	p=0.142
Neisseria flava	1	0	0	p=0.537
Neisseria subflava, N. elongata	14	14	1	p=0.157
Porphyromonas sp./Prevotella sp.(5)	13	2	1	**p=0.003**
Unassigned species	50	22	13	**p=0.001** (ANOVA)
Selenomonas sp.	0	0	0	
Streptomyces autolyticus, S. thermogriseus	0	0	0	
Fusobacterium periodonticum	11	22	1	**p<0.001**
Neisseria sp.	0	0	0	
Lachnospiraceae bacterium	0	0	0	
P.huttiensis	0	0	0	
Alcaligene xylosoxidans	0	0	0	
Granulicatella adiacens	12	4	1	**p=0.043**
Eubacterium sp.	0	0	0	
Megasphaera sp.	0	0	0	
Granulicatella sp.	0	0	0	
Burkholderia sp./ Granulicatella sp.	0	0	0	
Firmicutes sp.	0	0	0	
Total T-RF bands detected	181	95	27	

(1)Haemophilus sp - H. influenzae, H.segnis, H.parainfluenzae,Terrahaemophilus aromaticivorans
(2)Streptococcus sp. - S. pseudopneumoniae, S. pneumoniae, S. Mitis
(3)Veillonella sp. - V. atypical, V. parvular, V. criceti, V. ratti
(4)Prevotella sp - P.denticola, P.melanogenica, P. nigrescens, P. veroralis, P. intermedia
(5)Porphyromonas sp./Prevotella sp. - P. loescheii, P. denticola, P. veroralis, P. oris

Table 6 Numbers of species / T-RFLP bands detected in BAL samples

Table 7 Subject clinical characteristics (induced sputum sampling)

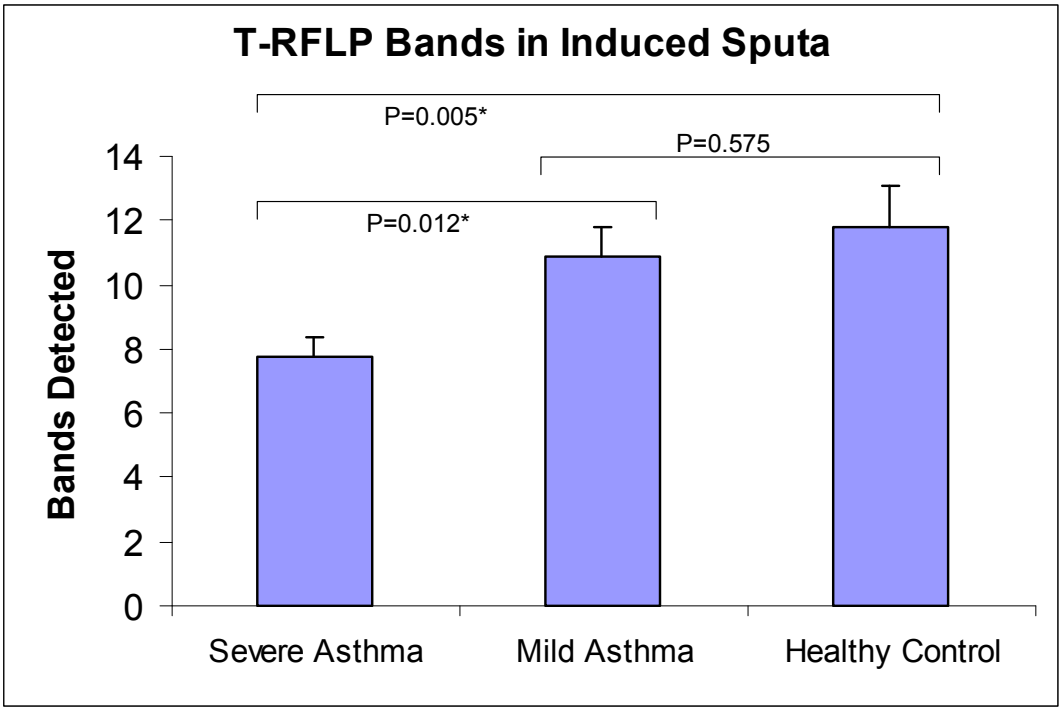
IS Subjects	Severe Asthma (N=36)	Mild Asthma (N=11)	Healthy Control (N=9)	
Age^	47.3 (15.5)	31.8 (10.0)	33.9 (3.9)	p<0.001 (ANOVA)
Sex M/F	6/3	26/10	7/4	p=0.093 (Chi-square)
Ex-Smokers	15	0	2	p=0.027 (Chi-square)
Pack years^^	0 (3.75)	0 (0)	0 (1.0)	p=0.032 (Kruskal-Wallis)
Asthma duration^	26.3 (19.3)	19.3 (13.8)	N/A	p=0.220 (t test)
FEV_1 Post BD (% predicted)^^	79.4 (33.1)	102.3 (20.3)	110 (18.7)	p<0.001 (Kruskal-Wallis)
FEV_1 Reversibility (%)^	5.5 (8.8)	2.6 (5.2)	N/A	p=0.305 (t test)
PEFR variability (%)^	25.7 (15.6)	15.3 (8.3)	N/A	P=0.56 (t test)
ACQ^	2.89 (1.11)	0.66 (0.47)	N/A	p<0.001 (t test)
ENO^	13.6 (9.9)	16.2 (11.6)	10.7 (11.4)	p=0.606 (ANOVA)
BDP mg eq/day^	2252 (1272)	145 (311)	N/A	p<0.001 (t test)
Maintenance Prednisolone^^ mg/day	N=19/36 0 (15)	N/A	N/A	
Squamous cells^^ (%cell diffential)	4.0 (16)	4.0 (6.5)	9.5 (19.6)	p=0.509 (Kruskal-Wallis)

^ Mean (SD) ^^ Median (IQR)

Table 8 Mean T-RFLP band identification in induced sputum

	Number	Mean T-RFLP Bands in Sputum	Std. Deviation	Std. Error Mean
Severe Asthma	36	7.78	3.59	0.60
Mild Asthma	11	10.91	2.95	0.89
Healthy Control	9	11.78	3.87	1.29

Figure 5 Mean T-RFLP band identification in induced sputum



Induced Sputum T-RF Bands Detected	Severe Asthma (N=36)	Mild Asthma (N=11)	Healthy Control (N=9)	Pearson Chi-squared
Haemophilus sp.(1)	28	10	9	p=0.209
Streptococcus sp.(2)	25	5	9	p=0.084
Moraxella catarrhalis	12	5	3	p=0.797
Pseudomonas aeruginosa	8	8	0	**p=0.001**
Staphylococcus aureus	4	8	0	**P<0.001**
Veillonella sp.(3)	26	9	9	p=0.314
Veillonella sp.	11	0	2	p=0.110
Prevotella sp.(4)	13	9	3	**p=0.022**
Neisseria flava	13	0	3	p=0.064
Neisseria subflava, N. elongata	18	11	5	**p=0.011**
Porphyromonas sp./ Prevotella sp.(5)	14	9	4	**p=0.043**
Unassigned species	86	31	39	p=0.128 (ANOVA)
Selenomonas sp.	2	0	3	**p=0.017**
Streptomyces autolyticus, S. thermogriseus	1	0	0	p=0.754
Fusobacterium periodonticum	8	11	5	**p<0.001**
Neisseria sp.	1	0	0	p=0.754
Lachnospiraceae bacterium	3	0	1	p=0.566
P.huttiensis	1	0	0	p=0.754
Alcaligene xylosoxidans	1	0	0	p=0.754
Granulicatella adiacens	1	4	5	**p<0.001**
Eubacterium sp.	1	0	0	p=0.754
Megasphaera sp.	2	0	0	p=0.562
Granulicatella sp.	1	0	0	p=0.754
Burkholderia sp./ Granulicatella sp.	0	0	3	**p<0.001**
Firmicutes sp.	0	0	3	**p<0.001**
Total T-RF bands detected	280	120	106	

(1)Haemophilus sp - H. influenzae, H.segnis, H.parainfluenzae,Terrahaemophilus aromaticivorans
(2)Streptococcus sp. - S. pseudopneumoniae, S. pneumoniae, S. Mitis
(3)Veillonella sp. - V. atypical, V. parvular, V. criceti, V. ratti
(4)Prevotella sp - P.denticola, P.melanogenica, P. nigrescens, P. veroralis, P. intermedia
(5)Porphyromonas sp./Prevotella sp. - P. loescheii, P. denticola, P. veroralis, P. oris

Table 9 Numbers of species / T-RFLP bands detected in induced sputa

4.3.3 Within group comparison of BAL and induced sputum species diversity

Within group comparison showed similar numbers of species detected in the IS and BAL from severe asthmatic subjects [Mean (SD) 7.78 (3.59) vs 6.70 (2.93), p=0.209]. However, significantly higher species diversity was seen in the IS when compared to BAL in both the mild asthma [10.90 (2.95) vs 3.89 (2.61), p<0.001] and healthy control [11.78 (3.87) vs 3.86 (2.91), p<0.001] subject groups.

Group	Specimen	N	Mean	SD	Sig (2-tailed)
Severe Asthma	IS	36	7.78	3.59	p=0.209
	BAL	27	6.70	2.93	
Mild Asthma	IS	11	10.90	2.95	p<0.001
	BAL	26	3.65	2.35	
Healthy Control	IS	9	11.78	3.87	p<0.001
	BAL	7	3.86	2.91	

Table 10 Within group comparison of IS and BAL species diversity

The same comparison of IS vs BAL species diversity was examined in paired, same subject, samples. This showed a significantly reduced number of species isolated from the distal airway in mild asthma (N=6, p<0.001). In severe asthma paired specimens there was no significant increase in species diversity in IS when compared to BAL [8.57 (4.64) vs 5.64 (3.27), p=0.086].

A. Paired Samples Severe Asthma (N=14)	Mean	SD	Sig (2 tailed)
IS T-RFLP bands	8.57	4.64	p=0.086
BAL T-RFLP bands	5.64	3.27	

B. Paired Samples Mild Asthma (N=6)	Mean	SD	Sig (2 tailed)
IS T-RFLP bands	11.83	2.23	p<0.001
BAL T-RFLP bands	3.00	1.10	

Table 11 A and B Mean T-RFLP profile band detection in paired IS and BAL samples from severe and mild asthmatics.

4.4 Discussion

BAL sampling of the distal lower airway showed a higher diversity of species within the severe asthma group (measured by number of species detected) when compared to mild asthmatics and healthy controls. However this effect was reversed in the more proximal airways when sampled using induced sputum. Here, the diversity of species within the severe asthmatic proximal airway demonstrated significantly fewer species of greater abundance than the more diverse, less abundant species found in the healthy and mild asthmatic airway.

Therefore in the proximal lower airway in severe asthma, a smaller number of species are making up the total airway flora, suggesting specific species may be overrepresented at the expense of species that may comprise a “normal”, diverse colonising flora. An immediate, and obvious analogy is with the flora of the gut where a diverse, mixed colonization is seen in health but where success and overgrowth of a single species is associated with disease[97].

Within the mild asthma and healthy groups significantly more species were identified in induced sputum than BAL samples. Rather than representing distinct proximal and distal compartments with differing flora it is likely that there is a spectrum of species diversity with a decreasing number of species represented the more distal the airway sampling.

This diminution in the number of species isolated as sampling moves more distal within the airways, however, was not seen in severe asthma. There was no significant decrease in the number of species identified proximally with induced sputum when compared to the more distal sampling with brochoalveolar lavage. This would seem to indicate a homogeneity in species diversity through the bronchial tree in treatment resistant, severe, disease.

Disease related change in the environment of the lower airway, for example failure of innate immune mechanisms, may enable the proliferation of selected species at the expense of other, less potentially pathogenic, micro-organisms. Treatment differences between the groups may also be aetiologically significant. Although subjects were excluded if they had a course of antibiotics within 6 weeks of

sampling, the severe asthma group had significantly higher numbers of courses of antibiotics in the preceding year. All subjects in the severe asthma group were on high dose inhaled corticosteroids which is a well recognised risk factor for pneumonia[98;99], and more than half were on maintenance oral prednisolone therapy.

No current smokers were recruited but 12/27 severe asthmatics in whom BAL was collected and 15/36 in whom sputum was induced, were ex-smokers. However numbers of pack years smoked in the severe asthmatic group was extremely low [median(IQR) 0 pack years (3.0) in the BAL group and 0(3.75) in the IS group]. This would suggest that smoking history would not account for differences seen in colonising flora

Due to small numbers of subjects who had contemporaneous paired IS and BAL sampling, comparison of mean species number was performed on a whole group basis. However, a small number of subjects (14 severe and 6 mild asthmatics) did have paired samples and comparison of distal and proximal species diversity validating the results from whole group comparisons.

Within the severe asthma group the reduced gradient in species number seen between the proximal and distal lower airway leads to the question of whether a homogenous colony made up of the same species exists in these subjects. 14 severe asthmatic subjects provided paired specimens, a number too small to lend statistical power to comparisons of specific species detection and abundance within the two samples. However *Haemophilus species*, the most commonly detected PPM (detected in 11/14 sputa and 11/14 BAL specimens), when found to be the dominant species in sputum was associated with dominance in BAL (dominant in 3/14 sputa, 4/14 BAL, p=0.011, Fisher's exact test). This may suggest that in severe asthma a similar species abundance as well as diversity may exist throughout the lower airway.
We will therefore need to examine the relative abundance of specific potentially pathogenic species within the lower airway, in disease and health, to look for overrepresentation of specific species that disrupt the normal flora.

Table 6 and Table 9 show the species identified within the BAL and induced sputum samples. A wide range of organisms are seen including the potential

respiratory pathogens *M. catarrhalis, S. aureus* and *P. aeruginosa*. *H. influenzae* is not identified as a single T-RFLP band but as part of the *Haemophilus sp.* genus. *S. pneumoniae* is included in the *Streptococcus sp.* T-RFLP band. Associations between the relative abundance of the PPMs and disease status is examined in chapter 5 and will not be discussed further here.

Of note, no T-RFLP signal was seen for *C. pneumoniae* or *M. pneumoniae* at a detection threshold of 0.01% of total bacterial signal. This may suggest that either these species are not seen in this severe asthma cohort or that their abundance is below the level of detection.

A variety of anaerobic Gram-negative species not typically associated with respiratory disease were detected including *Veillonella sp.*, a cocci found as normal flora of the intestine and oropharynx and rarely implicated in human disease; *Porphyromonas* and *Prevotella* species, anaerobes of the upper GI tract associated with periodontal disease[100;101]; and *Fusobacterium periodonticum*, a rod found as part of normal gut and dental flora[102].

Non-pathogenic *Neisseria* species were also commonly identified in all 3 subject groups. These species are also recognised members of the oral "microbiome"[103]. *Granulicatella adiacens*, previously known as a nutritionally variant streptococcus then as *Abiotrophia adiacens* before a genus redesignation, is an oral cavity commensal which has rarely been reported to cause bacteraemia and endocarditis[104;105].

These oropharyngeal species are not recognised as respiratory pathogens and have not previously been described as significant colonising flora in airways disease. The mechanism by which they are found in both asthmatic and healthy subject respiratory specimens cannot readily be explained. Aerosolised oral cavity bacteria may be inhaled through normal respiration or microaspiration may be a normal phenomenon. These species will then be "concentrated" by the mucociliary escalator. Identification of these species in BAL suggests that salivary contamination of induced sputum as it travels through the oropharynx does not explain the high rate of oral commensal identification in sputa.

The oral commensal species seen in significantly higher prevalence in severe asthmatic BAL than in mild asthmatic subjects were *Veillonella species* (*V. atypical, V. parvular, V. criceti, V. ratti*), *Porphyromonas sp./Prevotella sp.* (*P. loescheii, P. denticola, P. veroralis, P. oris*) and *Granulicatella adiacens*. Although *Fusobacterium periodonticum* showed a significantly higher prevalence in mild asthmatic samples.

Gastro-oesophageal reflux (GORD) has long been recognised as a trigger factor in persistent severe asthma. Both scintigraphic technetium monitoring[106] and simultaneous tracheal / oesophageal pH monitoring[107] have been used to demonstrate microaspiration in the presence of GORD in asthma which may deposit oropharyngeal or gastro intestinal species in the respiratory tract.

T-RF bands not corresponding to band size markers are designated unassigned species bands. No significant difference in numbers of unidentified species was seen between the 3 study populations in induced sputum. Mean (SD) unidentified species in BAL in both severe asthma [1.85 (1.1)] and health controls [1.86 (1.1)] was significantly higher than in mild asthma [0.85(0.6), $p<0.001$ and $p=0.003$ respectively]. Mean unidentified species were the same in severe asthmatic BAL samples as healthy controls. Although we cannot exclude unidentified species as being aetiologically significant in our asthma cohort, it is reassuring that these species are no more common in severe asthma than in healthy controls.

We have demonstrated a different distribution of airway flora in the severe asthmatic airway to the mild asthmatic and healthy airway. In severe asthma we see a lower number of species within the central airways sampled by induced sputum but preservation of species number moving more distally to the smaller airways sampled by BAL. This contrasts with the high species diversity found in proximal airways of mild asthmatics and healthy controls with diminishing numbers of species seen in distal airway sampling.
In later chapters we will look at differences in detection rates, dominance and relative abundance of specific species known to be pathogens within the respiratory tract.

5 Potentially pathogenic species abundance in asthmatic and healthy control subjects

5.1 Aim

To determine the frequency of detection, dominance and relative abundance of potentially pathogenic species in BAL and induced sputum from patients with severe asthma, mild asthma and healthy control subjects.

5.2 Methods

The recognised respiratory pathogens *Moraxella catarrhalis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Haemophilus sp.* (including *Haemophilus influenzae, segnis, parainfluenzae, Terrhaemophilus aromaticivorans*) and *Streptococcal sp.* (including *S. pseudopneumoniae, S. pneumoniae, S. mitis*) were identified in BAL and induced sputum from asthmatics and controls using T-RFLP profiling as previously described.

Rates of detection and abundance of specific species were recorded and compared between subject groups. In BAL specimens the total combined PPM abundance was calculated and compared between groups.

In induced sputum a total combined abundance of *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* was calculated and compared between subject groups.

Species were reported as detected if band signal was greater than 0.01% of the total electrophoretic strip.

5.3 Statistics

Detection rates between subject groups were compared using a Chi-Square test or Fisher's exact test if any cell had an expected cell count of less than 5. Species abundance, as described as a percentage of total bacteria load, was not normally distributed, therefore median species abundance were compared using the Mann-Whitney test.

5.4 Results

The clinical characteristics of the three subject groups are previously described in Table 4 and Table 7.

5.4.1 Bronchoalveolar lavage

PPMs were detected in 26/27 (96%) of severe asthma, 11/26 (42%) mild asthma and 3/7 (43%) of healthy control BAL specimens. PPM detection was significantly higher in severe asthma than mild asthma ($p<0.001$, Chi-square) and healthy control ($p=0.003$, Fischer's exact test) BAL.

Within the BAL specimens *Haemophilus sp.* detection was significantly higher in severe asthma (22/27, 81%) than in mild asthma (5/26, 19%) ($p<0.001$) and healthy controls (2/7, 29%) ($p=0.014$).

M. catarrhalis was detected in 3/27 (11%) of severe asthma specimens but no mild asthma or healthy control BAL.

Streptococcal sp. detection was significantly higher in severe asthma (16/27, 59%) than mild asthma (4/26, 15%) ($p=0.001$) but not healthy control BAL (2/7, 29%) ($p=0.214$).

S. aureus detection rates in severe (8/27, 30%), mild asthma (7/26, 27%) and healthy controls (2/7, 29%) were similar.

P. aeruginosa detection was highest in the mild asthma group (6/26, 23%) but did not differ significantly from severe asthma (2/27, 7%) or healthy control (1/7, 14%) groups.

Table 12 and Figure 7 show the relative percentage abundance of individual and total PPMs within the subject groups. Severe asthma BAL specimens demonstrated significantly higher median abundance of *Haemophilus sp* ($p<0.001$), *M. catarrhalis* ($p=0.043$), *Streptococcal sp.* ($p<0.001$), and total PPM ($p<0.001$), than mild asthmatic samples. Relative abundance of *S. aureus* was highest in severe asthma but was not statistically significantly higher than in mild asthma or healthy controls. Relative abundance of the *P. aeruginosa* was not significantly different between the three groups.

The dominant species was identified by the highest percentage abundance of all species detected within the sample. A PPM was the dominant species more often in severe asthma (15/27, 55.6%) than mild asthma (6/26, 23.1%, $p=0.016$) and healthy controls (1/7, 14.3%, $p=0.090$). *Haemophilus sp.* were the most common dominant species in severe asthma [9/27, 33.3% vs 1/26, 3.8% mild asthma ($p=0.011$) and 0/7, 0% of healthy controls ($p=0.151$)].

Bronchoalveolar Lavage Species	Group	N	Species Detected	Dominant Species	Mean	Std Deviation	Median	IQR
Haemophilus sp.	Severe asthma	27	22	9	21.77	25.2	12.7	26.1
	Mild asthma	26	5	1	1.91	6.1	0	0
	Healthy control	7	2	0	3.35	7.7	0	2.8
Moraxella catarrhalis	Severe asthma	27	3	1	3.87	16.1	0	0
	Mild asthma	26	0	0	0	0	0	0
	Healthy control	7	0	0	0	0	0	0
Streptococcus sp.	Severe asthma	27	16	2	8.91	10.5	5.1	20.1
	Mild asthma	26	4	0	0.93	3.4	0	0
	Healthy control	7	2	1	9.41	22.3	0	6.3
Staph. Aureus	Severe asthma	27	8	4	13.09	29.8	0	3.9
	Mild asthma	26	7	4	11.1	25.0	0	3.4
	Healthy control	7	2	0	0.45	0.8	0	1.3
Pseudomomas aeruginosa	Severe asthma	27	2	0	0.30	1.3	0	0
	Mild asthma	26	6	1	4.96	13.9	0	0.5
	Healthy control	7	1	0	0.33	.9	0	0
Total PPM abundance	Severe asthma	27	26	15	47.95	29.2	42.5	52.4
	Mild asthma	26	11	6	18.9	31.6	0	31.9
	Healthy control	7	3	1	13.5	22.7	0	20.6

Table 12 Mean and median percentage abundance of potentially pathogenic species in induced sputa from severe asthmatics, mild asthmatics and healthy controls.

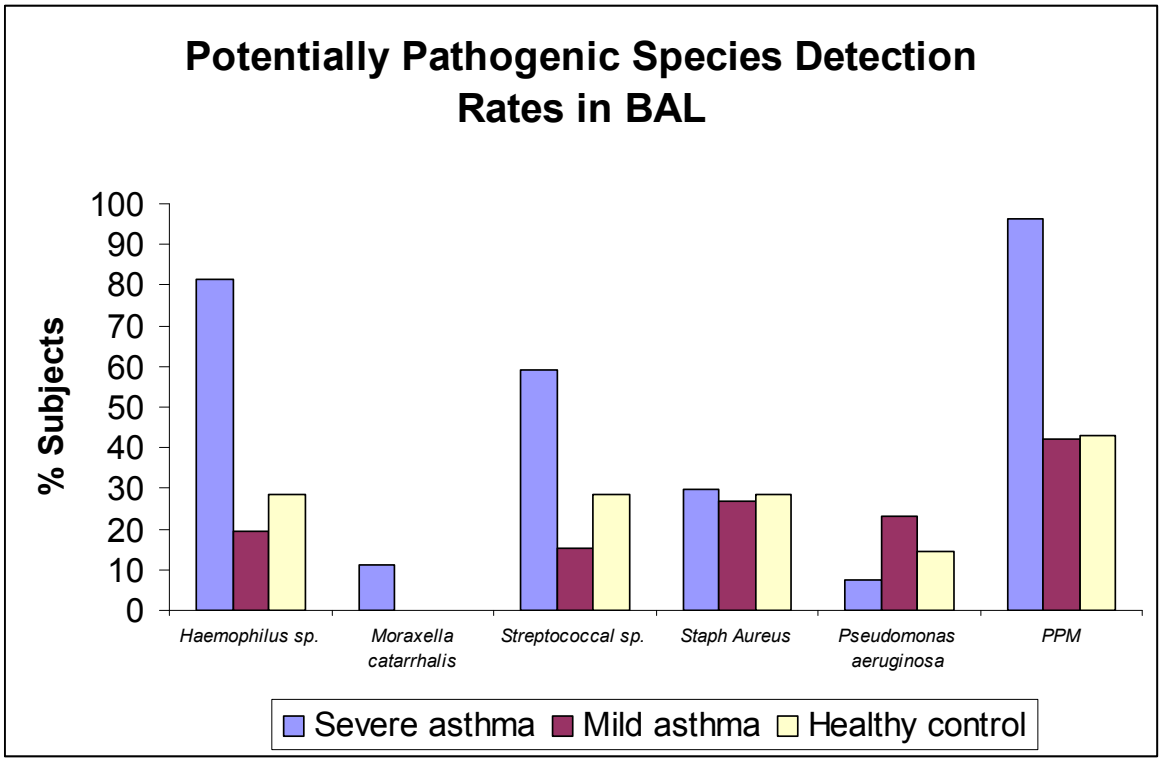


Figure 6 PPM detection in BAL

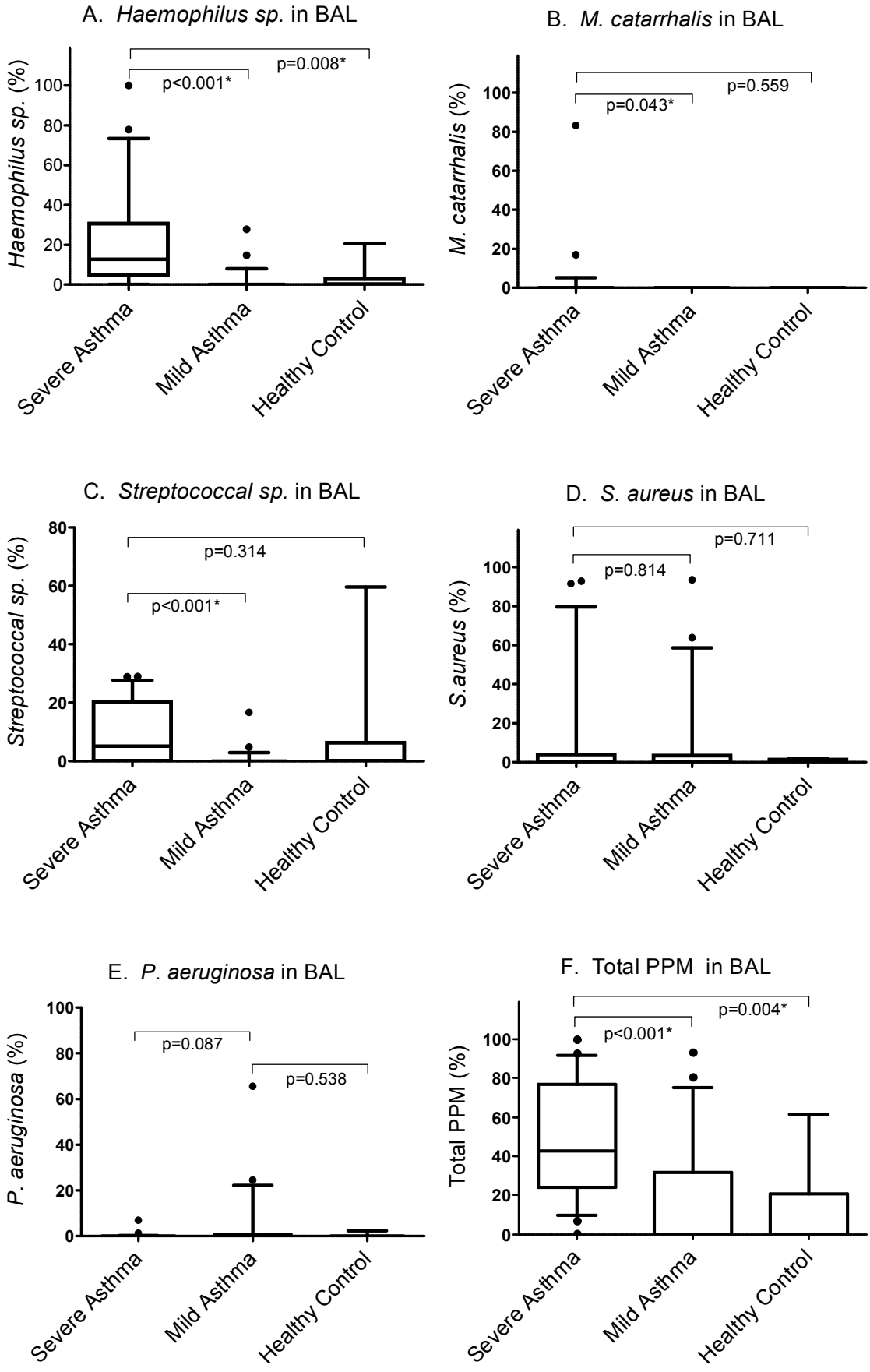


Figure 7A-F Median abundance of PPMs in BAL severe asthma, mild asthma and healthy control subjects

5.4.2 Induced sputum

Haemophilus sp. detection rates were high in induced sputum in all three subject groups with no significant difference between severe asthma (28/36, 78%), mild asthma (10/11, 91%) and healthy controls (9/9, 100%).

M. catarrhalis detection rates in severe asthma (10/36, 28%), mild asthma (5/11, 45%) and healthy subjects (3/9, 33%) were not significantly different.

Streptococcal sp. detection rates were also not significantly different between severe asthma (24/36, 67%), mild asthma (5/11, 45%) and healthy controls (9/9, 100%).

S.aureus was detected significantly more frequently in mild asthma (8/11, 73%) than in severe asthma (4/36, 11%) ($p<0.001$), and healthy control induced sputum (0/9, 0%) ($p=0.001$).

P. aeruginosa was detected significantly more frequently in mild asthma (8/11, 73%), than in severe asthma (10/36, 28%) ($p=0.012$), and healthy control sputum (0/9, 0%) ($p=0.001$). Co-colonization with *P.aeruginosa* and *S. aureus* was seen in 6/11 (55%) mild asthmatic subjects ($p=1.0$).

Haemophilus sp., M catarrhalis or *Streptococcal sp.* were detected in the majority of severe asthma (34/36, 94%), mild asthma (10/11, 91%) and healthy control (9/9, 100%) specimens. The total combined abundance of these species was significantly higher in severe than mild asthma (median 44.84% versus 17.57%, $p=0.002$).

Table 13 and Figure 9 show the relative abundance of individual and combined PPMs in induced sputum from the three subject groups.

Induced Sputum Species	Group	N	Species Detected	Dominant Species	Mean	Std Deviation	Median	IQR
Haemophilus sp.	Severe asthma	36	28	8	21.69	24.8	15.06	30.63
	Mild asthma	11	10	3	11.12	9.37	7.56	16.23
	Healthy control	9	9	2	17.33	12.76	17.56	27.48
Moraxella catarrhalis	Severe asthma	36	10	5	12.09	25.7	0	11.34
	Mild asthma	11	5	0	0.74	1	0	1.46
	Healthy control	9	3	0	1.21	2.58	0	1.57
Streptococcus sp.	Severe asthma	36	24	6	13.57	18.69	7.04	20.24
	Mild asthma	11	5	0	4.26	7.52	0	6.54
	Healthy control	9	9	1	11.04	9.22	10.61	14.35
Staph. Aureus	Severe asthma	36	4	0	0.38	1.38	0	0
	Mild asthma	11	8	1	8.8	11.05	4.86	12.85
	Healthy control	9	0	0	0	0	0	0
Pseudomomas aeruginosa	Severe asthma	36	10	1	4.14	11.77	0	1.17
	Mild asthma	11	8	1	6.87	8.31	4.58	9.78
	Healthy control	9	0	0	0	0	0	0
Total *Haemophilus sp.*, *M. catarrhalis* and *Streptococcal sp.*	Severe asthma	36	34	19	47.36	29.61	44.84	49.3
	Mild asthma	11	10	3	16.1	13.0	17.57	19.0
	Healthy control	9	9	3	29.59	14.1	28.36	23.6

Table 13 Mean and median abundance of potentially pathogenic species in induced sputa from severe asthmatics, mild asthmatics and healthy controls.

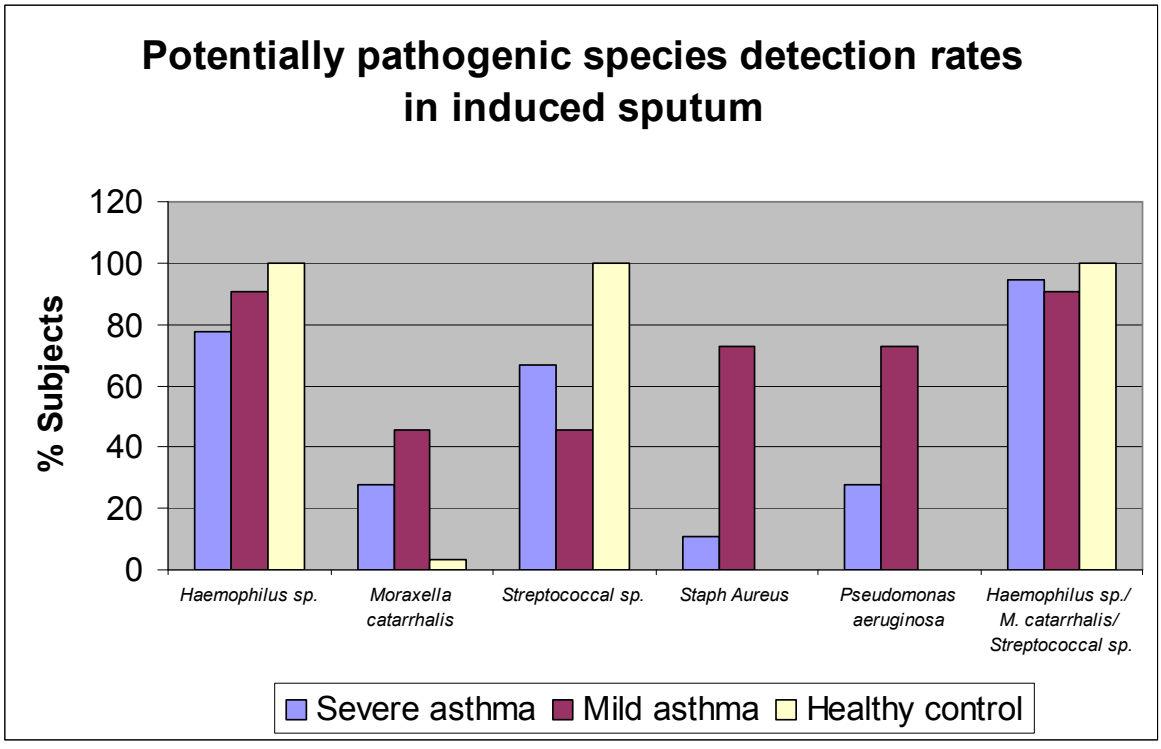


Figure 8 PPM detection in induced sputum

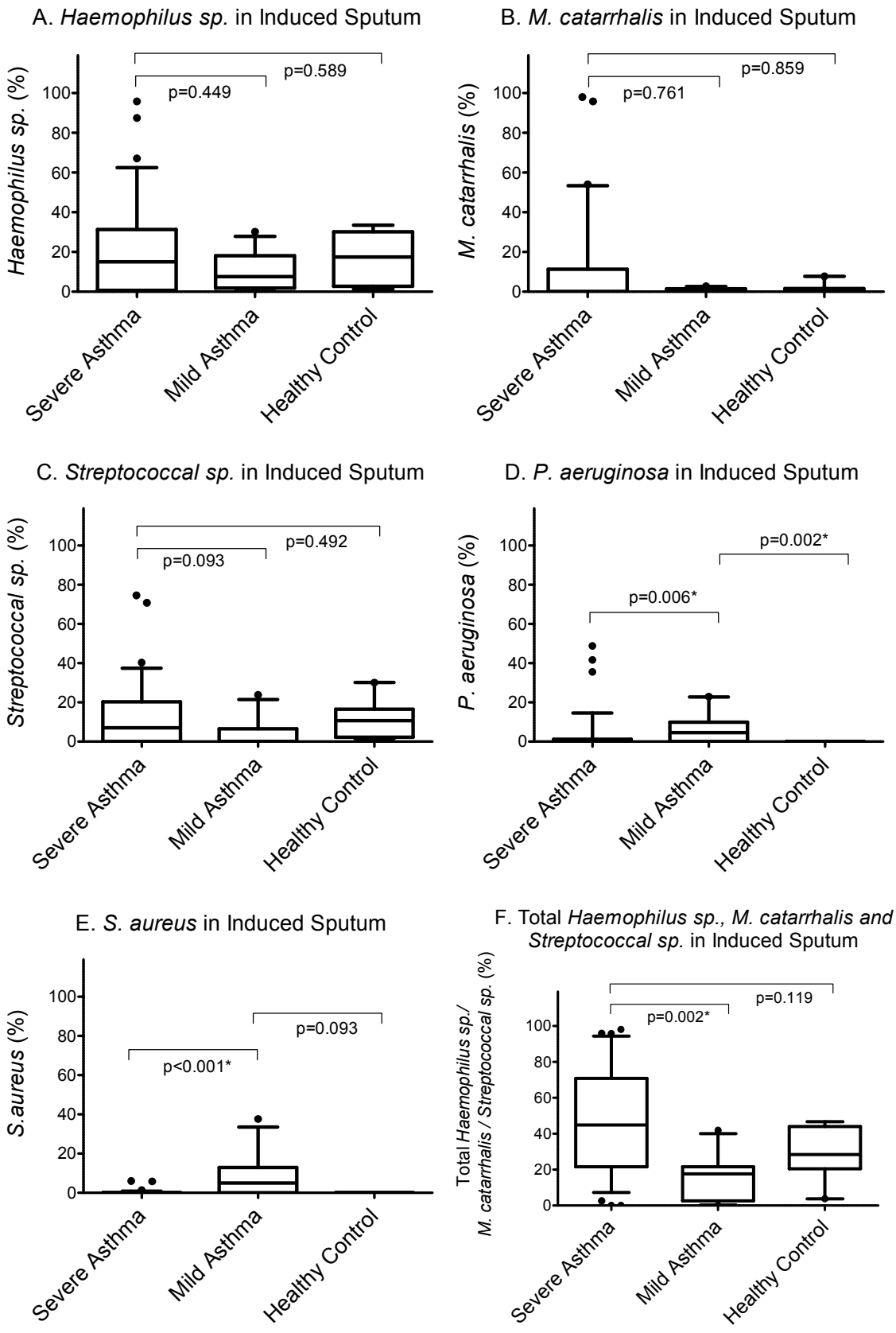


Figure 9 A-F Median abundance PPMs in induced sputa from severe asthmatics, mild asthmatics and healthy controls

5.4.3 Induced sputum and BAL dominant species abundance

In severe asthma BAL samples, potentially pathogenic species were the dominant (most abundant by percentage of total bacterial load) species in 15/27 subjects. The degree to which the first dominant species was the primary airway colonising organism was examined by comparison of median abundance of PPM and non PPM dominant species. The median abundance of the dominant species was significantly higher if it was found to be potentially pathogenic (p=0.036) (Table 14).

In severe asthma induced sputa, *Haemophilus sp./ M. catarrhalis /Streptococcal sp* .where the dominant species in 17/36 subjects. The median abundance of the dominant species in induced sputum was significantly higher if it was found to be one of these three potentially pathogenic species (p=0.013) (Table 15).

Severe Asthmatic Subjects	BAL	N	Median	IQR	
BAL Dominant Species Abundance (%) in severe asthma	Dominant PPM	15	68.5	54.4	p=0.036
	Dominant Non-PPM	12	32.4	22.6	

Table 14 Median abundance of dominant species in BAL

Severe Asthmatic Subjects	Sputum *Haemophilus sp./ M. catarrhalis /Streptococcal sp.* Dominant	N	Median	IQR	
Sputum Dominant Species Abundance (%) in severe asthma	Yes	19	54.0	38.4	p=0.013
	No	17	37.1	13.2	

Table 15 Median abundance of dominant species in induced sputa

5.5 Discussion

Patterns of detection of PPMs in BAL differed significantly between the three subject groups. BAL detection of any PPM was significantly higher in severe asthma than mild asthma and healthy controls. *Haemophilus sp.* and *Streptococcal sp.* were more commonly detected in severe asthma and showed significantly higher percentage abundance than in mild asthma. *M. catarrhalis* colonization of BAL specimens was only seen in severe asthma. *S. aureus* and *P. aeruginosa* detection rates were not significantly different in the lower airway specimens from the three subject groups.

A PPM was significantly more likely to be the dominant BAL airway species in severe asthma than in mild asthma. When comparing dominant species relative abundance within the severe asthma group, PPMs showed significantly higher abundance than non-PPM species.

This would suggest an overrepresentation of species that are potentially pathogenic within the respiratory tract in the BAL from severe asthmatic subjects. Furthermore, when these species are the dominant species, their relative abundance is greater, implying an excessive overgrowth when compared to non-PPMs.

Examination of induced sputa showed high detection rates for *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* with no significant difference between the three subject groups. Abundance of each of these three species was higher in severe than mild asthma but this did not reach significance. However, the combined total abundance of *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* was significantly higher in severe asthma when compared to the mild asthma group. When one of these three species was the dominant airway species within the severe asthma group, it showed significantly higher abundance as a percentage of total bacterial colonization than when compared to samples where non-PPMs were the dominant species.

Therefore, in the proximal lower airway sampled by induced sputum, *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* are the common potentially pathogenic species and are able to cause an overwhelming colonization when they become the dominant species.

These three species were selected for analysis as a group as they have previously been defined as the most clinically relevant colonising pathogenic species in the sputum of COPD patients[20;21;108], and have been implicated in the development of asthma in children following neonatal colonization[47]. In COPD, sputum

colonization with these species is associated with higher markers of neutrophilic inflammation including neutrophil elastase and myeloperoxidase and the neutrophil chemotractant IL-8[19;109]. This type of inflammation in asthma is associated with treatment resistant disease[110;111]. Therefore, the role of these three species needs to be determined in the context of asthma phenotype by comparison of clinical characteristics and airway inflammation within the severe asthma group.

Pseudomonas aeruginosa and S*taphylococcus aureus* colonization was only seen in asthmatic sputum specimens. Detection rates were significantly higher in mild asthma than severe asthma and median abundance of both *P. aeruginosa* and *S. aureus* was significantly higher in mild asthma than in severe asthma.

The increased incidence of these two pathogens together in mild disease is noteworthy. Correlation between the two species in the mild asthma group was not found to be significant, though this may be due to the small sample size. Synergy between *P. aeruginosa* and *S. aureus* has long been recognised in cystic fibrosis[112] where *S. aureus* is traditionally thought to sensitise the airway to *Pseudomonas* colonization. *P. aeruginosa* is now also believed to promote further colonization with other pathogenic species[113]. The mild and severe asthma groups investigated certainly represent different phenotypes. Some mild subjects may go on to develop a more severe phenotype, but without a longitudinal study of airway colonization we cannot know whether colonization with P. aeruginosa and S. aureus is an early feature of mild disease which can develop into state were *Haemophilus sp., M. catarrhalis* or *Streptococcal sp.* become the highly abundant dominant species as a more severe phenotype develops.

The process of T-RFLP profiling is able to detect the presence of, and estimate the relative abundance in relation to total bacterial signal, of single bacterial species such as *S.aureus* and *M.catarrhalis*. However *Haemophilus sp.* and *Streptococcal sp.* are each reported as a whole species genus. *Haemophilus influenzae* and *Streptococcus pneumoniae* are recognised respiratory pathogens playing a significant role in the pathogenesis of both stable disease and exacerbation in COPD[20;114]. However other *Haemophilus* species are also recognised respiratory pathogens including *H. parainfluenzae, H. hemolyticus* which have both been reported as airway colonising pathogens in COPD[22]. Non *S. pnemoniae* streptococcal species have previously been isolated in COPD[91].

Explanation of the apparent success of airway colonization by potentially pathogenic species needs to consider multiple factors including phenotypic, demographic and treatment differences.

None of the subjects recruited were current smokers. A significantly higher number of severe asthmatics were ex-smokers than in the healthy or mild asthma groups. Smoking has previously been associated with PPM airway colonization[115] through impairment of mucociliary clearance. The median number of pack years smoked was extremely low and within the severe asthmatic group smoking history did not influence PPM dominance rates. Therefore smoking history differences between the groups are not felt to be clinically relevant to between group differences in colonization rates.

The severe asthmatic group were on high dose inhaled corticosteroid (ICS) treatment and many were on oral prednisolone maintenance therapy (19/36 of induced sputum group and 15/27 BAL group). ICS impairs alveolar phagocytosis of bacteria[116] and its use in COPD is associated with increased risk of pneumonia[98;99]. The long term use of oral corticosteroids is also associated with an increased risk of pneumonia[117]. However, within the severe asthma group, there was no significant relationship between oral steroid use and PPM dominant airway colonization in BAL ($p=0.603$) or induced sputum ($p=0.709$).

Further investigation of the role of PPM airway colonization in asthma requires investigation of the airway inflammation and phenotype of colonized subjects.

6 Linking Bacterial Airway Colonization to the Innate immune response and Neutrophilic Inflammation

6.1 Aims

Severe asthma is a heterogenous disease with distinct pathological subphenotypes [7]. Induced sputum inflammatory differential cell counts can be used to classify airway inflammation as eosinophilic and non-eosinophilic [118]. Furthermore patients with greater than 61% neutrophil differential count can be described as having neutrophilic asthma[119]. Both non-eosinophilic and neutrophilic asthma have been associated with steroid resistant disease with poor response to inhaled corticosteroids[120;120-122].

The development of neutrophilic airways inflammation has been linked to the activation of the innate immune response[37;123].Endotoxins, soluble fragments of lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria, and Gram-positive cell wall fragments such as lipoteichoic acid (LTA), can act as pathogen associated molecular patterns (PAMPs) which are recognised by Toll-like receptors, CD14 and collectins. Activation of Toll-like receptors leads to an inflammatory cascade resulting in production of the pro-inflammatory cytokines IL-8, IL-1 and tumour necrosis factor α (TNF α)[35] leading to a shift toward a TH 1 response, neutrophil recruitment and a change in inflammatory cell differential profile.

Simpson et al. demonstrated increased mRNA expression of the innate immune receptors TLR2, TLR4 and CD14, as well as the cytokines IL-8 and IL-1β in induced sputum from patients with neutrophilic asthma[37].Neutrophilic airways inflammation was associated with higher airway LPS and potentially pathogenic bacteria were cultured in 43%, suggesting activation of the innate immune response by colonising airway bacteria may explain the heterogeneity of inflammatory response seen in severe asthma.

Few studies have looked at the role of airway colonising bacteria in stable asthma. However several studies have looked at the role of potentially pathogenic micro-organisms (PPMs), predominantly *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, in stable COPD[19-22]. Colonization of the

lower airway with these organisms is associated with increased levels of the neutrophil chemotractant IL-8[20], TNFα[19], absolute neutrophil counts and endotoxin[22].

Colonization with PPMs is also associated with worse clinical outcomes including health status as measured by the St. Georges Respiratory Questionnaire[19], exacerbation rates[21] and accelerated FEV1 decline[20].

We aimed to use T-RFLP to describe the abundance and dominance of *Haemophilus sp.*, *Streptococcus sp*., and *Moraxella catarrhalis* in induced sputum from stable severe asthmatics. Findings were correlated with clinical characteristics and markers of innate immune activation, interleukin-8 (IL-8) and neutrophil differential cell counts.

6.2 Methods

28 subjects were recruited with a clinical diagnosis of severe asthma as defined by the American Thoracic Society workshop on refractory asthma[77]. All subjects were at treatment step 4 or 5 of the British Thoracic Society asthma management guidelines[78] with 13 subjects on daily maintenance , oral corticosteroid therapy. All subjects were stable with a minimum of six weeks since last exacerbation or treatment with antibiotics.

History, including symptoms, exacerbation rate, medication and smoking history were recorded. Atopy was defined as skin prick positivity to one or more aeroallergens. Exhaled nitric oxide was measured using the Logan LR2500 (Logan Research, UK) trace gas and nitric oxide analyser system.

Pre and 15 minutes post salbutamol 200mcg FEV1 was recorded using a Vitalograph spirometer. Symptom scores were assessed using the Asthma Control Questionnaire[79]. Peak flow variability was calculated from a 2 week twice daily diary record (measured as maximum-minimum/ maximum).

6.2.1 Sample collection and processing

Induced sputum was collected in accordance with the European Respiratory Society guidelines[82]. Sputum was induced using 4.5% saline. In patient at risk of severe bronchoconstriction sputum was induced using a modified protocol using 0.9%, 3% or 4.5% saline. Sputum plugs were selected from expectorate and divided for differential cell counting and the remainder stored at -80^{0}c for subsequent T-RFLP.

Sputa for differential cell counts were immediately processed with dithioerythritol to separate cells from the fluid phase of sputum. The homogenised sample was then filtered using a 100µm cell strainer (BD Falcon) to remove mucus. Samples were centrifuged at 1500rpm for 10 minutes (Haraeus Labofuge 400R), the fluid phase supernatant was then removed and frozen at -80^{0}C, and the cell pellet resuspended in 1000µl phosphate buffered saline. A total cell count was made using a haemocytometer and cell viability determined using tryphan blue exclusion.

Cytospins were prepared using 70 x10^3 cells and centrifuged at 450rpm for 6 minutes (Shannon Cytospin 2). Cytospins were fixed with and stained using Rapi-Diff I, II and III (DiaCheM Int. Ltd, UK) and differential cell counts were obtained from 400 non-squamous cells.

6.2.2 Terminal Restriction Fragment Length Polymorphism Profiling

T-RFLP was performed on induced sputum samples as previously described[83]. In brief, bacterial DNA is extracted from the sample. The extracted nucleic acid undergoes PCR amplification using specific primers for the phylogenetically informative 16sRNA gene.

An IR dye primer is added and PCR amplicons are then cleaved using restriction endonuclease digestion. The ribosomal gene fragments are then resolved on a DNA sequencer to form a T-RFLP profile. T-RFLP profiles were analysed using Phoretix 1D Advanced software v.5.10 (Nonlinear Dynamics, Newcastle upon Tyne, UK), with band sizes determined by comparison to size markers (Microzone, Lewes, UK). Band volume (the product of the band area and signal intensity) is determined and expressed as a percentage of the total volume of bands in a given electrophoretic profile to give species abundance as a percentage of total bacteria load within the sample. The species with the highest percentage abundance within the sample was designated the dominant species.

6.2.3 Asthma Subtypes

Subjects were described as having neutrophilic asthma if the neutrophil differential cell count was greater than 61% of non-squamous cells.

6.2.4 Statistics

Data were analysed using SPSS. Non-parametric data are presented as median and inter-quartile range (IQR). Parametric data are presented as mean and

standard deviation. Non-normally distributed data were compared using the nonparametric Mann-Whitney U-test. Correlations between nonparametric data were undertaken using Spearman's rank correlation. Two-tailed tests were used and the level of significance was taken as p=0.05.

6.3 Results

Induced sputum was collected from 28 severe asthmatics for T-RFLP. Induced sputum sample size was insufficient for differential cell counts in 2 patients. Mean squamous cell counts at differential cell counting was 8.1% (SD 11.5). All patients were either ex-smokers (14) or never smokers (14).

The potentially pathogenic micro-organisms (PPMs) *Haemophilus sp.*, *Streptococcus sp.*, or *Moraxella catarrhalis* were identified as the most dominant colonising species in 17/28 patients.

Other dominant species (number) were, *Veillonella sp.* (5), *Neisseria sp.*(2), Unassigned species(1), *Prevotella sp*.(2) and *Pseudomonas aeruginosa*(1). Table 16 shows the number of subjects with each dominant airway species.

Dominant Species	Number	Mean % Abundance (Range)
Haemophilus sp	6	63.9 (26.5-95.8)
Streptococcus sp.	6	47.2 (25.6-74.6)
M. catarrhalis	5	68.5 (41.6-98.0)
Veillonella sp	5	43.8 (27.5-80.2)
Neisseria sp.	2	41.0 (39.6-42.3)
Prevotella sp.	2	30.5 (23.8-37.1)
Unassigned species	1	36.5
P. aeruginosa	1	48.8

Table 16 Induced Sputum dominant species rate and abundance in severe asthma

Table 17 shows the clinical characteristics and differential cell count results for subjects with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* as the most dominant airway species and subjects with other dominant species.

Severe asthma Induced Sputum	Dominant *Haemophilus sp./ Moraxella catarrhalis/ Streptococcus sp.*	Other dominant species	p value
Number	17	11	
Sex, M/F	6/11	1/10	0.191
Age^ [years]	51.7 (29-67)	41.6 (19-76)	0.083
Ex-smokers	8	6	0.699
Smoking pack years^	3.0 (0-30)	6.7 (0-28)	0.073
Maintenance Oral Prednisolone	9	4	0.390
Prednisolone mg/day*	8.4 (9.4)	6.3 (8.4)	0.623
ICS (BDP mcg Eq/day)*	2357 (936)	1927 (671)	0.285
Late onset asthma (after12)	12	6	0.444
Atopic	12	6	0.444
FEV_1 % predicted post-bronchodilator*	68.0 (24.0)	85.5 (19.7)	0.025**
% Reversibility*	5.5 (8.4)	8.8 (12.1)	0.664
PEFR Variability %*	30.0 (17.0)	18.0 (12.4)	0.268
Duration of asthma [years]*	31.8 (16.7)	15.6 (8.0)	0.008**
Exacerbations last 12 months Median (IQR)	4 (3-6)	2 (1-6)	0.290
ACQ Score*	3.03 (1.2)	2.87 (0.88)	0.723
Exhaled NO [ppb]*	12.5 (9.4)	12.5 (8.5)	0.851
% Neutrophil count^^	80 (16)	43 (38)	0.001**
% Eosinophil count^^	0.5 (1.8)	0.3 (1.8)	0.872
% Squamous cell count^^	5 (16)	2.8 (7)	0.418
Neutrophil >61% (number)	13	4	0.014**
Dominant species abundance (%)	54.0 (42.8)	37.1 (19.9)	0.047**
TRFLP bands (Number species detected)*	6.6 (3.3)	9.6 (4.2)	0.042**

* Values are mean (SD),^ Values are mean (Range),** Significant with $p<0.05$

^^ Values are median (IQR)

Table 17 Clinical characteristics (Severe asthma induced sputum)

Table 18 IS severe asthma supernatant cytokine measures

Severe Asthma Induced Sputum Supernatant Cytokine	Dominant *Haemophilus sp.*/ *Moraxella catarrhalis*/ *Streptococcus sp.*	Other dominant species	
Interleukin-8 (pg/ml)	5192 (9805)	1315 (2221)	p=0.08
ECP (ng/ml)	310 (1073)	149 (169)	p=0.298
Gro α (pg/ml)	3309 (4534)	1972 (9954)	p=0.951
α 2 Macroglobulin (ng/ml)	1832 (2380)	1170 (4199)	p=0.501

Median (IQR)

Subjects with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* as the most dominant airway colonising species by percentage abundance had significantly lower FEV_1 post bronchodilator percent predicted than subjects with any other dominant airway bacteria (Mean (SD) 68.0% (24.0) vs 85.5% (19.7), p=0.025). Dominance of these species was associated with longer disease duration (31.8 years (16.7) vs 15.6 years (8.0), p=0.008). Dominant airway colonization with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* was associated with higher neutrophil differential cell count (Median (IQR) 80% (67-83) vs 43% (29-67), p=0.001) and was significantly associated with neutrophilic asthma, defined as neutrophil differential cell count >61% (p=0.014).

Dominant airway colonisation with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* was associated with significantly higher abundance of the dominant species and significantly lower numbers of species detected.

Total abundance of *Haemophilus sp.*, *Streptococcus sp*., and *M. catarrhalis* within sputum samples correlated with neutrophil differential cell count (p=0.037) and with disease duration (p=0.037) (Figure 10 and Figure 11).

IL-8 measures from sputum supernatant were obtained in 26/28 subjects. IL-8 was higher in subjects with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* as the most dominant airway species, although this did not reach significance (p=0.08). However, IL-8 levels did correlate with the total abundance of *Haemophilus sp.*, *Streptococcus sp.,* and *M. catarrhalis* (p=0.027) (Figure 12). The strongest species correlation with IL-8 levels was with *M. catarrhalis* (p=0.014) (Figure 13). IL-8 also correlated with neutrophil count (p=0.001) (Figure 14) and lower FEV_1 post bronchodilator (p=0.03) (Figure 15).

ECP, GROα and α2-macroglobulin were measured in sputum supernatant from 24/28 subjects (16 PPM dominant, 8 other species dominant). Non significant trends toward higher levels in subjects with *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* as the dominant airway species were seen.

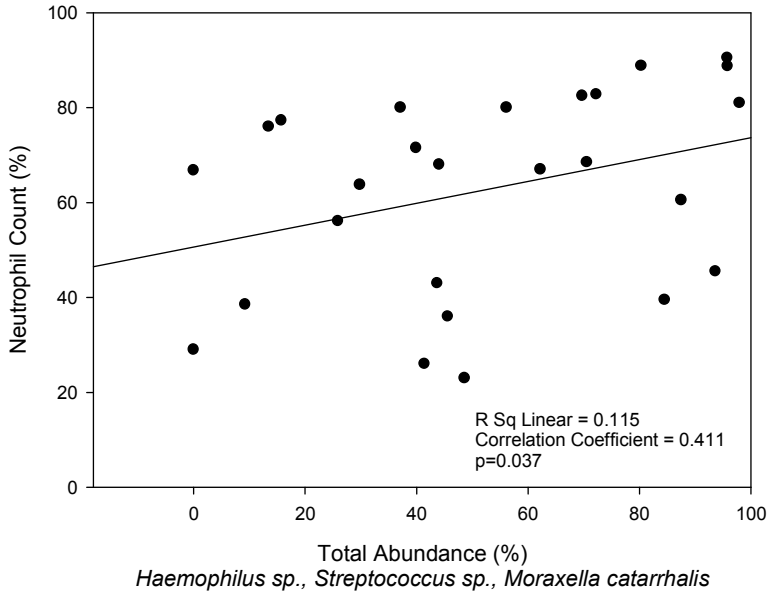


Figure 10 Species abundance and neutrophil count correlation in severe asthmatic induced sputum

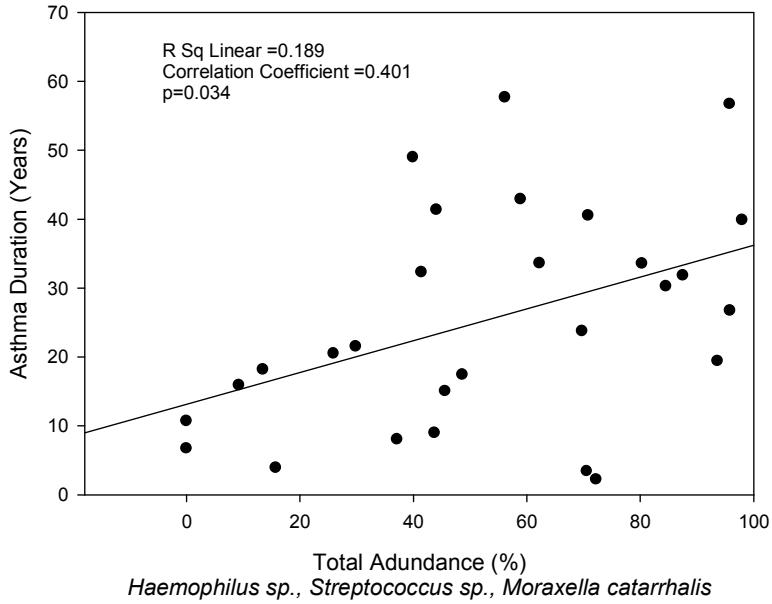


Figure 11 Species abundance and asthma duration in severe asthmatic induced sputum

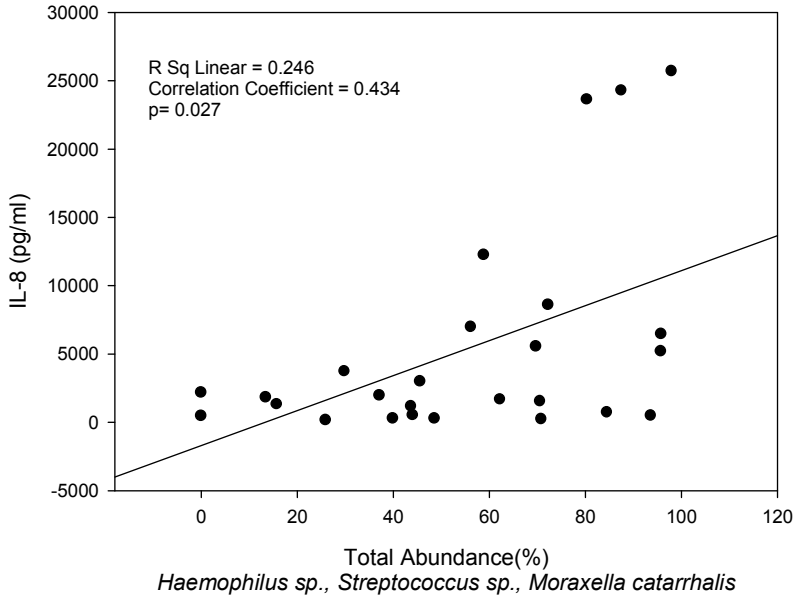


Figure 12 Species abundance and IL-8 correlation in severe asthma induced sputum

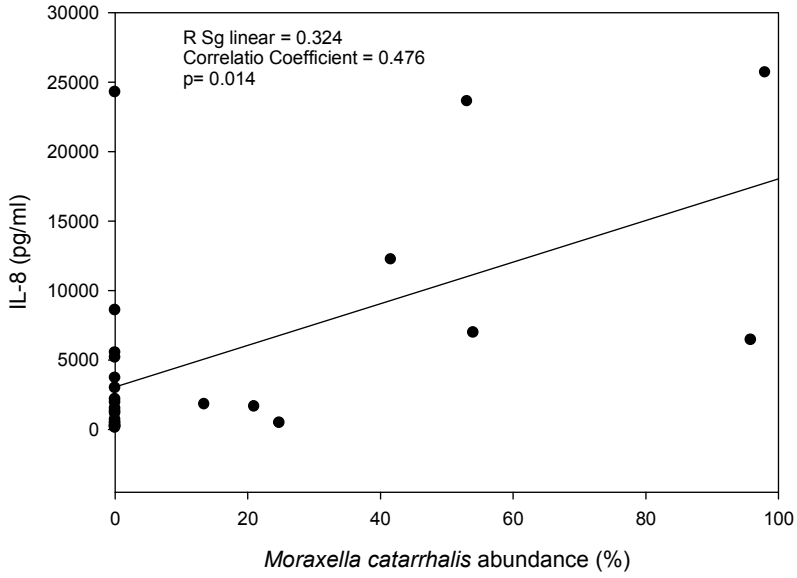


Figure 13 *M. catarrhalis* and IL-8 correlation in severe asthmatic induced sputum

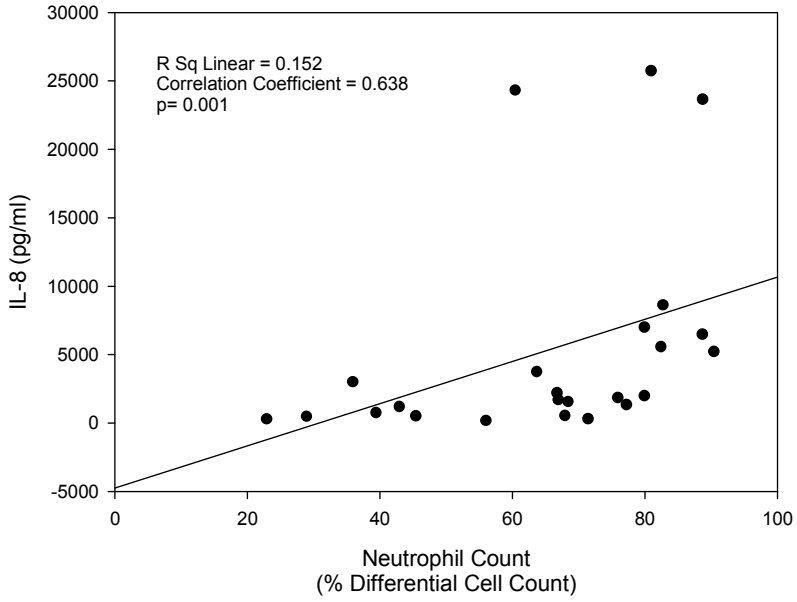


Figure 14 Neutrophil count and IL-8 correlation in severe asthmatic induced sputum

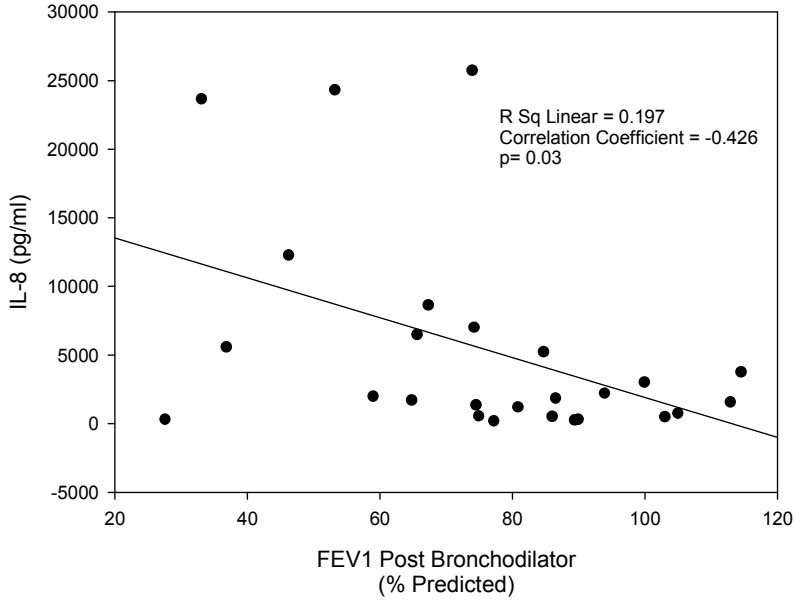


Figure 15 FEV1 post bronchodilator (%predicted) and IL-8 correlation in severe asthmatic induced sputum

6.4 Discussion

Molecular microbiological examination of lower airway specimens confirms that the lower airways are not sterile and can be considered to have a diverse colonising population. Multiple species are identifiable including potentially pathogenic bacteria such as *Haemophilus sp.*, *Streptococcus sp.,* and *M. catarrhalis* which are the most commonly identified dominant species.

We have shown that dominance of one of these potentially pathogenic bacteria is associated with worse airways obstruction and the development of a neutrophilic asthma phenotype. A longer disease duration is associated with one of these species becoming the dominant lower airway colonising species. Mean inhaled corticosteroid dose and oral prednisolone use was higher in this group than in those with non-PPMs as a dominant species, though this was not significant. Rather than reflecting causation in the development of more pathogenic bacteria as the dominant airway species, it is likely that higher ICS dose and maintenance oral steroid therapy reflects treatment of more severe, refractory disease. There was no significant difference in smoking history between the groups, suggesting cigarette smoke exposure was not relevant in the development of a PPM as the dominant airway species or neutrophilic inflammation in this small study population.

Exacerbation rate was higher in the PPM dominant group though this did not reach significance.

Dominance of *Haemophilus sp.*, *Streptococcus sp.,* or *M. catarrhalis* was associated with significantly higher abundance than when non pathogenic species were found as the dominant organism. Dominance of these species was also associated with significantly lower total numbers of species detected suggesting that these pathogens “overcolonize” the airway at the expense of other species.

Non-eosinophilic and neutrophilic asthmatics represent up to 25% of symptomatic asthma patients and 59% of patients on high dose inhaled corticosteroids attending asthma clinics[121;123]. Whilst asthma is a predominantly a T Helper 2 (TH-2) lymphocyte driven disease, with TH-2 cytokines driving eosinophilic inflammation[34], innate immune mechanisms may lead to a shift towards TH-1 and TH-17 mediated neutrophilic inflammation in a significant group of patients with severe asthma.

Increased airway bacterial load and specific bacterial species will lead to increased soluble bacterial cell wall fragments such as LPS from the outer cell membrane of Gram-negative bacteria within the airway. The lipid portion of the molecule, unlike the polysaccharide is not antigenically specific and is able to bind to pattern recognition receptors to trigger the innate response and neutrophil recruitment. Previous studies in COPD have shown that *Haemophilus sp.*, *Streptococcus sp.* and *M. catarrhalis* are associated with increased markers of innate immune activation and there is biological plausibility in the idea that these species may have a role in neutrophilic asthma.

IL-8 levels correlated with the total abundance of PPMs, particularly *M. catarrhalis*. As a strong neutrophil chemotractant it is not surprising to see levels correlated with neutrophil differential cell counts but it was also associated with evidence of worse airways obstruction.

Low squamous cell contamination in the induced sputum samples implies low salivary contamination and there is unlikely to be a significant contribution to T-RFLP profile results from oropharyngeal bacteria. A previous comparison of T-RFLP profiles from spontaneously expectorated sputum and mouthwash samples in cystic fibrosis patients showed little evidence of oropharyngeal contamination[96], suggesting molecular microbiological examination of sputum can give an accurate account of lower airway colonization.

Asthma is a heterogenous disease with neutrophilic asthma representing a more steroid resistant phenotype. With greater understanding of the molecular mechanisms involved in the development of this phenotype, targeted therapies can be developed either through manipulation of colonising species using vaccination and antibiotic therapy, or through treatment targeting the chemotractants involved in neutrophil recruitment.

Macrolide antibiotics are now understood to have an anti-inflammatory “class” effect. Since erythromycin was first used to treat Japanese panbronchiolitis, a condition characterised by neutrophilic bronchial inflammation, newer macrolides have been employed for their actions which extend beyond antimicrobial activity.
In a mouse model of cystic fibrosis, treatment with low dose azithromycin significantly reduced LPS induced neutrophilic airway inflammation when

compared to untreated mice[124]. It has also been shown to significantly reduce nuclear factor-kappa B (NF-kB) expression and TNFα secretion in a CF-derived airway epithelial cell line[125]. In vivo studies of azithromycin show reduced BAL IL-8 and neutrophilia in bronchiolitis obliterans syndrome post lung transplantion[126] and, in a small study of childhood asthma, a reduction in neutrophilic airway infammation[127]. Although NF-κB inhibition is strong candidate, the exact immunomodulatory mechanism of macrolides is not yet fully understood. Non antibiotic macrolides which inhibit NF-κB are in development which will answer the question as to whether beneficial effects are due to changes in airway flora or anti-inflammatory effects.

A Cochrane review of antibiotic therapy in chronic stable asthma identified 7 randomised controlled trials of macrolide use for greater than 4 weeks. Although improvements in symptom scores were seen, there was insufficient evidence to support this treatment strategy. The overall disappointing results of antibiotic therapy in stable asthma are likely to reflect the selection of patient cohorts with differing underlying pathology. Selection of patients with neutrophilic asthma where bacterial colonization and innate immune activation are more likely to be important is needed. In a placebo controlled study Simpson *et al.* gave clarithromycin 500mg bd for 8 weeks to severe, refractory asthmatics. They found treatment with this macrolide reduced airway IL-8 and neutrophil counts and improved quality of life scores when compared to placebo. However anti-inflammatory effects and improvements in AQLQ were significantly greater in patients with non-eosinophilic asthma[37].

Inhibition of neutrophil chemotaxis represents a therapeutic target in the group of severe patients with non-eosinophilic inflammation. A human monoclonal antibody to IL-8 has undergone phase II trials in COPD[128]. The primary outcome demonstrated a small but significant improvement in dyspnoea scores.

The neutrophil chemotactic effects of IL-8 (CXCL8), CXCL1 (GRO-α) and CXCL5 (epithelial-derived neutrophil attractant- 78) are mediated by a common receptor, the CXC receptor (CXCR). CXCR1/2 receptor antagonists are in development with ongoing phase II trials in asthma and COPD. One such chemokine antagonist, ADZ8309 has been shown to significantly reduce sputum neutrophil count and neutrophil elastase in LPS challenged healthy volunteers[129]

These strategies will reduce the inflammatory effect of innate immune activation and excess IL-8 production but will not address the underlying pathogenic bacterial colonization, which would suggest targeted antibiotic use or immunisation to specific bacterial species may be more beneficial in neutrophilic asthma and provide an alternative treatment strategy in a patient group where there is currently an unmet need for new therapies.

7 The clinical relevance of potentially pathogenic bacteria in BAL in severe asthma

7.1 Aim

To examine the effect of lower airway colonization with the respiratory pathogens, *Haemophilus sp.*, *Streptococcal sp.*, *Moraxella catarrhalis*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* on clinical outcomes and peripheral airways inflammation in severe asthmatic subjects.

7.2 Methods

Severe asthma subjects were recruited from the Southampton University Hospitals NHS Trust severe asthma service as previously described.

Current smokers and subjects who had exacerbations or antibiotic treatment within the previous six weeks were excluded. Bronchoalveolar lavage was collected as previously described into a sterile trap and processed immediately. BAL was performed in the right upper lobe in all subjects. Symptoms permitting, the same volume of sterile saline (120mls) and dwell time was used in all subjects. Un-centrifuged BAL was frozen and stored at -80^{o}C for subsequent batch T-RFLP profiling. BAL supernatant was stored at -80^{o}C and cytospins prepared for differential cell counting.

7.3 Statistics

Between-group comparisons of normally distributed data were performed using a 2 sample t test. Non parametric group comparisons were made using the Mann-Whitney U test. Total abundance of PPM in the group was not normally distributed and all correlations were performed with Spearman's rank correlations.

7.4 Results

Bronchoalveolar lavage specimens were collected from 27 severe asthmatic subjects. All subjects were at BTS/SIGN asthma guidelines treatment step 4 or 5[78]and all subjects met the ATS workshop on refractory asthma consensus criteria for definition of severe asthma[77]. All were on high dose inhaled corticosteroid and 15 were on oral maintenance prednisolone. Mean age [43.7 years (SD12.7)] and asthma duration [24.8 (16.0)] were high and 17 subjects were atopic.

The dominant colonising bacterial species by percentage abundance of total bacterial load was identified. Subjects were then divided into two groups on the basis of T-RFLP findings of either a dominant PPM or non-PPM. Subjects with dominant *Haemophilus sp.*, *Streptococcal sp.*, *Moraxella catarrhalis* *Staphylococcus aureus* or *Pseudomonas aeruginosa* (N=15) were designated PPM dominant, and subjects with all other dominant species (N=12) were designated non-PPM dominant.

Baseline characteristics, differential cell count, cytokine measures and dominant species grouping data are presented in Table 19.

Dominance of a pathogenic species in severe asthma BAL samples was associated with a significantly lower number of species being detected [mean (SD) 5.7 (3.2) vs 7.9 (2.1), p=0.043] and a higher abundance of the dominant species [median (IQR) 68.5% (54.4) vs 32.4% (22.6), p=0.036].

Dominance of pathogenic species was associated with significantly higher IL-8 [median (IQR) 195pg/ml (355) vs 53pg/ml (126), p=0.046] and a higher BAL neutrophilia [median (IQR) 14.8% (30.5) vs 6.5 (13.43), p=0.114], though this did not reach statistical significance.

The total abundance (as a percentage of total bacterial load within the sample) of PPM was calculated by the addition of abundance of *Haemophilus sp.*, *Streptococcal sp.*, *Moraxella catarrhalis* *Staphylococcus aureus* and *Pseudomonas aeruginosa* . Table 21 shows the correlations between clinical characteristics and total PPM abundance scores.

The total abundance of potentially pathogenic bacterial species in severe asthmatic BAL samples correlated with decline in FEV_1 post bronchodilator percent predicted (Spearman's correlation coefficient r=0.389, p=0.045) and increasing asthma duration (r=0.401, p=0.038).

There was a positive correlation seen between the total percentage abundance of pathogenic species and BAL neutrophil counts (r=0.439, p=0.022) and BAL IL-8 (r=0.491, p=0.011). Total BAL PPM correlations are presented in Table 21 and Figure 16

	Potentially pathogenic species Dominant (N=15)	Non-respiratory pathogen as dominant species (N=12)	
Age (years)^	47.9 (38.6)	38.6 (11.8)	p=0.916
Asthma Duration (years)^	28.7 (16.7)	19.9 (14.1)	p=0.483
Age asthma onset (years)^	19.2 (18.6)	18.7 (15.0)	p=0.381
ACQ^	2.95 (1.1)	2.79 (1.0)	p=0.336
PEFR Variability^	28.0 (14.3)	31.4 (17.9)	p=0.673
ICS dose (BDP mcg eq/day)^	1925 (820)	2548 (1839)	p=0.245
Ex-Smokers	7	5	p=0.759
Pack Years^^	0 (3.0)	0 (3.3)	p=0.867
Prednisolone maintenance mg/day^^	10 (17.5)	5 (20.0)	p=0.867
FEV_1 post BD % predicted^	75.3 (25.4)	83.1 (27.5)	p=0.746
Exacerbations last 12 months ^^	4 (4.0)	6 (7.5)	p=0.200
ENO ppb^^	7.5 (8.2)	12.1 (19.8)	p=0.560
BAL neutrophils (% total cell count)^^	14.8 (30.5)	6.5 (13.43)	p=0.114
BAL eosinophils (% total cell count)^^	0.8 (1.5)	0.63 (5.5)	p=0.867
BAL IL-8 (pg/ml)^^	195 (355)	53 (126)	**p=0.046***
Number Species Detected^	5.7 (3.2)	7.9 (2.1)	**p=0.043***
Dominant species abundance (%)^^	68.5 (54.4)	32.4 (22.6)	**p=0.036***

^ Mean (Standard deviation)
^^Median (Inter-quartile range)

Table 19 PPM dominant vs non-PPM dominant BAL, baseline characteristics and BAL results

Table 20 Severe asthmatic BAL supernatant cytokine measurements

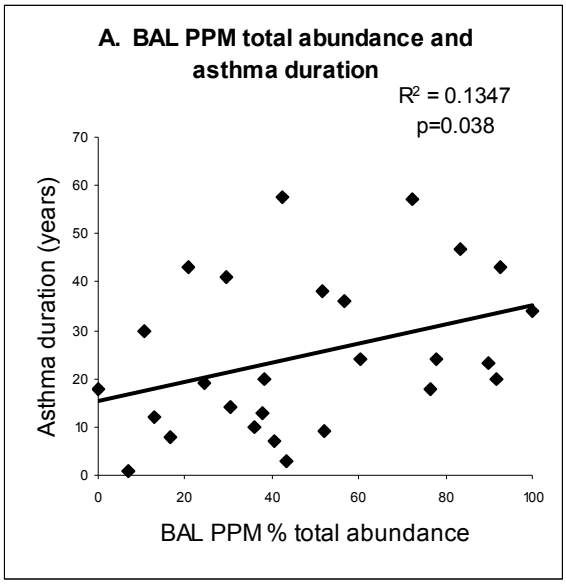
Severe Asthma BAL Supernatant Cytokine	Dominant *PPM* N=15	Other dominant species N=12	
IL-8 (pg/ml)	195 (355)	53 (126)	**p=0.046***
ECP (ng/ml)	18.0 (42.4)	5.88 (38.6)	p=0.614
Gro α (pg/ml)	4615 (4563)	2943 (5391)	p=0.277
ENA 78 (pg/ml)	58.1 (85.1)	24.5 (66.0)	p=0.200
α 2 Macroglobulin (ng/ml)	999 (1817)	644 (3718)	p=0.683
IL 1β (pg/ml)	6.0 (6.6)	0.8 (6.1)	p=0.149
IL6 (pg/ml)	7.1 (4.7)	3.7 (7.7)	p=0.204

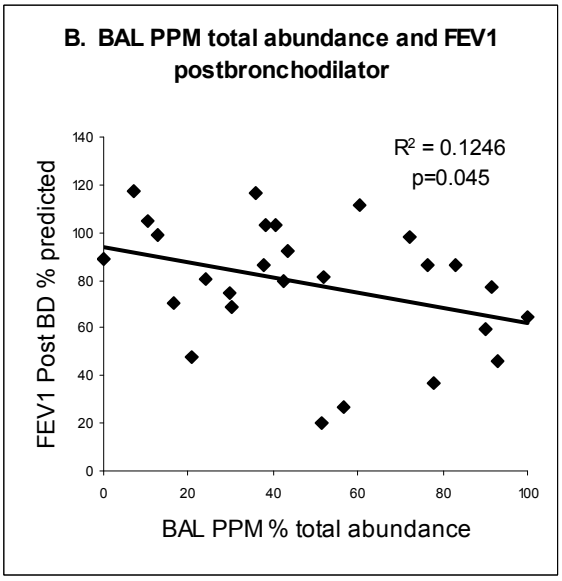
Median (IQR)

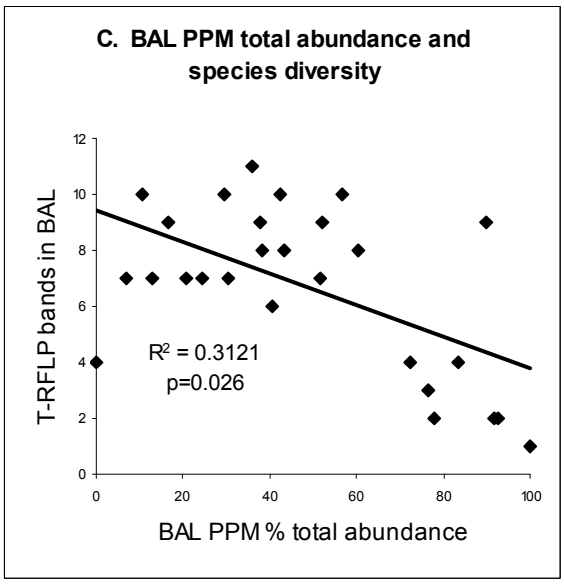
	Spearman's Correlation BAL PPM total (% abundance)	Sig. (2-tailed)
Age	0.216	p=0.278
Asthma duration	0.401(*)	**p=0.038***
Age asthma onset	-0.261	p=0.189
FEV_1 (% predicted)	-0.254	p=0.201
FEV_1 Post Bronchodilator (% predicted)	-0.389(*)	**p=0.045***
Exacerbations in the last 12 months	-0. 287	p=0.147
ACQ	0. .325	p=0.106
PEFR variability	0.003	p=0.990
Inhaled Corticosteroid dose (BDP mg eq/day)	-0.105	p=0.603
Prednisolone maintenance	0. 008	p=0.970
Exhaled nitric oxide (ppb)	-0. 187	p=0.361
Pack years	0. 199	p=0.320
BAL T-RFLP bands	-0. 427(*)	**p=0.026***
BAL IL-8	0. 491(*)	**P=0.011***
BAL neutrophils	0. 439(*)	**p=0.022***
BAL eosinophils	-0. 058	p=0.774
BAL macrophages	-0. 205	p=0.305
BAL lymphocytes	-0. 198	p=0.322
BAL epithelial cells	-0. 093	p=0.643

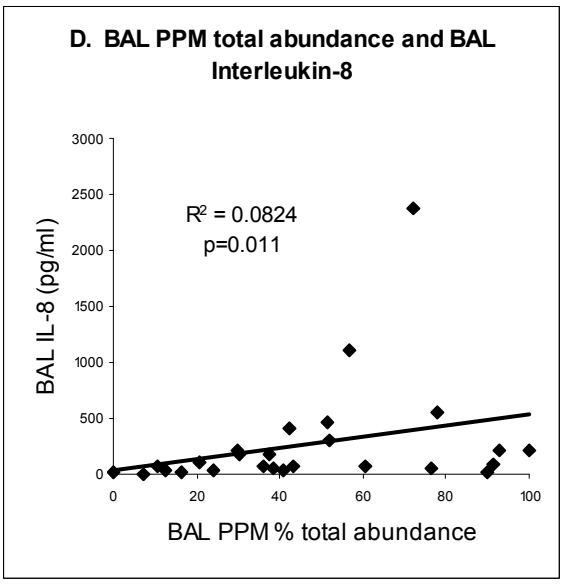
* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

Table 21 Correlations of total BAL PPM abundance









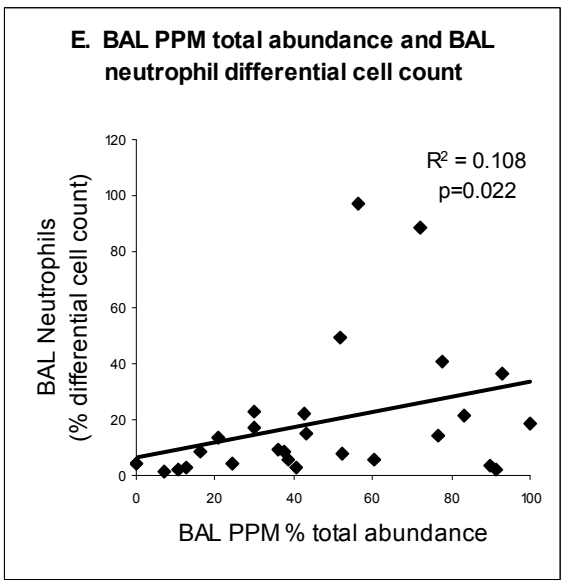


Figure 16 A-E Correlations of BAL PPM total abundance

7.5 Discussion

Potentially pathogenic organisms were commonly the most dominant airway bacterial species in the BAL of this group of severe asthmatics. When a PPM was the dominant species, abundance was significantly higher than when a non-PPM was the dominant species and the total number of species identified, *ie*. the species diversity, was significantly lower. This would imply that potentially pathogenic species can more successfully dominate the colonising flora of the lower airway at the expense of other species.

PPM dominance was associated with significantly higher supernatant IL-8 and a trend toward higher neutrophil counts suggesting that, as seen in examination of induced sputa, potentially pathogenic species are associated with innate immune activation leading to increased IL-8 production and neutrophil chemotaxis.

Trends toward higher levels of multiple other cytokines were seen in BAL from asthmatics with dominant PPM airway colonisation. GRO-α and ENA-78 are CXCR2 (cysteine-X-cysteine receptor 2) chemokines. Growth related oncogene-α, GRO-α (also known as CXCL1), like IL-8 (CXCL8) is a neutrophil chemotractant[130]. Epithelial-derived neutrophil attractant-78, ENA-78 (CXCL5) is also recognised as a neutrophil chemotractant in the setting of COPD exacerbation and is elevated in asthma exacerbation in the presence of airway eosinophilia[131;132]. IL-6 and IL1β are released by alveolar macrophages in response to PAMPs and also contribute to neutrophil chemotaxis[133;134].

α2-macroglobulin is a plasma protein extravasated into the airway as a result of microvascular injury and airway inflammation[135] and has been linked to severe asthma[136]

There were 7 ex-smokers in the PPM dominant group (47%) and 5 in the non-PPM dominant group (42%). Amongst these ex-smokers the mean number of pack years smoked was low [4.4 (SD4.0) in the PPM group versus 10.3 (12.2) in the non-PPM group, p= 0.350] allowing us to conclude that smoking history does not account for the differences seen.

Inhaled corticosteroid dosage was high but not significantly different between the two groups. 9/15 (60%) of the PPM dominant group were also on maintenance oral prednisolone treatment compared to 6/12 (50%) of the non-PPM group (p=0.603). Of those subjects on maintenance oral steroids the mean dose did not differ between the two groups [14.9mg/day prednisolone (4.7) in PPM group vs

20.0 (7.1) in the non-PPM dominant group] suggesting that steroid therapy does not explain differences seen in colonising flora.

The mucociliary escalator, if functioning normally deposits sputum from all lobes of the lung into the major bronchi and trachea where it can be collected using induced sputum. Induced sputum therefore can be relied upon to collect sputum from all lobes[137]. However BAL sampling involves wedging of the bronchoscope in a third generation segmental bronchus, therefore only sampling a limited lung area. Whether single lobe sampling can be representative of whole lung chronic colonization is not known. CF studies suggest bacterial load differs between lobes but cultured species are concordant[138]. The T-RFLP profile itself does not provide a marker of bacterial load but it is likely that species detection and abundance is representative of the entire distal lung.

These results independently support the findings from examination of induced sputa from severe asthmatic subjects that potentially pathogenic species are commonly the dominant colonising species in the severe asthmatic airway. Dominance of a PPM is at the expense of the abundance and diversity of other colonising flora. Furthermore dominance of these organisms is associated with worse airways obstruction, higher levels of neutrophil chemotractants and higher neutrophil counts.

8 Species Synergism

Haemophilus sp. and *Streptococcal sp.* commonly coexist in human disease including otitis media and lower respiratory tract infection[139;140]. Non- typeable *Haemophilus influenzae* and *Streptococcus pneumonia* are known to act synergistically in increasing mucin production[141]

In cystic fibrosis synergism between *S. aureus* and *P. aeruginosa* is recognised and there is suggestion that Staph aureus may sensitize the lung to subsequent *P. aeruginosa* colonization[112;142].

8.1 Aim

We looked for correlation between airway colonization with *Haemophilus sp.* and *Streptococcal sp.* in induced sputum and BAL.

8.2 Results

In severe asthmatic subjects detection of *Haemophilus sp.* was associated with detection of Streptococcal species in both induced sputum (N=36, Chi-Square test, p=0.047) and BAL (N=27, Chi-Square test, p=0.003)

In all subjects (mild, severe and healthy) Haemophilus airway colonization was associated with streptococcal carriage in BAL (N=61 Chi square test, p=0.0001) and in induced sputum (N=56, Chi-Square test, p=0.015) (Table 22).

8.3 Discussion

Complex interactions occur between species in mixed bacterial colonies. Species commonly co-colonize in cystic fibrosis where synergism is thought to aid the sequential addition of colonizing species to the CF lung flora[142].

We have found an association between the two most common colonising PPM species seen in severe asthma, but other associations are likely to exist which are not detectable with our sample size.

Whether the damage within the severe asthmatic airway provides an environment which favours these species, or whether co-colonization is enabled by species synergism is beyond the scope of this work.

A. Induced Sputum Severe Asthma		*Streptococcal sp.* Detected		Total
		No	Yes	
Haemophilus sp. Detected	No	5	3	8
	Yes	7	21	28
Total		12	24	36

B. BAL Severe Asthma		*Streptococcal sp.* Detected		Total
		No	Yes	
Haemophilus sp. Detected	No	5	0	5
	Yes	6	16	22
Total		11	16	27

C. BAL All Subjects		*Streptococcal sp.* Detected		Total
		No	Yes	
Haemophilus sp. Detected	No	30	2	32
	Yes	8	21	29
Total		38	23	61

D. Induced Sputum All Subjects		*Streptococcal sp.* Detected		Total
		No	Yes	
Haemophilus sp. Detected	No	6	3	9
	Yes	12	35	47
Total		18	38	56

Table 22 A - D Co-colonization of *Haemophilus sp*. and *Streptococcal sp.*

9 Innate and adaptive immune effect of airway colonization with *Haemophilus species*

Both Gram-negative and Gram-positive colonising species can initiate an innate immune response in the airway through the interaction of PAMPs with pattern recognition receptors. However, it is likely that some pathological species act as classic antigens leading the specific IgE production. In one study of 190 asthmatics, specific IgE to *H. influenzae*, *S. pneumoniae* or both was detected in 29% of subjects[143]. Serum specific IgE to Non-typeable *Haemophilus influenzae* (NTHi) is detectable by ELISA in patients with asthma, COPD and healthy controls. Asthmatic patients sensitised to indoor inhaled allergens have significantly higher serum NTHi IgE than non allergic asthmatics. This effect is more marked in severe asthma suggesting NTHi IgE may contribute to disease severity[144].

9.1 Aim

Serum NTHi IgE was measured by ELISA from subjects who underwent either bronchoscopy, induced sputum or both. Levels were correlated with measures of disease severity and airway colonization with *Haemophilus sp.* measured using T-RFLP.

9.2 Methods

Serum NTHi IgE was measure by ELISA (Hunter Immunology Ltd/University of Newcastle, Australia). Sputum induction and bronchoscopy were performed as previously described.

9.3 Results

Serum NTHi IgE was obtained in a total of 35 severe asthmatics, 7 healthy controls and 4 mild asthmatics.

Serum NTHi IgE results across all three patient groups were positively skewed. Log NTHi IgE was calculated and correlated positively, in all 3 subject groups with both induced sputum (N=31, Pearson correlation 0.656, $p<0.001$) and BAL *Haemophilus sp.* percentage abundance (N=16, Pearson correlation 0.505, $p=0.046$).

In severe asthma subjects log serum NTHi IgE correlated with the percentage abundance of *Haemophilus sp.* in induced sputum (N=28, Pearson's coefficient 0.665, p<0.001, R Sq = 0.443) (Figure 17).

In severe asthma subjects log serum NTHi IgE correlated with the percentage abundance of *Haemophilus sp.* in bronchoalveolar lavage (N=11, Pearson's correlation 0.639, p= 0.034, R Sq = 0.409) (Figure 18).

A significant correlation was seen between serum log NTHi IgE and sputum eosinophilia in severe asthma (N=23, Pearson correlation 0.530, p=0.009, R Sq 0.281).
In severe asthmatics serum log NTHi IgE correlated with BAL IL-8 (N=11, Pearson correlation 0.763, R Sq 0.582, p=0.006) and BAL neutrophil differential cell counts (N=11, Pearson's correlation 0.706, R Sq = 0.499, p=0.006).

Although mean NTHi IgE was higher in the severe asthma group [N=35, mean 3152EU, (SD)6174] than the combined mild asthma and healthy control group [N=11, mean 2661EU, (SD)2529], median serum NTHi IgE was lower in the severe group [798EU, IQR1497 vs 1370EU, IQR 2615 (p=0.055)]
There was no significant correlation between markers of disease severity, disease duration or atopy and serum NTHi IgE in the severe asthmatic group.

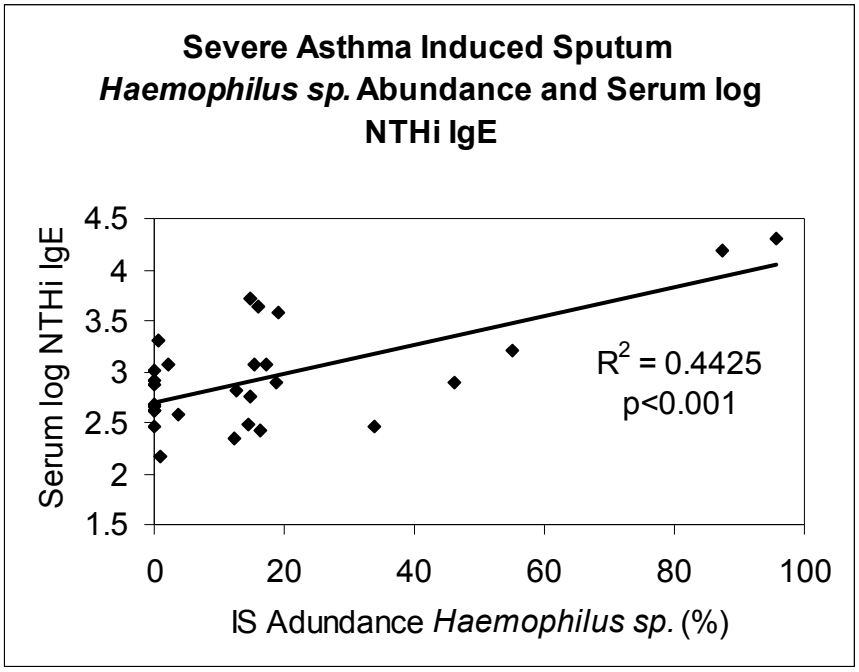


Figure 17 log NTHi IgE correlation with IS *Haemophilus sp.* abundance

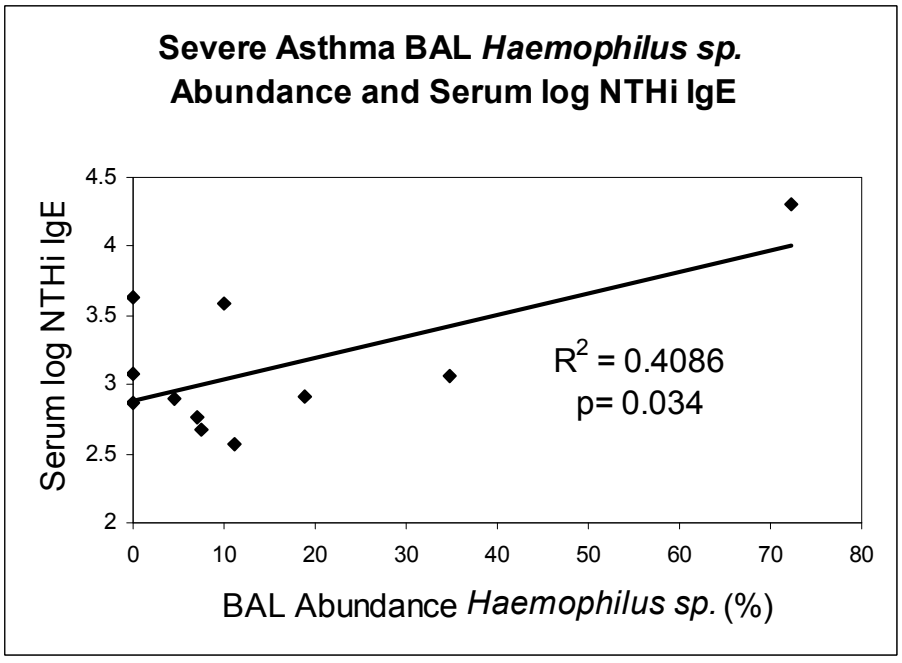


Figure 18 log NTHi IgE correlation BAL *Haemophilus sp.* abundance

9.4 Discussion

Serum NTHi IgE level correlates with the percentage abundance of lower airway *Haemophilus sp.* detected on T-RFLP profiling in severe asthmatics. This would suggest an active adaptive immune response to lower airway colonization. No correlation could be found with disease severity although numbers of mild asthmatic and healthy controls were low.

NTHi is known to increase proinflammatory cytokine expression including TNF-α, IL-6, MCP-1 and IL-8 in respiratory epithelial cells[145] and a correlation was seen between NTHi IgE and BAL IL-8 and neutrophilia.

The *Haemophilus sp.* band seen on T-RFLP represents a diverse family of Haemophilus species but these results would suggest that non-typeable *H. influenzae* is represented within this band.

NTHi is known to bind eosinophils through the (1–3)-β-D-glucan receptor leading to the release of proinflammatory cytokines[146] and further inflammatory cell recruitment. This may explain the correlation seen between NTHi IgE and sputum eosinophilia in the severe asthma group.

Therefore NTHi colonisation of the lower airway may be able to drive asthma, not only directly through TH-1 mediated IL-8 production and neutrophil recruitment, but also through specific IgE and TH-2 mediated inflammation.

10 T lymphocyte receptor Vβ repertoire profiling in severe asthma, mild asthma and healthy controls

10.1 Background

The Gram-positive coccus *S. aureus* is frequently detected as part of the normal microflora of the upper respiratory tract, with 20% of the population thought to be chronic carriers. However, rates of *S. aureus* carriage are significantly higher in diseases such as allergic rhinitis and nasal polyposis than in healthy controls[59;147] and carriage is linked to more severe disease[57]. *S. aureus* species are able to release a group of high molecular weight pyrogenic proteins known as enterotoxins[148]. These enterotoxins can act as superantigens by cross-linking the variable β-chain (Vβ) of the T cell receptor (TCR) and the class II MHC molecule outside the peptide binding grove, thus bypassing conventional, MHC-restricted, antigen processing[53]. This leads to an exaggerated activation of up to 25 % of naïve T cells compared with <0.01% when specific antigens are presented within the antigen binding groove[149]. The Vβ region of the TCR has limited variation and there are around 60 Vβ subsets currently described[150]. *S. aureus* species are able to produce more than one enterotoxin simultaneously and each toxin may recognise more than one Vβ segment [49]. Activation of T cells via this route leads a clonal expansion of the specific enterotoxin binding subsets[50] resulting in a skewing of the TCR Vβ repertoire and a characteristic Vβ profile "signature".

Serum measurement of specific IgE to *S. aureus* enterotoxin (SAE) has been used as a marker of exposure to these superantigens. Specific IgE to the major SAE serotypes SAE-A, SAE-C and TSST-1 was found at a significantly increased frequency in severe asthmatics compared to mild asthmatics and healthy controls and has been associated with increased total IgE, eosinophilic cationic protein (ECP) and steroid dependency[72;73]. In vitro, dexamethasone inhibition of phytohaemagglutinin (PHA) induced PBMC proliferation is significantly impaired in the presence of SAE-B. Furthermore, SAE-B increases the expression of glucocorticoid receptor β which acts as an inhibitor to glucocorticoid action[68].

Therefore, there is a growing body of evidence to suggest that *S. aureus* and SAEs may have a role in steroid resistance in asthma.

10.2 Aims

We have used flow cytometry to document the TCR Vβ repertoire profiles in bronchoalveolar lavage and blood from a group of well characterised severe asthmatics, mild asthmatics and healthy controls and correlated our findings with the identification of *S. aureus* species within the lower airway using terminal restriction fragment length polymorphism (T-RFLP) profiling.

10.3 Methods

Bronchoalveolar lavage (BAL) was collected from severe asthmatic, mild asthmatic and healthy control subjects. Paired blood samples were taken from severe asthmatics.

BAL was processed immediately as previously described. The cell solution was kept on ice and antibodies applied within 1 hour of bronchoscopy.

Cell surface expression of T lymphocyte Vβ receptor subsets was detected by flow cytometry using a six colour FACS Aria (BD Biosciences) and analysed using BD FACSDiva software. The following monoclonal antibodies were applied in combination to fresh BAL and blood cells:

CD3 Per-CP flourochrome-conjugated monoclonal antibody
(Becton Dickinson Catalog No.345766)
CD4 PE-Cy7 flourochrome-conjugated monoclonal antibody
(Becton Dickinson Catalog No.348809)
CD8 APC-Cy7 flourochrome-conjugated monoclonal antibody
(Becton Dickinson Catalog No.341110)
Aqua-fluorescent reactive dye fixable dead cell stain kit
(Component A and B) (Invitrogen Molecular Probes).
TCR Vβ Repertoire kit
(IO Test Beta Mark, Beckman Coulter PN IM3497).

The TCR Vβ Repertoire kit comprises 8 vials (A-H) each containing a mixture of 3 different conjugated TCR Vβ antibodies, making a total of 24 subclasses (approximately 70 % coverage of the normal human TCR Vβ repertoire) In each vial, of the 3 Vβ specific antibodies, one was phycoerythrin (PE) conjugated, one fluorescein isothiocyanate (FITC) conjugated and the third was both PE and FITC conjugated. This enabled quantitative analysis of 24 Vβ subsets from 8 sample

tubes using 2 flourochromes. Table 25 shows Vβ monoclonal antibody subclass and flourochrome combination in each of the 8 FACS tubes.

10.3.1 TCR Vβ Repertoire Antibody Acquisition

Table 23 below illustrates the tube set up for BAL. BAL tubes were left on ice in the dark for 30 minutes for antibody acquisition. Cells were then washed with 2000µl phosphate buffered saline/ bovine serum (autoMACS Rinsing Solution 1450ml + MACS 10% Bovine Serum Albumin Stock Solution 75ml) and centrifuged at 1500rpm for 5 minutes. The resultant cell pellet was resuspended for a second PBS/BSA wash and centrifuged for a further 5 minutes. The final cell pellet was resuspended in 250µl PBS/BSA for FACS analysis.

Table 24 below illustrates the tube set up for blood. After application of antibodies tubes were left in the dark at room temperature for 30 minutes. 1000 µl of lysing buffer (BD Pharm Lyse, BD Biosciences) was added to each tube and left for 15 minutes then centrifuged at 200G for 5 minutes. The cell pellet was then washed with 2000µl PBS/BSA and centrifuged at 1500rpm for 5 minutes. The final cell pellet was resuspended in 250µl PBS/BSA for FACS analysis.

FACS Tube	BAL cells	TCRVβ PE, FITC (Vial, µl)	CD3 PerCp (µl)	CD4 PE-Cy7 (µl)	CD8 APC-Cy7 (µl)	Aqua Live/Dead Stain (µl)
A	$0.3x10^6$	Vial A 20	10	1	1	1
B	$0.3x10^6$	Vial B 20	10	1	1	1
C	$0.3x10^6$	Vial C 20	10	1	1	1
D	$0.3x10^6$	Vial D 20	10	1	1	1
E	$0.3x10^6$	Vial E 20	10	1	1	1
F	$0.3x10^6$	Vial F 20	10	1	1	1
G	$0.3x10^6$	Vial G 20	10	1	1	1
H	$0.3x10^6$	Vial H 20	10	1	1	1

Table 23 Antibodies and Flourochromes applied to BAL

FACS Tube	Whole Blood (µl)	TCRVβ PE, FITC (Vial, µl)	CD3 PerCp (µl)	CD4 PE-Cy7 (µl)	CD8 APC-Cy7 (µl)	Aqua Live/Dead Stain (µl)
A	50	Vial A 20	10	1	1	1
B	50	Vial B 20	10	1	1	1
C	50	Vial C 20	10	1	1	1
D	50	Vial D 20	10	1	1	1
E	50	Vial E 20	10	1	1	1
F	50	Vial F 20	10	1	1	1
G	50	Vial G 20	10	1	1	1
H	50	Vial H 20	10	1	1	1

Table 24 Antibodies and flourochromes applied to blood

Tube	Histogram Quadrant	Vβ Subclass	Flourochrome
A	Q1 Q2 Q4	Vβ 5.3 Vβ 7.1 Vβ 3	PE PE+FITC FITC
B	Q1 Q2 Q4	Vβ 9 Vβ 17 Vβ 16	PE PE+FITC FITC
C	Q1 Q2 Q4	Vβ 18 Vβ 5.1 Vβ 20	PE PE+FITC FITC
D	Q1 Q2 Q4	Vβ 13.1 Vβ 13.6 Vβ 8	PE PE+FITC FITC
E	Q1 Q2 Q4	Vβ 5.2 Vβ 2 Vβ 12	PE PE+FITC FITC
F	Q1 Q2 Q4	Vβ 23 Vβ 1 Vβ 21.3	PE PE+FITC FITC
G	Q1 Q2 Q4	Vβ 11 Vβ 22 Vβ 14	PE PE+FITC FITC
H	Q1 Q2 Q4	Vβ 13.2 Vβ 4 Vβ 7.2	PE PE+FITC FITC

Table 25 TCR Vβ repertoire kit tube set up

10.3.2 T Lymphocyte Vβ Repertoire Gating strategy for BAL

Figure 19 shows an example of the gating strategy used for each of the 8 BAL tubes used (Tube D shown). Large cells and debris were gated out from the forward scatter/side scatter plot using the lymphoctye polygon gate R1. CD3+ T lymphocytes were gated positively by their staining with anti-CD3 antibody conjugated to Per-Cp from the events displayed in R1. Aqua stain negative CD3+ cells were designated live (histogram gate). Live CD3+ cells were displayed in PE-Cy7 area / APC-Cy7 area plot. CD4+ (PE-Cy7+/APC-Cy7-) and CD8+ cells (APC-Cy7+/ PE-Cy7-) were gated and displayed in separate plots with quadrant gates. Quadrants 1, 2 and 4 represent the TCRVβ subgroups PE+/FITC-, PE+/FITC+ and FITC+/PE- respectively.

10.3.3 T Lymphocyte Vβ Repertoire Gating Strategy for Blood

The gating strategy for blood samples was as for BAL. However no live/dead staining was used. Therefore Per-Cp positive staining CD3+ T cells were displayed in the PE-Cy7 area / APC-Cy7 area plot in order to gate CD4+ and CD8+ T cells (Figure 20).

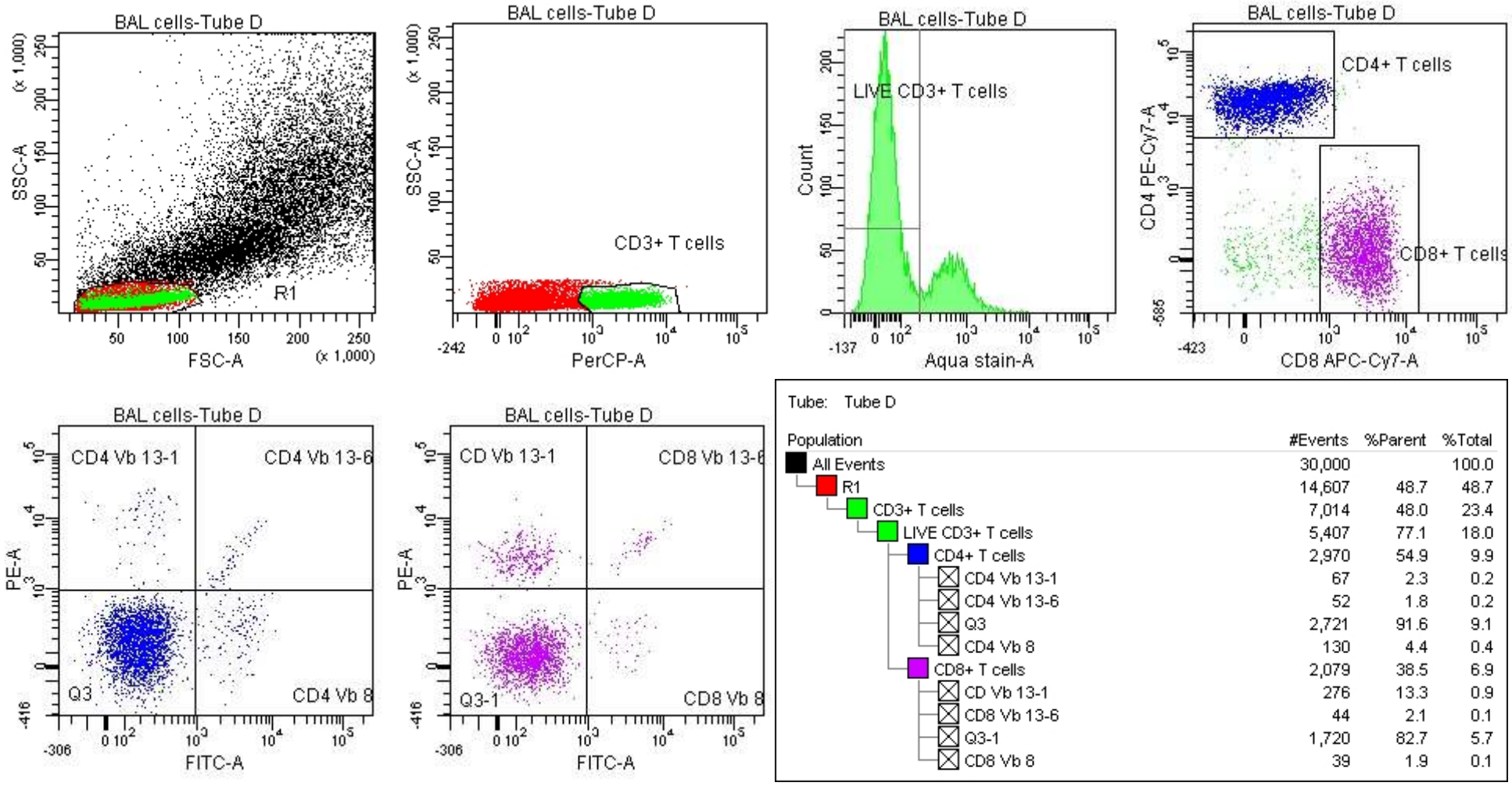
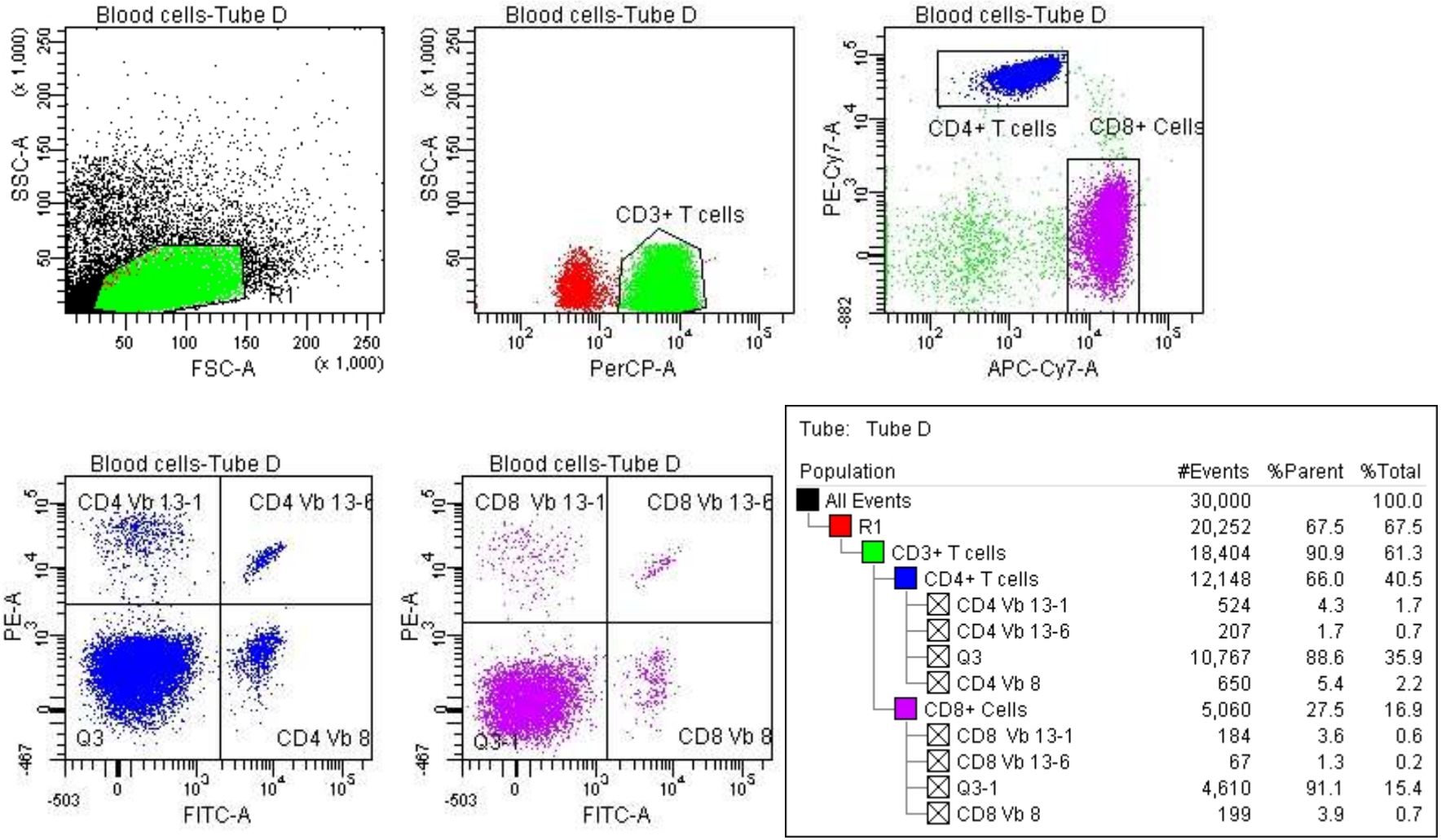


Figure 19 Bronchoalveolar lavage TCR Vβ repertoire gating strategy



Tube: Tube D

Population	#Events	%Parent	%Total
All Events	30,000		100.0
R1	20,252	67.5	67.5
CD3+ T cells	18,404	90.9	61.3
CD4+ T cells	12,148	66.0	40.5
CD4 Vb 13-1	524	4.3	1.7
CD4 Vb 13-6	207	1.7	0.7
Q3	10,767	88.6	35.9
CD4 Vb 8	650	5.4	2.2
CD8+ Cells	5,060	27.5	16.9
CD8 Vb 13-1	184	3.6	0.6
CD8 Vb 13-6	67	1.3	0.2
Q3-1	4,610	91.1	15.4
CD8 Vb 8	199	3.9	0.7

Figure 20 Blood TCR Vβ repertoire gating strategy

10.4 Results

10.4.1 TCR Vβ expression and *S. aureus* colonization in severe asthma

BAL was obtained from 21 severe asthmatic subjects. Within this group *S. aureus* species were detected in BAL from 8/21 (38%) subjects. BAL CD4+ TCR Vβ repertoire profiles were obtained from all subjects and CD8+ profiles were obtained from 15. Paired blood TCR Vβ repertoire profiles were obtained in 10 subjects.

Subjects were divided into those in whom *S. aureus* was detected in BAL by T-RFLP profiling. Table 26 below shows the clinical characteristics and BAL supernatant cytokine results for severe asthmatic subjects with detectable and undetectable *S. aureus* DNA in BAL.

BAL CD4+ and CD8+ T Cell Receptor Vβ expression was compared between the 2 groups (*S. aureus* colonized and uncolonized). Significantly increased expression of CD4+ TCR Vβ 5.3, 5.1, 13.1 and 2 was seen in *S.aureus* colonized (colonized N=8, un-colonized N=13) severe asthmatic BAL(
Table 27, Figure 21).

There was no significantly increased expression of any Vβ subclass when comparing BAL CD8+ T cells from severe asthmatic subjects with BAL *S. aureus* detected (N=6) and not detected (N=9) (Table 28).

Severe Asthmatic Subjects	*S. aureus* detected in BAL	*S. aureus* not detected in BAL	p value
Number	8	13	
Sex, M/F	4/4	2/11	p=0.146
Age* [years]	44.3 (13.2)	42.7 (14.7)	p=0.810
Ex-smokers	5	3	p=0.164
Smoking pack years^	2 (4.25)	0 (3.5)	p=0.097
Maintenance Oral Prednisolone	6	5	p=0.183
Prednisolone mg/day^	13.8 (17.9)	0 (17.5)	p=0.281
ICS (BDP mcg Eq/day)*	1625 (324)	2589 (1850)	p=0.164
Late onset asthma (after12)	4	8	p=0.673
Atopic	5	7	p=1.00
FEV_1 % predicted post-bronchodilator^	78.5 (23.9)	89.0 (32.9)	p=0.111
% Reversibility*	7.9 (7.4)	10.9 (9.6)	p=0.461
PEFR Variability %*	24.6 (15.8)	32.0 (12.3)	p=0.347
Duration of asthma [years]*	23.2 (18.3)	21.9 (17.0)	p=0.875
Severe Exacerbations last 12 months ^	6 (3.8)	4 (5.5)	p=0.256
ACQ Score*	2.45 (1.35)	3.11 (0.92)	p=0.191
Exhaled NO [ppb]*	10.0 (8.9)	15.4 (12.8)	p=0.320
BAL Interleukin-8 [pg/ml]^	76.5 (170.0)	72.6 (245.2)	p=0.939
BAL ENA-78 [pg/ml]^	84.0 (97.8)	21.9 (34.4)	**p=0.024**
BAL GROα [pg/ml]^	3292 (3235)	3053 (4429)	p=0.885
BAL α2 Macroglobulin [ng/ml]^	1037 (1997)	318 (1735)	p=0.612
BAL IL-1β [pg/ml]^	4.25 (7.23)	3.39 (11.46)	p=0.883
BAL IL-6 [pg/ml]^	5.01 (6.82)	3.83 (7.13)	p=0.828
BAL ECP ng/ml^	6.88 (40.1)	7.44 (45.6)	p=0.885
BAL % Neutrophil count ^	11.5 (15.4)	8.0 (18.5)	p=0.717
BAL % Eosinophil count^	0.8 (3.6)	0. (4.5)	p=0.365
BAL *S. aureus* % abundance^	39.9 (82.9)	N/A	

* Mean (SD) ^ Median (IQR)

Table 26 Clinical and BAL characteristics of severe asthmatics

CD4+ TCR Vβ subset	*Staph. aureus* detected in BAL in severe asthma (% T cells) (N=8)	*Staph. aureus* not detected in BAL in severe asthma (% T cells) (N=13)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median (IQR)	Median (IQR)	
Vβ5.3	2.80 (2.28)	0.70 (0.75)	**p=0.001***
Vβ7.1	2.45 (1.98)	1.40 (2.05)	p=0.345
Vβ3	5.70 (6.65)	5.60 (4.50)	p=0.942
Vβ9	5.20 (3.23)	4.10 (3.00)	p=0.111
Vβ17	3.65 (2.88)	4.60 (2.95)	p=0.562
Vβ16	1.05 (0.98)	1.00 (1.05)	p=0.971
Vβ18	1.10 (1.90)	0.60 (0.45)	p=0.54
Vβ5.1	7.90 (2.95)	3.70 (2.45)	**p=0.010***
Vβ20	3.80 (5.80)	5.30 (3.60)	p=0.328
Vβ13.1	8.40 (4.03)	3.50 (0.90)	**p<0.001***
Vβ13.6	2.20 (1.58)	2.00 (2.85)	p=0.856
Vβ8	3.75 (2.38)	4.80 (4.7)	p=0.111
Vβ5.2	1.10 (0.85)	1.40 (1.20)	p=0.772
Vβ2	8.05 (2.23)	5.50 (2.8)	**p=0.022***
Vβ12	2.40 (2.23)	2.50 (1.05)	p=0.245
Vβ23	2.20 (4.20)	1.70 (6.75)	p=0.781
Vβ1	4.20 (4.70)	3.30 (2.60)	p=0.781
Vβ21.3	1.80 (1.80)	1.80 (1.55)	p=0.781
Vβ11	0.65 (0.78)	0.90 (0.95)	p=0.327
Vβ22	2.10 (3.03)	2.20 (3.00)	p=0.772
Vβ14	2.55 (1.13)	3.00 (2.85)	p=0.346
Vβ13.2	2.05 (2.15)	2.60 (2.45)	p=0.328
Vβ4	0.20 (1.05)	0.30 (1.45)	p=0.176
Vβ7.2	2.00 (1.90)	0.70 (0.85)	p=0.111

Table 27 CD4+ TCR Vβ profiling in *S.aureus* colonized and uncolonized severe asthmatics

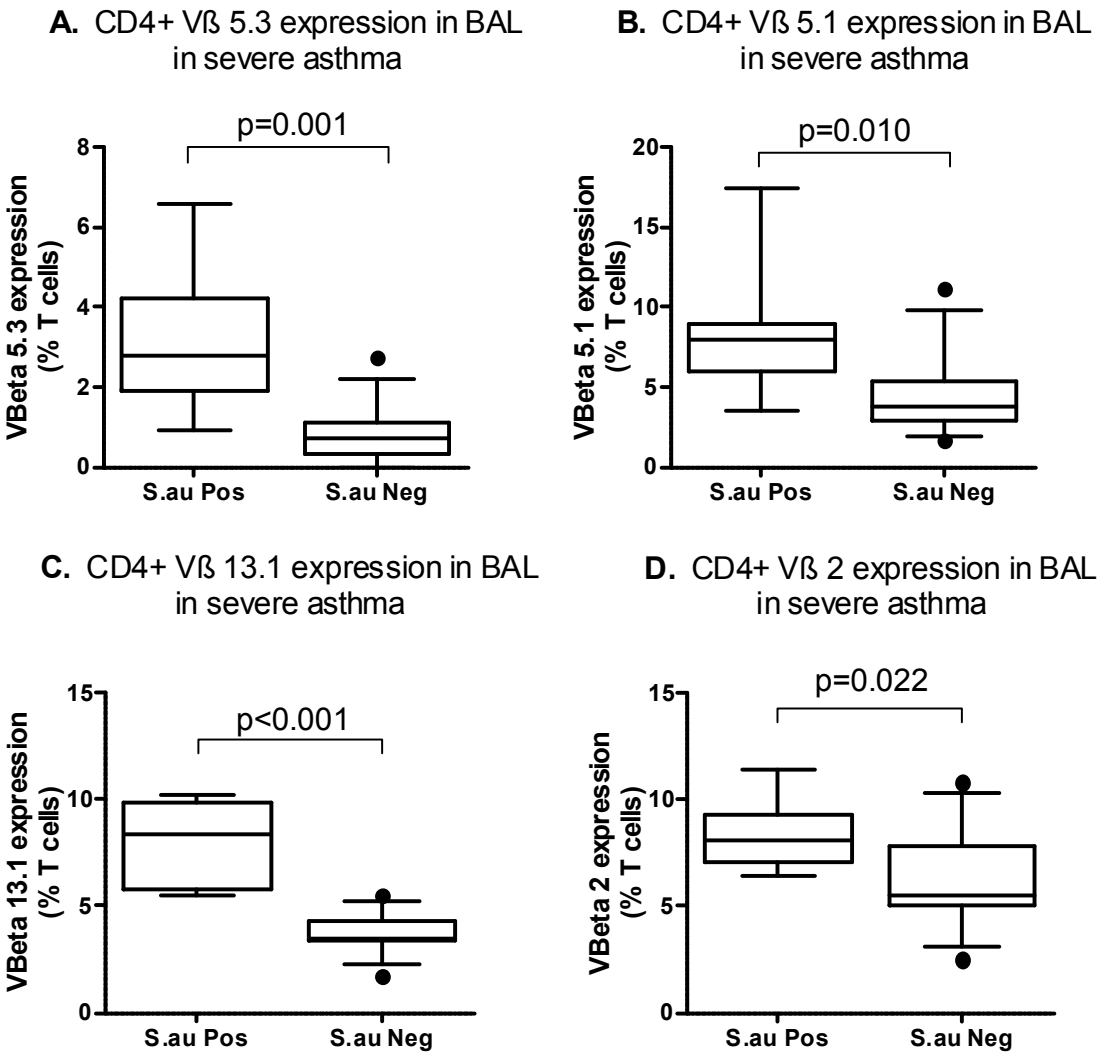


Figure 21 A-D CD4+ TCR Vβ profiling in *S.aureus* colonized and uncolonized severe asthmatics

CD8 TCR Vβ subset	*Staph. aureus* detected in BAL in severe asthma (% T cells) (N=6)	*Staph. aureus* not detected in BAL In severe asthma (% T cells) (N=9)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median (IQR)	Median (IQR)	
Vβ5.3	0.95 (1.98)	0.70 (0.50)	p=0.722
Vβ7.1	1.75 (3.08)	2.80 (0.90)	p=0.409
Vβ3	6.25 (14.68)	4.60 (7.6)	p=0.724
Vβ9	3.00 (4.83)	3.50 (6.30)	p=0.555
Vβ17	1.95 (4.60)	2.60 (2.90)	p=0.680
Vβ16	1.60 (3.13)	0.70 (0.85)	p=0.478
Vβ18	0.20 (0.98)	0.40 (0.55)	p=0.677
Vβ5.1	5.50 (5.30)	4.20 (5.00)	p=0.376
Vβ20	3.35 (1.93)	3.10 (2.30)	p=0.813
Vβ13.1	2.65 (5.00)	3.50 (7.55)	p=0.556
Vβ13.6	1.80 (3.48)	1.0 (1.90)	p=0.813
Vβ8	4.45 (5.60)	2.30 (2.80)	p=0.814
Vβ5.2	0.60 (1.38)	1.00 (0.85)	p=0.344
Vβ2	3.60 (2.88)	5.00 (5.35)	p=0.175
Vβ12	0.25 (1.08)	0.80 (0.50)	p=0.110
Vβ23	3.80 (3.75)	2.10 (6.65)	p=1.000
Vβ1	6.30 (5.80)	2.80 (6.80)	p=0.161
Vβ21.3	1.10 (2.55)	1.20 (4.20)	p=0.285
Vβ11	0.15 (2.35)	0.60 (0.45)	p=0.123
Vβ22	2.10 (2.00)	4.20 (4.85)	p=0.723
Vβ14	6.20 (6.83)	6.70 (4.10)	p=0.480
Vβ13.2	4.10 (8.58)	3.30 (7.20)	p=0.768
Vβ4	0.05 (0.43)	0.10 (0.55)	p=0.582
Vβ7.2	1.25 (3.48)	1.10 (4.15)	p=0.555

Table 28 CD8+ TCR Vβ profiling in *S.aureus* colonized and uncolonized severe asthmatics

10.4.2 TCR Vβ expression and *Streptococcal sp.* colonization in severe asthma

Streptococcal species were detected by T-RFLP profiling in BAL from 11/21 (52%) severe asthmatic subjects. Of the 24 TCR Vβ subsets profiled on CD4+ cells only Vβ 20 showed statistically higher expression in the *Streptococcal sp.* colonized subjects (median [IQR] 7.3% [7.0] vs 4.2% [3.5], p=0.029).

CD4+ TCR Vβ subset	*Streptococcal sp.* not detected in severe asthma BAL (% T cells) (N=10)	*Streptococcal sp.* detected in severe asthma BAL (% T cells) (N=11)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median (IQR)	Median (IQR)	
Vβ5.3	1.2 (2.25)	0.80 (1.6)	p=0.572
Vβ7.1	2.5 (2.68)	1.4 (2.0)	p=0.120
Vβ3	5.35 (5.55)	7.2 (4.5)	p=0.231
Vβ9	4.6 (2.63)	4.7 (3.2)	p=0.751
Vβ17	5.0 (4.6)	3.6 (2.3)	p=0.057
Vβ16	0.85 (1.03)	1.3 (1.0)	p=0.216
Vβ18	0.75 (1.33)	0.7 (0.5)	p=0.723
Vβ5.1	5.0 (4.68)	5.1 (5.6)	p=0.778
Vβ20	4.2 (3.53)	7.30 (7.0)	**p=0.029***
Vβ13.1	4.3 (5.2)	4.9 (2.8)	p=0.860
Vβ13.6	2.35 (2.83)	2.0 (1.4)	p=0.597
Vβ8	4.8 (5.23)	4.1 (2.1)	p=0.259
Vβ5.2	1.3 (0.8)	1.1 (1.3)	p=0.778
Vβ2	6.6 (3.13)	7.3 (4.3)	p=0.860
Vβ12	2.5 (1.48)	2.2 (1.0)	p=0.549
Vβ23	1.65 (4.68)	2.5 (6.4)	p=0.140
Vβ1	3.85 (3.78)	2.15 (2.88)	p=0.212
Vβ21.3	2.3 (2.0)	1.6 (0.98)	p=0.240
Vβ11	0.9 (0.93)	0.9 (0.93)	p=0.290
Vβ22	3.45 (2.98)	1.2 (1.4)	p=0.015*
Vβ14	2.6 (1.83)	2.9 (3.0)	p=0.549
Vβ13.2	2.15 (1.78)	3.0 (3.1)	p=0.778
Vβ4	1.35 (1.48)	0.2 (0.2)	p=0.025*
Vβ7.2	0.9 (1.13)	1.1 (1.8)	p=0.751

Table 29 CD4+ TCR Vβ profiling in severe asthmatic subjects colonized or uncolonized by *Streptococcal sp.*

10.4.3 TCR Vβ expression in severe asthma, mild asthma and healthy controls

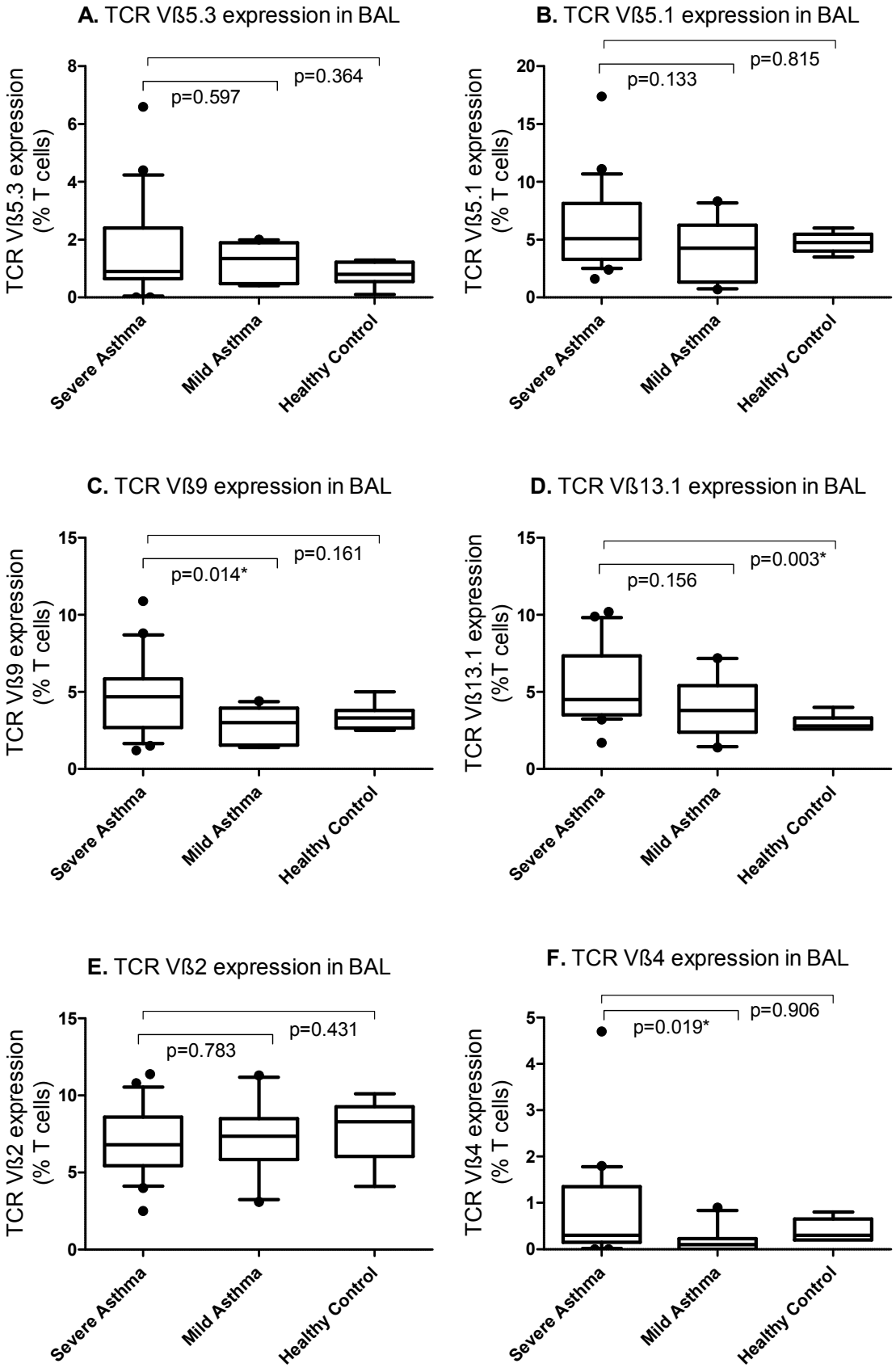
BAL CD4+ TCR Vβ profiles were obtained in 21 severe asthmatics, 10 mild asthmatics and 6 healthy controls. Significantly increased expression of Vβ9, and Vβ4 was seen in BAL from severe asthmatic subjects when compared to mild asthmatic subjects and Vβ13.1 when compared to healthy controls.

CD4+ TCR Vβ subset	Mild Asthma (N=10)	Severe Asthma (N=21)	Healthy Control (N=6)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median% (IQR)	Median% (IQR)	Median% (IQR)	
Vβ5.3	1.35 (0.70)	0.9 (2.05)	0.80 (0.83)	NS
Vβ7.1	1.30 (1.43)	1.7 (1.75)	1.05 (0.68)	NS
Vβ3	4.05 (3.63)	5.60 (4.75)	4.30 (3.08)	NS
Vβ9	3.00 (2.40)	4.70 (3.15)	3.30 (1.15)	p=0.014^
Vβ17	4.55 (0.93)	4.00 (1.90)	4.95 (1.85)	NS
Vβ16	1.45 (0.93)	1.0 (1.05)	1.15 (0.83)	NS
Vβ18	0.80 (0.48)	0.70 (0.75)	0.85 (0.48)	NS
Vβ5.1	4.25 (4.93)	5.1 (4.85)	4.75 (1.45)	NS
Vβ20	3.95 (4.93)	5.1 (4.15)	5.30 (3.73)	NS
Vβ13.1	3.80 (3.03)	4.50 (3.85)	2.80 (0.73)	p=0.003^^
Vβ13.6	1.80 (3.50)	2.10 (2.60)	1.80 (2.50)	NS
Vβ8	4.35 (2.60)	4.60 (3.85)	7.45 (4.68)	NS
Vβ5.2	1.25 (1.10)	1.20 (0.73)	1.15 (0.73)	NS
Vβ2	7.35 (2.65)	6.80 (3.15)	8.30 (3.23)	NS
Vβ12	2.55 (1.83)	2.50 (1.10)	1.85 (1.50)	NS
Vβ23	3.85 (4.63)	2.15 (5.78)	1.95 (1.95)	NS
Vβ1	2.75 (0.90)	3.35 (3.10)	3.05 (1.88)	NS
Vβ21.3	2.50 (1.23)	1.80 (1.43)	2.10 (0.50)	NS
Vβ11	0.75 (0.50)	0.80 (0.90)	0.70 (0.68)	NS
Vβ22	2.50 (1.98)	2.20 (3.25)	1.90 (2.45)	NS
Vβ14	2.10 (1.25)	2.70 (2.20)	2.35 (2.08)	NS
Vβ13.2	2.75 (3.30)	2.30 (2.35)	3.70 (1.28)	NS
Vβ4	0.55 (0.23)	1.0 (1.20)	0.95 (0.45)	p=0.019^
Vβ7.2	0.10 (1.33)	0.30 (1.55)	0.30 (1.18)	NS

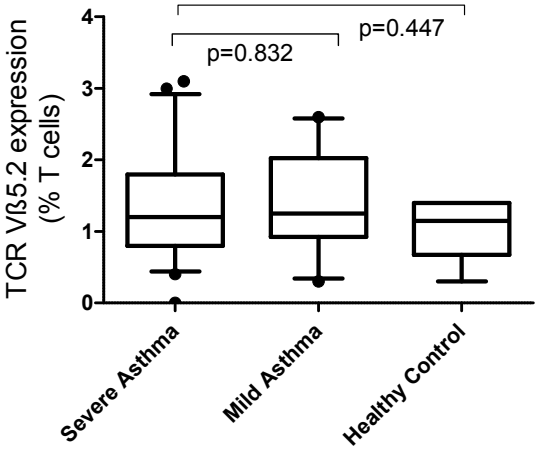
^Severe asthma versus mild asthma ^^Severe asthma versus healthy control

Table 30 TCR Vβ expression in asthmatic and healthy groups

Figure 22 (A-G) TCR Vβ expression in severe asthmatics, mild asthmatic and healthy control BAL



G. TCR Vß5.2 expression in BAL



10.4.4 TCR Vβ expression and *S. aureus* colonization all subject groups

CD4+ TCR Vβ BAL profiles were obtained in 37 asthmatic and healthy subjects (22 CD8+ TCR Vβ BAL profiles).

8/21 (38%) severe asthmatics, 3/10 (30%) mild asthmatics and 2/6 (33%) healthy controls were *S. aureus* colonized in BAL. CD4+ TCR Vβ profiles were compared between *S.aureus* colonized BAL in all subject groups (Table 31). Significantly higher expression of Vβ 5.3, 5.1 and 13.1 was demonstrated in subjects with detectable BAL *S. aureus*.

CD8+ TCR Vβ BAL profiles showed no significant differences in Vβ subset expression when comparing S. aureus colonized (N=7) and un-colonized (N=15) subjects (Table 33).

In *S. aureus* colonized subjects, the percentage abundance of *S.aureus* as a total of all bacterial DNA detected was higher in the severe asthma group than the combined mild asthma and healthy control group [severe asthma N=8 vs combined group N=5, median (IQR), 39.9% (82.9) vs 1.9% (73.3), p=0.464], though this difference did not reach statistical significance.

Within these *S. aureus* colonized subjects the median percentage representation of Vβ 5.3 and TCR Vβ 13.1 was significantly higher in the severe asthma group than the combined group (see Table 32).

When TCR Vβ profiles were compared between the combined group of 5 *S.aureus* positive subjects (mild asthmatics and healthy controls) and all *S.aureus* negative subjects, there were no significantly overexpressed CD4+ TCR Vβ subsets and a single CD8+ TCR Vβ subset with higher median percentage expression (Vβ 12, p=0.049)

CD4+ TCR Vβ subset	Staph. aureus detected in BAL in all subject groups (% T cells) (N=13)	Staph. aureus not detected in BAL in all subject groups (% T cells) (N=24)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median (IQR)	Median (IQR)	
Vβ5.3	2.0 (2.5)	0.7 (1.0)	**p<0.001***
Vβ7.1	2.0 (2.0)	1.3 (0.6)	p=0.126
Vβ3	5.2 (4.2)	5.1 (4.1)	p=0.738
Vβ9	4.4 (2.3)	3.3 (2.4)	p=0.101
Vβ17	4.4 (4.5)	4.6 (2.2)	p=0.836
Vβ16	1.3 (1.0)	1.1 (0.9)	p=0.523
Vβ18	0.8 (0.8)	0.7 (0.6)	p=0.146
Vβ5.1	6.7 (4.0)	3.9 (2.8)	**p=0.011***
Vβ20	4.2 (4.9)	5.3 (3.7)	p=0.239
Vβ13.1	6.3 (4.5)	3.5 (1.5)	**P<0.001***
Vβ13.6	2.0 (1.6)	2.0 (1.3)	p=0.937
Vβ8	4.0 (4.6)	4.9 (3.4)	p=0.119
Vβ5.2	1.0 (0.8)	1.4 (0.9)	p=0.503
Vβ2	7.8 (1.8)	6.4 (4.0)	p=0.054
Vβ12	2.2 (2.3)	2.5 (1.4)	p=0.691
Vβ23	2.9 (3.9)	2.0 (4.7)	p=0.374
Vβ1	3.9 (3.0)	2.6 (1.5)	p=0.240
Vβ21.3	2.0 (0.7)	2.3 (1.2)	p=0.382
Vβ11	0.8 (0.6)	0.8 (0.7)	p=0.962
Vβ22	2.3 (3.4)	2.3 (1.8)	p=0.714
Vβ14	2.7 (1.6)	2.5 (1.8)	p=0.924
Vβ13.2	3.0 (2.3)	3.1 (2.7)	p=0.494
Vβ4	0.2 (0.6)	0.2 (0.6)	p=0.987
Vβ7.2	1.2 (1.8)	0.8 (1.0)	p=0.238

Table 31 CD4+ TCR Vβ profiling in *S.aureus* colonized and uncolonized subjects

S. aureus colonized BAL	Severe Asthma (N=8)	Mild asthma or Healthy Control (N=5)	Mann-Whitney U Sig (2tail)
Vβ 5.3	2.8 (2.3)	1.3 (1.2)	**p=0.033***
Vβ 5.1	7.9 (3.0)	4.9 (4.1)	p=0.057
Vβ13.1	8.4 (4.0)	4.0 (2.7)	**p=0.013***

Median (IQR) % of total T cell population

Table 32 Median (IQR) CD4+ TCR Vβ subsets (%) in *S. aureus* colonized subjects

CD8+ TCR Vβ subset	*Staph. aureus* detected asthmatic BAL (% CD8 T cells) (N=7)	*Staph. aureus* not detected asthmatic BAL (% CD8 T cells) (N=15)	Mann-Whitney U Asymp. Sig. (2 tailed)
	Median (IQR)	Median (IQR)	
Vβ5.3	1.2 (1.6)	0.7 (1.1)	p=0.479
Vβ7.1	1.8 (1.7)	2.9 (0.9)	p=0.010
Vβ3	6.3 (13.2)	4.6 (7.9)	p=0.724
Vβ9	3.0 (5.2)	5.0 (7.1)	p=0.525
Vβ17	3.3 (3.9)	3.4 (2.2)	p=0.832
Vβ16	0.5 (2.1)	0.5 (0.6)	p=0.832
Vβ18	0.3 (1.6)	0.2 (0.5)	p=0.393
Vβ5.1	6.0 (4.1)	4.2 (4.5)	p=0.217
Vβ20	2.0 (3.0)	3.9 (3.7)	p=0.090
Vβ13.1	2.6 (0.5)	3.5 (6.0)	p=0.359
Vβ13.6	0.7 (1.5)	1.4 (1.5)	p=0.180
Vβ8	3.5 (4.0)	2.4 (3.5)	p=0.459
Vβ5.2	0.7 (1.40)	0.9 (0.9)	p=0.621
Vβ2	4.5 (1.9)	5.0 (5.3)	p=0.503
Vβ12	1.1 (2.4)	0.8 (0.4)	p=0.620
Vβ23	3.55 (12.4)	2.8 (7.8)	p=0.668
Vβ1	5.65 (5.98)	3.7 (3.5)	p=0.459
Vβ21.3	0.45 (2.65)	1.8 (3.2)	p=0.087
Vβ11	0.3 (0.3)	0.7 (0.8)	p=0.096
Vβ22	1.9 (5.9)	2.6 (4.3)	p=0.525
Vβ14	2.2 (6.5)	5.5 (3.0)	p=0.192
Vβ13.2	4.9 (9.6)	3.7 (5.2)	p=0.972
Vβ4	0.1 (0.2)	0.1 (0.3)	p=0.536
Vβ7.2	0.3 (3.6)	1.1 (4.2)	p=0.129

Table 33 CD8+ TCR Vβ profiling in *S. aureus* colonized and uncolonized (all subject groups)

10.4.5 Paired TCR Vβ profiling in blood and BAL

In all subject groups, same subject, paired blood and BAL were collected for CD4+ TCR Vβ profiling in 22 subjects and CD8+ TCR Vβ profiling in 19 subjects.

Of the 24 TCR Vβ subclasses profiled, correlation was seen between CD4+ TCR Vβ expression in contemporaneous paired blood and BAL samples from the same subjects in only Vβ3 and Vβ2 expressing T cells. Mean CD4 TCR Vβ expression in paired blood and BAL samples showed significant difference in Vβ20, Vβ2, Vβ23, Vβ22 and Vβ4.

Correlation was seen between CD8 TCR Vβ expression in contemporaneous paired blood and BAL samples from the same subjects in Vβ17,

Vβ13.6 and Vβ4. Mean CD8 TCR Vβ expression in paired blood and BAL samples showed significant difference in Vβ17, Vβ13.6, and Vβ4. Table 34 and Table 35 show mean percentage abundance and correlation of TCR Vβ subclass expression in paired blood and BAL in CD4+ and CD8+ T cell populations.

CD4+ T Cells Paired Samples (N=22)	Mean	Std. Deviation	Paired t test Sig (2 tailed)	Correlation	Sig.
BAL Vβ5.3	1.54	1.40	p=0.077	0.021	p=0.927
Blood Vβ5.3	0.98	0.19			
BAL Vβ7.1	1.62	1.05	p=0.483	-0.320	p=0.146
Blood Vβ7.1	1.83	0.57			
BAL Vβ3	5.35	4.05	p=0.323	0.438	**p=0.041**
Blood Vβ3	4.56	2.24			
BAL Vβ9	3.64	1.61	p=0.195	0.222	p=0.321
Blood Vβ9	3.19	0.65			
BAL Vβ17	4.75	2.34	p=0.193	0.308	p=0.163
Blood Vβ17	5.40	0.93			
BAL Vβ16	1.20	1.21	p=0.220	0.342	p=0.119
Blood Vβ16	0.89	0.29			
BAL Vβ18	1.06	1.65	p=0.654	0.278	p=0.211
Blood Vβ18	0.91	0.35			
BAL Vβ5.1	4.78	3.49	p=0.062	-0.049	p=0.827
Blood Vβ5.1	6.36	1.29			
BAL Vβ20	5.01	3.22	p=0.006	0.206	p=0.357
Blood Vβ20	2.92	1.19			
BAL Vβ13.1	4.65	2.41	p=0.594	0.223	p=0.319
Blood Vβ13.1	4.38	0.73			
BAL Vβ13.6	2.37	1.42	p=0.104	-0.102	p=0.651
Blood Vβ13.6	1.83	0.35			
BAL Vβ8	5.25	2.49	p=0.698	0.338	p=0.124
Blood Vβ8	5.45	1.51			
BAL Vβ5.2	1.18	0.64	p=0.602	0.419	p=0.053
Blood Vβ5.2	1.25	0.43			
BAL Vβ2	7.01	2.03	p<0.001	0.644	**p=0.001**
Blood Vβ2	9.36	1.17			
BAL Vβ12	2.19	1.10	p=0.191	0.346	p=0.115
Blood Vβ12	1.89	0.55			
BAL Vβ23	2.87	2.72	p<0.001	0.135	p=0.550
Blood Vβ23	0.43	0.14			
BAL Vβ1	3.50	1.68	p=0.300	0.154	p=0.493
Blood Vβ1	3.11	0.64			
BAL Vβ21.3	2.84	1.58	p=0.111	-0.071	p=0.755
Blood Vβ21.3	2.25	0.32			
BAL Vβ11	1.15	1.04	p=0.091	0.136	p=0.546
Blood Vβ11	0.76	0.18			
BAL Vβ22	2.64	1.59	p=0.001	0.183	p=0.414
Blood Vβ22	4.01	0.83			
BAL Vβ14	2.73	1.44	p=0.743	0.119	p=0.598
Blood Vβ14	2.62	0.54			
BAL Vβ13.2	3.09	1.46	p=0.065	0.199	p=0.388
Blood Vβ13.2	2.42	0.92			
BAL Vβ4	0.59	1.03	p<0.001	-0.035	p=0.879
Blood Vβ4	1.98	0.43			
BAL Vβ7.2	1.24	0.82	p=0.332	0.365	p=0.103
Blood Vβ7.2	1.05	0.73			

Table 34 Paired blood and BAL CD4+ TCR Vβ subclass means and correlations

CD8+ T Cells Paired Samples (N=19)	Mean	Std. Deviation	Paired t test Sig (2 tailed)	Correlation	Sig.
BAL Vβ5.3	1.32	1.25	p=0.156	0.339	p=0.156
Blood Vβ5.3	1.13	0.39			
BAL Vβ7.1	2.95	1.93	p=0.870	0.040	p=0.870
Blood Vβ7.1	3.72	1.15			
BAL Vβ3	8.08	5.84	p=0.158	0.337	p=0.158
Blood Vβ3	5.00	3.62			
BAL Vβ9	5.59	4.19	p=0.960	-0.012	p=0.960
Blood Vβ9	2.36	0.64			
BAL Vβ17	3.19	1.60	p=0.007	-0.593	**p=0.007**
Blood Vβ17	5.48	1.49			
BAL Vβ16	0.83	0.94	p=0.737	-0.083	p=0.737
Blood Vβ16	0.83	0.71			
BAL Vβ18	0.61	1.27	p=0.791	0.065	p=0.791
Blood Vβ18	0.25	0.17			
BAL Vβ5.1	4.89	2.98	p=0.328	0.237	p=0.328
Blood Vβ5.1	3.19	1.14			
BAL Vβ20	3.43	2.03	p=0.613	0.124	p=0.613
Blood Vβ20	2.13	1.06			
BAL Vβ13.1	4.28	4.11	p=0.939	0.019	p=0.939
Blood Vβ13.1	3.99	1.04			
BAL Vβ13.6	1.82	1.73	p=0.037	0.481	**p=0.037**
Blood Vβ13.6	1.36	0.49			
BAL Vβ8	3.85	3.43	p=0.884	0.036	p=0.884
Blood Vβ8	4.11	1.30			
BAL Vβ5.2	0.90	0.71	p=0.293	0.255	p=0.293
Blood Vβ5.2	1.36	0.77			
BAL Vβ2	6.13	3.97	p=0.994	-0.002	p=0.994
Blood Vβ2	6.47	2.27			
BAL Vβ12	1.09	1.15	p=0.940	-0.018	p=0.940
Blood Vβ12	1.39	0.56			
BAL Vβ23	6.17	6.07	p=0.101	-0.388	p=0.101
Blood Vβ23	1.53	0.52			
BAL Vβ1	4.84	3.12	p=0.519	-0.158	p=0.519
Blood Vβ1	4.83	2.16			
BAL Vβ21.3	2.38	1.96	p=0.461	0.180	p=0.461
Blood Vβ21.3	2.17	0.50			
BAL Vβ11	0.99	1.95	p=0.849	-0.047	p=0.849
Blood Vβ11	0.84	0.40			
BAL Vβ22	3.07	2.54	p=0.862	0.043	p=0.862
Blood Vβ22	3.36	0.93			
BAL Vβ14	5.58	2.90	p=0.577	-0.137	p=0.577
Blood Vβ14	6.65	2.08			
BAL Vβ13.2	5.52	5.85	p=0.661	-0.111	p=0.661
Blood Vβ13.2	3.74	1.41			
BAL Vβ4	0.16	0.24	p=0.006	-0.620	**p=0.006**
Blood Vβ4	1.88	0.71			
BAL Vβ7.2	2.19	2.73	p=0.334	0.242	p=0.334
Blood Vβ7.2	1.36	1.12			

Table 35 Paired blood and BAL CD8+ TCR Vβ subclass means and correlations

10.5 Discussion

Around 40 Staphylococcal and Streptococcal superantigens have been described. Structural similarities have led to the belief that these proteins confer an evolutionary advantage to the microorganism by interfering with the adaptive immune response, suppressing TH-2 response and promoting TH-1 type responses, increasing non-specific T cell proliferation and reducing high-affinity cytotoxic antibody production[151].

Genetic studies suggest the majority of *S. aureus* clinical isolates have superantigen encoding loci[152]. 80% of human nasal isolates demonstrate at least one SAg gene[52] with the majority having multiple genes .

Within the severe asthma group in whom Vβ profiles were obtained 8/21 subjects had *S. aureus* detected in the BAL. Significantly higher representation of Vβ5.1, Vβ5.3, Vβ13.1 and Vβ2 regions were found in the *S. aureus* colonized group. Vβ5.1 is known to act as a specific binding site for a wide range of staphylococcal superantigens including SEE, SEK, SEL, SEN, SEO, SEP, SEQ and SER. Vβ5.3 is the specific TCR binding region for SEA, SED, SEK, SEL and SEN[54]. Vβ13.1 is bound by SEG. Vβ2 is recognised as the specific binding region for TSST-1[53].

Vβ repertoire nomenclature gives numbers to related subfamilies. Subfamilies share >75% gene sequence homology[153]. Therefore a theoretical overlap between members of the same family may exist.

Skewing of the TCR Vβ repertoire provides evidence of a T cell mitogenic effect.

These results provide surrogate evidence of the presence of superantigens within the lower airway in severe asthma.

The expected cytokine response to T cell activation through superantigenic stimulation would include release of TNFα, IFNγ and IL-6 as well as the release then inhibition of IL-2[48]. Subsequent recruitment of T cells and activation of macrophages leads to further release of IL-1 and TNFα[154].

IL-6 was higher in BAL supernatant from *S. aureus* colonized severe asthmatics, but not significantly so. The only cytokine measured that was significantly raised in *S. aureus* colonized severe asthmatics was epithelial-derived neutrophil attractant-78. ENA-78 (CXCL5) is a known neutrophil chemotractant in the setting of COPD exacerbation and is raised in exacerbation of asthma where it is associated with airway eosinophilia[131;132]. Higher eosinophils counts were seen in the *S. aureus* colonized group but this difference did not reach significance.

SEA and SEB administration to human nasal epithelial cells leads to significantly increased IL-8 production in asthmatic subjects[155] and in mouse studies, intranasal administration of SEB leads to a dose dependent increase in BAL neutrophils[156]. In our small sample size, we did not see a significant difference in BAL supernatant IL-8 in *S. aureus* colonized severe asthmatics when compared to un-colonized subjects though a non-significant increase in BAL neutrophils was demonstrated.

The binding of CD4 to MHC II stabilises TCR-mediated recognition and aids signalling. By analogy with conventional T cell recognition, it has long been assumed that CD4:MHC class II interaction would give a selective advantage to CD4+ cells in response to bacterial superantigen binding when compared to CD8+ (MHC class I restricted) cells[157;158]. This seems to be consistent with our observed results, with no increase in any Vβ subclass seen in CD8+ T cells.
However, clonal expansion in both CD4+ and CD8+ Vβ 8+ subsets has been demonstrated in vitro with SEB[159;160], therefore this result needs to be interpreted with caution.

Within the severe asthma group, isolation of *S. aureus* within the BAL by T-RFLP profiling was associated with a trend toward worse airways obstruction (median FEV_1 post bronchodilator % predicted [IQR] 78.5% [23.9] vs 89.0% [32.9], p=0.111) and higher exacerbation rate (median severe exacerbation rate [IQR] 6 [3.8] vs 4 [5.5], p=0.256) but due to the small sample size these did not reach significance. 6/8 (75%) of *S. aureus* colonized severe asthmatic were on oral maintenance corticosteroid therapy with a median (IQR) dose of 13.8 (17.9) mg/day compared to 5/13 (38%) of un-colonized subjects with a median dose of 0 (17.5) mg/day. Again, due to the small sample size neither of these differences reached statistical significance (p=0.183 , p= 0.281 respectively), however, this may support the theory that *S. aureus* colonization is associated with a degree of steroid resistance[68;72;73].
No significant difference was seen in age of asthma onset, asthma duration, atopy smoking history or symptom scores between *S. aureus* colonized and un-colonized subjects.

Rates of *S. aureus* colonization were not significantly higher in severe asthma than in mild asthmatics or healthy controls (38% vs 30% vs 33%, p= 0.903). Of the 24

CD4+ TCR Vβ subclasses profiled, only two showed significantly higher expression in severe than mild asthma (Vβ4 and Vβ9) and one when severe subjects were compared with healthy controls (Vβ 5.1).
When all three subject groups were analysed together, significantly higher expression of Vβ5.3, Vβ5.1 and Vβ13.1 was seen in *S. aureus* colonized subjects. This would suggest a limited group, disease specific, effect on TCR Vβ expression and strengthen the case for a superantigenic cause for skewing of Vβ profiles.

Of the 24 TCR Vβ repertoires examined there was correlation between paired blood and BAL T cell profiles in only 2 subclasses for CD4+ and 3 for CD8+ T cells. The correlated CD4+ TCR Vβ subclasses were not those that showed significant differences between *S. aureus* colonized and un-colonized subjects. Significant mean differences were seen in subclass abundance in 5 CD4+ TCR Vβ subclasses and 3 CD8+ TCR Vβ subclasses. Therefore blood TCR Vβ profiling could not be used as a surrogate marker for BAL examination. The findings of skewing of the Vβ profile in BAL are lung specific supporting a localised, organ specific, superantigenic cause.

Streptococcal species, like *Staphylococcus aureus*, are known to produce a range of superantigenic proteins. *Streptococcus pyogenes*, also known as Lancefield group A streptococcus (GAS) colonizes the respiratory tract in 5-15% of healthy individuals[151] and is recognised for its superantigen generation. Streptococcal superantigens, like staph enterotoxins, can bind more than one Vβ region in the human TCR repertoire and therefore act as powerful T cell mitogens, activating up to 25% of the T cell population[161] and causing a massive proinflammatory response. *Streptococcal species* were detected in 11/21 severe asthmatic subjects in whom TCR Vβ profiles were obtained. From the 24 major Vβ subclasses measured, only Vβ20 showed significantly increased frequency in Strep. colonized subjects. Vβ20 has not previously been demonstrated to have specificity for commonly recognised GAS superantigens. Therefore the clinical relevance of this result is in doubt.

Oligoclonal expansion of Vβ8 and Vβ5.1 in peripheral blood has previously been linked to allergy to house dust mite in adult asthmatics[162]. Hauk *et al.* reported a significantly higher expression of T cells expressing Vβ8 in BAL from poorly controlled asthmatics when compared to a combined group of well controlled

asthmatics and healthy controls[75]. Rates of atopy in their study were 67% in the poorly controlled asthmatic group and 33% in the combined control group.
In our data the severe asthmatic group showed no increased expression of Vβ 8 when compared to mild asthmatics or healthy controls. However, atopy, as determined by skin prick positivity to one or more aeroallergens was associated with significantly increased expression of CD4+ TCR Vβ8 in BAL amongst severe asthmatics (median [IQR] atopic subjects 4.9% [4.5] vs non-atopics 3.4% [1.9] p=0.046), both mild and severe asthmatics (median [IQR] atopics 4.85% [2.8] vs non-atopics 3.25% [2.1], p=0.035), and in all healthy and asthmatic subjects (median [IQR] atopics 5.0% [3.7] vs non-atopics 3.4%[1.9], p=0.013).
Therefore the overexpression of TCR Vβ8 previously reported by Hauk may be as a result of over representation of atopic subjects in the poorly controlled asthmatic group.

Phenotypic differences between *S. aureus* colonized and un-colonized severe asthmatic subjects were not demonstrated in this small sample size (n=21). Trends were seen in markers of disease severity such as FEV_1 percent predicted post bronchodilator toward more severe disease in the *S. aureus* colonized group but these did not reach statistical significance.

The oligoclonal expansion of specific TCR Vβ subsets has been demonstrated in the airways of severe asthmatics who are *S. aureus* colonized. Expansion of 3 TCR Vβ subsets was seen in a combined severe asthma/ mild asthma/ healthy control group who were *S. aureus* colonized. However, within these subjects median percentage representation of these Vβ subsets was significantly higher in the severe asthmatics than the combined control group. This would suggest the skewing of TCR Vβ profiles is more profound, and may be limited to, the severe asthmatic lower airway.

These data provide evidence of an immunological effect of *S. aureus* colonization but further work is required to establish this group as a phenotypic entity in whom superantigens trigger or maintain chronic airway inflammation, within the heterogenous severe asthma patient population.

11 Discussion

11.1 Conclusions

We have demonstrated that potentially pathogenic bacterial species are commonly detected in induced sputa from severe asthmatics and that a molecular microbiological method of detection is more sensitive than standard culture based detection methods.

Patterns of species detection differ significantly between severe asthmatic subjects, mild asthmatics and healthy controls. A homogeneity of species number is seen in the proximal and distal airways of severe asthmatics subjects. This is in contrast to mild asthmatic subjects and healthy controls who demonstrate significantly higher species diversity in the proximal lower airway and lower species number in the distal lower airway.

The total abundance and detection rates of potentially pathogenic microorganisms (PPMs) is significantly higher in severe asthmatic BAL when compared to mild asthmatic specimens.

Haemophilus sp., M. catarrhalis and *Streptococcal sp*. were selected as the genera of greatest interest in induced sputa. These species are found in significantly higher abundance in severe asthma than other subject groups.

When a potentially pathogenic species is the dominant airway species in severe asthma it has a significantly higher abundance than non-pathogenic dominant species. In addition, dominance of a PPM is associated with a lower species diversity. This would suggest that species such as *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* are able to significantly “overcolonize” the airway at the expense of other flora.

In a severe asthmatic population, dominant colonization of induced sputum with any of these three species was associated with worse airways obstruction and a neutrophilic asthma phenotype. Dominance of these species was not significantly associated with smoking history or steroid therapy. Longer disease duration was associated with dominant colonization with these species.

Examination of induced sputum and BAL specimens from severe asthmatics demonstrate similar patterns of detection and abundance of *Haemophilus sp., M. catarrhalis* and *Streptococcal sp.* suggesting a homogeneity of colonization throughout the severe asthmatic airway.
We have identified the neutrophilic asthma phenotype as being associated with airway colonization with potentially pathogenic bacterial species more commonly linked to lower airway carriage in COPD. This asthma phenotype responds poorly to corticosteroid therapy and there are currently few therapeutic options.

The Gram-positive coccus *S. aureus* is detected in higher abundance in the distal airways on BAL sampling than in induced sputum in severe asthmatic subjects ($p=0.041$). This species has previously been linked with the production of superantigens and steroid resistance.
Surrogate evidence of the action of *Staphylococcal* enterotoxins is seen in severe asthmatic subjects who are colonized by *S. aureus* with skewing of Vβ repertoire and oligoconal expansion of specific Vβ subsets known to act as ligands for *S.aureus* superantigens. This effect was more marked in colonized severe asthmatics than in a *S. aureus* colonized mild asthmatic and healthy control group. Trends were seen towards worse airways obstruction and higher maintenance steroid requirement in *S. aureus* colonized severe asthmatics when compared to uncolonized severe subjects, but these did not reach significance.

Therefore colonisation of the severe asthmatic lower airway with potentially pathogenic species is likely to alter asthma phenotype. These results suggest that targeted intervention, either with antibiotics or vaccine therapy, may, in future, provide additional therapeutic strategies.

11.2 Asthmatic airway susceptibility to bacterial colonization.

Bacterial colonization in the lower respiratory tract leads to a proinflammatory cytokine response, neutrophil recruitment, mucous hypersecretion, impaired mucociliary clearance and epithelial damage[163]. This can, in turn, impair innate immune defence within the airway. However, impaired innate immunity may be a cause of bacterial colonization as well as an effect in severe asthma.

Mucociliary clearance is impaired in chronic, stable asthma[164;165]. The mechanism of impaired ciliary function is not clear and is likely to be multifactorial due to reduced ciliary beat frequency and function, mucus hypersecretion and viscosity, shedding of airway ciliated epithelium and asthma associated airway inflammation[166-168]. Supernatants from *P. aeruginosa* and *H. influenzae* cultures have been shown to reduce ciliary beat frequency and cause dyskinesia in healthy human nasal cilia[169]. In addition to this *P. aeruginosa, S. pneumoniae* and *H. influenzae* enhance mucus secretion in animal airway models[170].

In our work we have not looked for evidence of viral infection, but, viral and bacterial infection cannot be considered in isolation. The role of co-infection in COPD has been demonstrated with higher bacterial load, worse symptom scores and lower FEV_1 associated with exacerbations where both viral and bacterial pathogens are identified[171].

Bronchial epithelial viral infection predisposes to colonization and infection from bacteria by disruption of barrier function[172], disruption of ciliated cells leading to reduction in mucociliary clearance[173] and alteration of epithelial cell membranes facilitating bacterial adherence[174-176].

TLR2 and TLR4 show increased expression in stable neutrophilic asthma[37]. Whether innate immune dysregulation precedes, or results from, bacterial colonization is not known. Genetic variation in TLR2 has been identified as an asthma susceptibility gene in the children of European farmers[177]

TLR7 and TLR8 are Toll-like receptors that recognise single stranded RNA[178]. Unlike TLR2 and 4 they are not expressed at the cell surface but are found in the endosomal compartment, therefore activation requires endocytosis by the immune cell. A Danish, family based genetic association study has identified SNPs of both TLR7 and 8 which have a significant association with asthma[179]. Although these

TLRs are not essential in the innate response to bacterial infection, it does demonstrate how innate immune deficiency may have a role in asthma pathogenesis.

In a mouse model, ovalbumin induced allergic airway inflammation and the TH-2 cytokines IL-4 and IL-13 significantly impaired TLR2 and IL-6 expression in response to *M. pneumoniae* infection[180] suggesting that asthma related inflammation can impair clearance of bacterial pathogens.

Alveolar macrophages (AM), a key cellular component of the airway innate immune defence, show decreased phagocytosis in moderate and severe childhood asthma when compared to healthy subjects. Furthermore, Increased AM apoptosis is seen in asthmatic subjects and is accelerated in the presence of LPS[181].

Human ageing is understood to be associated with changes in lung structure and function[182]. Of particular interest is the age related decline in ciliary beat frequency and function of the mucociliary escalator[183;184]. Asthma duration was significantly associated with the emergence of pathogenic species as the dominant airway species in asthma. Mean age was higher in the PPM dominant group but not significantly so. Similarly the age of severe asthmatic subjects was higher than mild subjects and healthy controls. The elderly are more likely to develop pneumonia and have a fatal outcome[182], and age related decline in innate immune function may have a role in changing colonization patterns but this would apply in subjects over the age of 65 who were not, by and large the age group recruited.

Thus the innate immune system, the first line of defence from airway bacterial infection can be compromised in asthma through different mechanisms. PAMPs from viruses or bacteria, reactive oxygen species from tobacco smoke or pollution and allergens in sensitised individuals can compromise the epithelial barrier, damage epithelial tight junctions and lead to abnormal mucociliary clearance further worsening innate immune function. Colonising species shed antigen, activating pattern recognition receptors leading to a chemokine response, recruiting further neutrophils, macrophages and T cells to the site of inflammation which may be contributing to treatment resistance in severe disease.

11.3 Airway colonization

The healthy airway has previously been considered sterile[76;90;91] however our culture independent analysis shows a variety of species within the proximal lower airway with a decreasing number of detectable species moving distally. Colonization of the airway, although un-associated with the systemic signs and symptoms of infection, is not a benign condition. Failure of the initial host inflammatory response to clear an organism leads to the vicious circle of airway tissue damage, a process more familiar in bronchiectasis[185]. Asthmatic patients with a diagnosis of bronchiectasis were not selected as subjects for investigation. However findings of bronchiectatic change are commonly found if HRCT is used in the routine evaluation of the severe asthmatic patient[186]. Bronchial wall thickening, hyperlucency, centrilobular prominence, bronchiectasis, thick linear opacities and mucoid impaction are features seen in both eosinophilic asthma and neutrophilic asthma and correlate with disease severity, FEV_1 decline and duration of disease[187]. These radiological findings, seen when looking for evidence of remodelling, would suggest that if bacterial colonization is not aetiologically significant, airway structural change over time lends itself to the persistence of airway pathogenic organisms.

11.4 Proinflammatory effect of airway lipopolysaccharide

Inhalation of endotoxin in asthmatic subjects leads to significant, transient airways obstruction and increase in bronchial hyperresponsiveness[188], an effect not seen in non asthmatic subjects[189].

Direct, controlled segmental instillation of endotoxin, allergen or both into the airways of house dust mite sensitive asthmatic subjects has shown that LPS enhances allergic inflammation[190]. Lung segments treated with both LPS and allergen showed increased inflammatory cell infiltrate, with increased numbers of lymphocytes, neutrophils, eosinophils, monocytes, and myeloid dendritic cells (mDCs), increased IL-1α, IL-6 and TNF α in BAL fluid when compared to segments treated with allergen alone. Increased mDC number is of interest as these antigen presenting cells express TLR2 and TLR4 and are potent inducers of T cell proliferation.

Chronic LPS exposure in mice results in a chronic peribronchial lymphocytic inflammation, macrophage accumulation, mucus cell metaplasia and airway wall

thickening[191]. Therefore, resident airway bacteria may play a role in persistence of airways inflammation in the chronically colonized asthmatic subject.

11.5 Vaccination use in asthma

Antibiotic use in early childhood has been linked to disruption of intestinal flora and subsequent development of childhood wheeze and asthma[192;193]. Change in intestinal flora could well be associated with change in respiratory tract colonization. However, whether antibiotic use in childhood is a cause of asthma or a result of prescribing for early onset asthma like symptoms in those that would go on to develop the disease anyway, is yet to be resolved[194].

Immunomodulatory therapy with intra-tracheal, killed *Streptococcus pneumoniae* in a mouse model of allergic asthma showed a reduction in TH-2 driven inflammation, mucus hypersecretion and airway hyperreactivity[195]. The effect of current pneumococcal vaccination programmes on asthma prevalence is not known and there is no evidence that currently available pneumoncoccal vaccines, which are associated with low antibody production, are able to suppress TH-2 related inflammation or allergic airways disease[196].

Oral non-typeable *Haemophilus influenzae* vaccination has been trialled in COPD with short term reductions seen in carriage rates and acute exacerbations[197]. Multicomponent vaccines, or bacterial lysate, are immunostimulatory agents made from mixtures of antigens derived from multiple bacterial species. These oral agents present PAMPs via TLR receptors to antigen presenting cells leading to induction of T helper cells and production of B lymphocytes and specific IgA within the respiratory tract[198]. These agents have been demonstrated to shorten exacerbation duration and symptoms in COPD[199]. However, little is known about the long term effects on airway bacterial diversity or load and there is a lack of randomised controlled trials of their use in asthma.

11.6 Use of T-RFLP profiling in asthma

In vitro culture of specimens is tailored to detect a limited range of species[200] and will only grow a fraction of a diverse, complex microbial community[201]. T-RFLP profiling was used for detection of bacterial species. The advantage of this technique is that it gives a profile of all bacterial species found within a respiratory sample, regardless of assumptions made a priori about which species will be present. It also provides an estimate of the percentage abundance of a bacterial

species, therefore the dominant species are identified. The drawbacks of this molecular technique, however, have been its inability to identify individual species within the *Streptococcus* and *Haemophilus* genera and the lack of estimate of overall bacterial load. Bacterial load has been estimated in previous COPD studies by calculation of numbers of colony forming units on culture[20;202]. This technique however, is not entirely satisfactory as it is under the influence of "culture bias".
The detection of bacterial DNA using T-RFLP profiling will not distinguish between live and dead bacteria. Colonisation with actively replicating, metabolically active, species could more accurately be detected using Reverse Transcriptase Terminal Restriction Fragment Length Polymorphism profiling (RT-T-RFLP) which detects bacteria RNA. However this technology was not available to us at the time of study.

Previous studies of long term antibiotic use in asthma have had disappointing results. However these results suggest that a placebo controlled trial of long term macrolide therapy in severe asthmatic subjects with proven dominant airway PPM colonisation and neutrophilic inflammation would be justified.
T-RFLP profiling is a useful tool to obtain a semi-quantitative indication of all genera within specimens. However more specific PCR for individual species such as *H. influenzae* or *S. pneumoniae* is needed to justify the use of targeted vaccine therapy in subjects in whom bacterial colonisation is felt to have a significant role.

12 References

Reference List

1. Beasley R. The Global Burden of Asthma Report; Global Initiative for Asthma (GINA) www.ginasthma.org. 2008.

   Ref Type: Generic

2. Asthma UK. Where do we stand? Asthma in the UK today. 2004.

   Ref Type: Report

3. Joint Health Surveys Unit (2002). Health Survey for England 2001. TSO London . 2002.

   Ref Type: Generic

4. Chanez, P., S. E. Wenzel, G. P. Anderson, J. M. Anto, E. H. BEL, L. P. Boulet, C. E. Brightling, W. W. Busse, M. Castro, B. Dahlen, S. E. Dahlen, L. M. Fabbri, S. T. Holgate, M. Humbert, M. Gaga, G. F. Joos, B. Levy, K. F. RABE, P. J. STERK, S. J. Wilson, and I. Vachier. 2007. Severe asthma in adults: What are the important questions? *Journal of Allergy and Clinical Immunology* 119:1337-1348.

5. Antonicelli, L., C. Bucca, M. Neri, F. De Benedetto, P. Sabbatani, F. Bonifazi, H. G. Eichler, Q. Zhang, and D. D. Yin. 2004. Asthma severity and medical resource utilisation. *Eur Respir J* 23:723-729.

6. Serra-Batlles, J., V. Plaza, E. Morejon, A. Comella, and J. Brugues. 1998. Costs of asthma according to the degree of severity. *Eur Respir J* 12:1322-1326.

7. Wenzel, S. 2005. Severe Asthma in Adults. *Am.J.Respir.Crit.Care Med.* 172:149-160.

8. Chobot R, Uvitskt I H, and Dundy H. 1951. The relationship of the etiologic factors in asthma in infants and children. *J Allergy* 22:106-110.

9. Rackemann, F. M. 1920. The Relation of Sputum Bacteria to Asthma. *J Immunol* 5:373-377.

10. Nicholson, K. G., J. Kent, and D. C. Ireland. 1993. Respiratory Viruses and Exacerbations of Asthma in Adults. *British Medical Journal* 307:982-986.

11. Johnston, S. L., P. K. Pattemore, G. Sanderson, S. Smith, F. Lampe, L. Josephs, P. Symington, S. O'Toole, S. H. Myint, D. A. Tyrrell, and . 1995. Community study of role of viral infections in exacerbations of asthma in 9-11 year old children. *BMJ* 310:1225-1229.

12. Martin, R. J., M. Kraft, H. W. Chu, E. A. Berns, and G. H. Cassell. 2001. A link between chronic asthma and chronic infection. *Journal of Allergy and Clinical Immunology* 107:595-601.

13. Von, H. L., T. Vasankari, K. Liippo, E. Wahlstrom, and M. Puolakkainen. 2002. Chlamydia pneumoniae and severity of asthma. *Scand.J Infect.Dis.* 34:22-27.

14. Cook, P. J., P. Davies, W. Tunnicliffe, J. G. Ayres, D. Honeybourne, and R. Wise. 1998. Chlamydia pneumoniae and asthma. *Thorax* 53:254-259.

15. Huittinen, T., D. Hahn, T. Anttila, E. Wahlstrom, P. Saikku, and M. Leinonen. 2001. Host immune response to Chlamydia pneumoniae heat shock protein 60 is associated with asthma. *Eur Respir J* 17:1078-1082.

16. Black, P. N., R. Scicchitano, C. R. Jenkins, F. Blasi, L. Allegra, J. Wlodarczyk, and B. C. Cooper. 2000. Serological evidence of infection with Chlamydia pneumoniae is related to the severity of asthma. *Eur Respir J* 15:254-259.

17. Rogers, G., T. Daniels, A. Tuck, M. Carroll, G. Connett, G. David, and K. Bruce. 2009. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC Pulmonary Medicine* 9:14.

18. Murray A, Mostafa S van Saene H. Essentials in clinical microbiology. Baillieres's: Clinical Anaesthesiology 5: pp. 1-23. 1991.
Ref Type: Generic

19. Banerjee, D., O. A. Khair, and D. Honeybourne. 2004. Impact of sputum bacteria on airway inflammation and health status in clinical stable COPD. *Eur Respir J* 23:685-691.

20. Wilkinson, T. M. A., I. S. Patel, M. Wilks, G. C. Donaldson, and J. A. Wedzicha. 2003. Airway Bacterial Load and FEV1 Decline in Patients with Chronic Obstructive Pulmonary Disease. *Am.J.Respir.Crit.Care Med.* 167:1090-1095.

21. Patel, I. S., T. A. R. Seemungal, M. Wilks, S. J. Lloyd-Owen, G. C. Donaldson, and J. A. Wedzicha. 2002. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax* 57:759-764.

22. Sethi, S., J. Maloney, L. Grove, C. Wrona, and C. S. Berenson. 2006. Airway Inflammation and Bronchial Bacterial Colonization in Chronic Obstructive Pulmonary Disease. *Am.J.Respir.Crit.Care Med.* 173:991-998.

23. ten BRINKE, A. N. N. E., C. I. N. D. de LANGE, A. H. ZWINDERMAN, K. F. RABE, P. J. STERK, and E. H. BEL. 2001. Sputum Induction in Severe Asthma by a Standardized Protocol . Predictors of Excessive Bronchoconstriction. *Am.J.Respir.Crit.Care Med.* 164:749-753.

24. Pizzichini, E., M. M. Pizzichini, A. Efthimiadis, F. E. Hargreave, and J. Dolovich. 1996. Measurement of inflammatory indices in induced sputum: effects of selection of sputum to minimize salivary contamination. *Eur Respir J* 9:1174-1180.

25. Gershman, N. H., H. H. Wong, J. T. Liu, M. J. Mahlmeister, and J. V. Fahy. 1996. Comparison of two methods of collecting induced sputum in asthmatic subjects. *Eur Respir J* 9:2448-2453.

26. Leader of the Working Group:, Members of the Working Group:, Q. Hamid, M. M. Kelly, M. Linden, R. Louis, M. M. M. Pizzichini, E. Pizzichini, C. Ronchi, F. Van Overveld, and R. Djukanovic. 2002. Methods of sputum processing for cell counts, immunocytochemistry and in situ hybridisation. *Eur Respir J* 20:19S-23.

27. Murray, P. R. and J. A. Washington. 1975. Microscopic and baceriologic analysis of expectorated sputum. *Mayo Clin Proc.* 50:339-344.

28. ALEXIS, N. E., S. C. HU, K. I. R. B. ZEMAN, T. O. D. D. ALTER, and W. D. BENNETT. 2001. Induced Sputum Derives from the Central Airways . Confirmation Using a Radiolabeled Aerosol Bolus Delivery Technique. *Am.J.Respir.Crit.Care Med.* 164:1964-1970.

29. Thomson, R. B., Jr. 1999. Laboratory diagnosis of respiratory infections. *Curr.Opin.Infect.Dis.* 12:115-119.

30. Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol.Rev.* 59:143-169.

31. Bruce KD and M. R. Hughes. 2000. Terminal restriction fragment length polymorphism monitoring of genes amplified directly from bacterial communities in soils and sediments. *Mol Microbiol* 16:261-269.

32. Liu, W. T., T. L. Marsh, H. Cheng, and L. J. Forney. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl.Environ.Microbiol.* 63:4516-4522.

33. Rogers, G. B., C. A. Hart, J. R. Mason, M. Hughes, M. J. Walshaw, and K. D. Bruce. 2003. Bacterial Diversity in Cases of Lung Infection in Cystic Fibrosis Patients: 16S Ribosomal DNA (rDNA) Length Heterogeneity PCR and 16S rDNA Terminal Restriction Fragment Length Polymorphism Profiling. *J.Clin.Microbiol.* 41:3548-3558.

34. Djukanovic, R., W. R. Roche, J. W. Wilson, C. R. Beasley, O. P. Twentyman, R. H. Howarth, and S. T. Holgate. 1990. Mucosal inflammation in asthma. *Am Rev.Respir Dis.* 142:434-457.

35. Reed, C. E. and D. K. Milton. 2001. Endotoxin-stimulated innate immunity: A contributing factor for asthma. *J Allergy Clin.Immunol.* 108:157-166.

36. Sabroe, I., C. M. Lloyd, M. K. B. Whyte, S. K. Dower, T. J. Williams, and J. E. Pease. 2002. Chemokines, innate and adaptive immunity, and respiratory disease. *Eur Respir J* 19:350-355.

37. Simpson, J. L., T. V. Grissell, J. Douwes, R. J. Scott, M. J. Boyle, and P. G. Gibson. 2007. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* 62:211-218.

38. Peden, D. B., K. Tucker, P. Murphy, L. Newlin-Clapp, B. Boehlecke, M. Hazucha, P. Bromberg, and W. Reed. 1999. Eosinophil influx to the nasal airway after local, low-level LPS challenge in humans. *J Allergy Clin Immunol* 104:388-394.

39. Schwartz, D. A. 2001. Does Inhalation of Endotoxin Cause Asthma? *Am.J.Respir.Crit.Care Med.* 163:305-306.

40. von, M. E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, S. Maisch, M. Waser, and D. Nowak. 2000. Exposure to endotoxin or other bacterial components might protect against the development of atopy. *Clin Exp.Allergy* 30:1230-1234.

41. Gereda, J. E., D. Y. M. Leung, A. Thatayatikom, J. E. Streib, M. R. Price, M. D. Klinnert, and A. H. Liu. 2000. Relation between house-dust endotoxin exposure, type 1 T-cell development, and allergen sensitisation in infants at high risk of asthma. *The Lancet* 355:1680-1683.

42. Braun-Fahrlander, C., J. Riedler, U. Herz, W. Eder, M. Waser, L. Grize, S. Maisch, D. Carr, F. Gerlach, A. Bufe, R. P. Lauener, R. Schierl, H. Renz, D. Nowak, E. von Mutius, and the Allergy and Endotoxin Study Team. 2002. Environmental Exposure to Endotoxin and Its Relation to Asthma in School-Age Children. *N Engl J Med* 347:869-877.

43. Gehring, U., W. Bischof, B. Fahlbusch, H. E. Wichmann, and J. Heinrich. 2002. House Dust Endotoxin and Allergic Sensitization in Children. *Am.J.Respir.Crit.Care Med.* 166:939-944.

44. Martin, M., J. Katz, S. N. Vogel, and S. M. Michalek. 2001. Differential Induction of Endotoxin Tolerance by Lipopolysaccharides Derived from Porphyromonas gingivalis and Escherichia coli. *J Immunol* 167:5278-5285.

45. Cleveland, M. G., J. D. Gorham, T. L. Murphy, E. Tuomanen, and K. M. Murphy. 1996. Lipoteichoic acid preparations of gram-positive bacteria induce interleukin-12 through a CD14-dependent pathway. *Infect.Immun.* 64:1906-1912.

46. Hoogerwerf, J. J., A. F. de Vos, P. Bresser, J. S. van der Zee, J. M. Pater, A. de Boer, M. Tanck, D. L. Lundell, C. Her-Jenh, C. Draing, S. von Aulock, and T. van der Poll. 2008. Lung Inflammation Induced by Lipoteichoic Acid or Lipopolysaccharide in Humans. *Am.J.Respir.Crit.Care Med.*200708-1261OC.

47. Bisgaard, H., M. N. Hermansen, F. Buchvald, L. Loland, L. B. Halkjaer, K. Bonnelykke, M. Brasholt, A. Heltberg, N. H. Vissing, S. V. Thorsen, M. Stage, and C. B. Pipper. 2007. Childhood Asthma after Bacterial Colonization of the Airway in Neonates. *N Engl J Med* 357:1487-1495.

48. Llewelyn, M. and J. Cohen. 2002. Superantigens: microbial agents that corrupt immunity. *The Lancet Infectious Diseases* 2:156-162.

49. Gould, H. J., P. Takhar, H. E. Harries, E. Chevretton, and B. J. Sutton. 2007. The Allergic March from Staphylococcus aureus Superantigens to Immunoglobulin E. *Chem.Immunol.Allergy* 93:106-136.

50. Choi, Y., B. Kotzin, L. Herron, J. Callahan, P. Marrack, and J. Kappler. 1989. Interaction of Staphylococcus aureus Toxin "Superantigens" with Human T Cells. *Proceedings of the National Academy of Sciences* 86:8941-8945.

51. Kappler, J., B. Kotzin, L. Herron, E. W. Gelfand, R. D. Bigler, A. Boylston, S. Carrel, D. N. Posnett, Y. Choi, and P. Marrack. 1989. V beta-specific stimulation of human T cells by staphylococcal toxins. *Science* 244:811-813.

52. Omoe, K., Hu DL, H. Takahashi-Omoe, A. Nakane, and K. Shinagawa. 2005. Comprehensive analysis of classical and newly described staphylococcal superantigenic toxin genes in Staphylococcus aureus isolates. *FEMS Microbiol Lett* 246:191-198.

53. Marrack, P. and J. Kappler. 1990. The Staphylococcal Enterotoxins and Their Relatives. *Science* 248:705-711.

54. Thomas, D., Chou S, O. Dauwalder, and G. Lina. 2007. Diversity in Staphylococcus aureus enterotoxins. *Chem.Immunol.Allergy* 93:24-41.

55. Shiomori, Teruo, Yoshida, Shin ichi, Miyamoto, Hiroshi, and Makishima, Kazumi. Relationship of nasal carriage of Staphylococcus aureus to pathogenesis of perennial allergic rhinitis. Journal of Allergy and Clinical Immunology 105[3], 449-454. 2000.
Ref Type: Abstract

56. Rossi, R. E. and G. Monasterolo. 2004. Prevalence of serum IgE antibodies to the Staphylococcus aureus enterotoxins (SAE, SEB, SEC, SED, TSST-1) in patients with persistent allergic rhinitis. *Int.Arch.Allergy Immunol.* 133:261-266.

57. Riechelmann, H., A. Essig, T. Deutschle, A. Rau, B. Rothermel, and M. Weschta. 2005. Nasal carriage of Staphylococcus aureus in house dust mite allergic patients and healthy controls. *Allergy* 60:1418-1423.

58. Okano, M., H. Hattori, T. Yoshino, Y. Sugata, M. Yamamoto, T. Fujiwara, A. A. Satoskar, A. R. Satoskar, and K. Nishizaki. 2005. Nasal exposure to Staphylococcal enterotoxin enhances the development of allergic rhinitis in mice. *Clinical & Experimental Allergy* 35:506-514.

59. Van Zele, T., P. Gevaert, J. B. Watelet, G. Claeys, G. Holtappels, C. Claeys, P. van Cauwenberge, and C. Bachert. 2004. Staphylococcus aureus colonization and IgE antibody formation to enterotoxins is increased in nasal polyposis. *Journal of Allergy and Clinical Immunology* 114:981-983.

60. Hauser, C., B. Wuethrich, L. Matter, J. A. Wilhelm, W. Sonnabend, and K. Schopfer. 1985. Staphylococcus aureus skin colonization in atopic dermatitis patients. *Dermatologica* 170:35-39.

61. Leung, D. Y. 2003. Infection in atopic dermatitis. *Curr.Opin.Pediatr.* 15:399-404.

62. Postlethwaite, A. E., M. A. Holness, H. Katai, and R. Raghow. 1992. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* 90:1479-1485.

63. Lin, Y. T., C. T. Wang, and B. L. Chiang. 2007. Role of bacterial pathogens in atopic dermatitis. *Clin Rev.Allergy Immunol* 33:167-177.

64. Zollner, T. M., T. A. Wichelhaus, A. Hartung, M. C. Von, T. O. Wagner, V. Brade, and R. Kaufmann. 2000. Colonization with superantigen-producing Staphylococcus aureus is associated with increased severity of atopic dermatitis. *Clin.Exp.Allergy* 30:994-1000.

65. Bunikowski, R., M. E. Mielke, H. Skarabis, M. Worm, I. Anagnostopoulos, G. Kolde, U. Wahn, and H. Renz. 2000. Evidence for a disease-promoting effect of Staphylococcus aureus-derived exotoxins in atopic dermatitis. *J.Allergy Clin.Immunol.* 105:814-819.

66. Wachs, G. N. and H. I. Maibach. 1976. Co-operative double-blind trial of an antibiotic/corticoid combination in impetiginized atopic dermatitis. *Br.J Dermatol.* 95:323-328.

67. Corrigan, C. J., P. H. Brown, N. C. Barnes, J. J. Tsai, A. J. FREW, and A. B. Kay. 1991. Glucocorticoid resistance in chronic asthma. Peripheral blood T lymphocyte

activation and comparison of the T lymphocyte inhibitory effects of glucocorticoids and cyclosporin A. *Am.Rev.Respir Dis.* 144:1026-1032.

68. Hauk, P. J., Q. A. Hamid, G. P. Chrousos, and D. Y. M. Leung. 2000. Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. *Journal of Allergy and Clinical Immunology* 105:782-787.

69. Leung, D. Y. M., Q. Hamid, A. Vottero, S. J. Szefler, W. Surs, E. Minshall, G. P. Chrousos, and D. J. Klemm. 1997. Association of Glucocorticoid Insensitivity with Increased Expression of Glucocorticoid Receptor beta á. *J.Exp.Med.* 186:1567-1574.

70. Hellings, P. W., G. Hens, I. Meyts, D. Bullens, J. Vanoirbeek, P. Gevaert, M. Jorissen, J. L. Ceuppens, and C. Bachert. 2006. Aggravation of bronchial eosinophilia in mice by nasal and bronchial exposure to Staphylococcus aureus enterotoxin B. *Clin Exp.Allergy* 36:1063-1071.

71. Herz, U., R. Ruckert, T. Tschernig, K. Wollenhaupt, U. Neuhaus-Steinmetz, R. Pabst, and H. Renz. 1999. Airway exposure to bacterial superantigen (SEB) induces airway inflammation associated with airway hyperresponsiveness - A model for non-allergic asthma. *Faseb Journal* 13:A1126.

72. Bachert, C., P. Gevaert, P. Howarth, G. Holtappels, C. P. van, and S. G. Johansson. 2003. IgE to Staphylococcus aureus enterotoxins in serum is related to severity of asthma. *J.Allergy Clin.Immunol.* 111:1131-1132.

73. Bachert, C., P. Gevaert, N. Zhang, Z. T. van, and C. Perez-Novo. 2007. Role of staphylococcal superantigens in airway disease. *Chem.Immunol.Allergy* 93:214-236.

74. Wahlstr&ouml, m J.[1] [2], D. Gigliotti, A. Roquet, H. Wigzell, A. Eklund, and J. Grunewald. 2001. T Cell Receptor Vbeta Expression in Patients with Allergic

Asthma before and after Repeated Low-Dose Allergen Inhalation. *Clinical Immunology* 100:31-39.

75. Hauk, P. J., S. E. Wenzel, A. E. Trumble, S. J. Szefler, and D. Y. Leung. 1999. Increased T-cell receptor vbeta8+ T cells in bronchoalveolar lavage fluid of subjects with poorly controlled asthma: a potential role for microbial superantigens. *J Allergy Clin.Immunol* 104:37-45.

76. Laurenzi GA, P. R. K. E. 1961. Bacteriologic flora of the lower respiratory tract. *N.Engl.J Med.* 265:1273-1278.

77. Wenzel SE, F. J. I. C. P. S. S. S. S. SJ. 2000. Proceedings of the ATS Workshop on Refractory Asthma . Current Understanding, Recommendations, and Unanswered Questions. *Am.J.Respir.Crit.Care Med.* 162:2341-2351.

78. 2008. British Guideline on the Management of Asthma. *Thorax*1-121.

79. Juniper, E. F., P. M. O'Byrne, G. H. Guyatt, P. J. Ferrie, and D. R. King. 1999. Development and validation of a questionnaire to measure asthma control. *Eur Respir J* 14:902-907.

80. Quanjer PH. Standardized lung function testing. Bull Eur Physiopathol Respir 19 suppl. 5, 45-51. 1-1-1983.
Ref Type: Generic

81. Chai, H., R. S. Farr, L. A. Froehlich, D. A. Mathison, J. A. McLean, R. R. Rosenthal, A. L. Sheffer, S. L. Spector, and R. G. Townley. 1975. Standardization of bronchial inhalation challenge procedures. *Journal of Allergy and Clinical Immunology* 56:323-327.

82. Paggiaro PL., P. Chanez, O. Holz, P. W. Ind, R. Djukanovic, P. Maestrelli, and P. J. Sterk. 2002. Sputum induction. *Eur Respir J* 20:3S-8.

83. Rogers, G. B., M. P. Carroll, D. J. Serisier, P. M. Hockey, G. Jones, and K. D. Bruce. 2004. Characterization of Bacterial Community Diversity in Cystic Fibrosis

Lung Infections by Use of 16S Ribosomal DNA Terminal Restriction Fragment Length Polymorphism Profiling. *J.Clin.Microbiol.* 42:5176-5183.

84. ten BRINKE, A. N. N. E., C. I. N. D. de LANGE, A. H. ZWINDERMAN, K. F. RABE, P. J. STERK, and E. H. BEL. 2001. Sputum Induction in Severe Asthma by a Standardized Protocol . Predictors of Excessive Bronchoconstriction. *Am.J.Respir.Crit.Care Med.* 164:749-753.

85. FAHY, J. V., H. A. BOUSHEY, S. C. LAZARUS, E. A. MAUGER, R. M. CHERNIACK, V. M. CHINCHILLI, T. J. CRAIG, J. M. DRAZEN, J. G. FORD, J. E. FISH, E. L. L. I. ISRAEL, M. O. N. I. KRAFT, R. F. LEMANSKE, R. J. Martin, D. I. A. N. McLEAN, S. P. PETERS, C. H. R. I. SORKNESS, and S. J. Szefler. 2001. Safety and Reproducibility of Sputum Induction in Asthmatic Subjects in a Multicenter Study. *Am.J.Respir.Crit.Care Med.* 163:1470-1475.

86. Djukanovic, R., P. J. Sterk, J. V. Fahy, and Hargreave{section}. 2002. Standardised methodology of sputum induction and processing. *Eur Respir J* 20:1S-2.

87. Geckler, R. W., D. H. Gremillion, C. K. McAllister, and C. Ellenbogen. 1977. Microscopic and bacteriological comparison of paired sputa and transtracheal aspirates. *J.Clin.Microbiol.* 6:396-399.

88. Joyce, S. M. 1986. Sputum analysis and culture. *Annals of Emergency Medicine* 15:325-328.

89. Health Protection Agency. 2008. Investigation of bronchoalveolar lavage, sputum and associated specimens, BSOP 57.

90. LEES, A. W. and W. MCNAUGHT. 1959. Bacteriology of lower-respiratory-tract secretions, sputum, and upper-respiratory-tract secretions in "normals" and chronic bronchitics. *Lancet* 19;2:1112-1115.

91. Cabello, H., A. Torres, R. Celis, M. El-Ebiary, J. Puig de la Bellacasa, A. Xaubet, J. Gonzalez, C. Agusti, and N. Soler. 1997. Bacterial colonization of distal airways in healthy subjects and chronic lung disease: a bronchoscopic study. *Eur Respir J* 10:1137-1144.

92. GENCAY, M. E. S. U., J. J. RUDIGER, M. I. C. H. TAMM, M. A. R. K. SOLER, A. P. PERRUCHOUD, and M. I. C. H. ROTH. 2001. Increased Frequency of Chlamydia pneumoniae Antibodies in Patients with Asthma. *Am.J.Respir.Crit.Care Med.* 163:1097-1100.

93. Keatings, V. M., D. J. Evans, B. J. O'Connor, and P. J. Barnes. 1997. Cellular profiles in asthmatic airways: a comparison of induced sputum, bronchial washings, and bronchoalveolar lavage fluid. *Thorax* 52:372-374.

94. ALEXIS, N. E., S. C. HU, K. I. R. B. ZEMAN, T. O. D. D. ALTER, and W. D. BENNETT. 2001. Induced Sputum Derives from the Central Airways . Confirmation Using a Radiolabeled Aerosol Bolus Delivery Technique. *American Journal of Respiratory and Critical Care Medicine* 164:1964-1970.

95. Holz, O., J. Kips, and H. Magnussen. 2000. Update on sputum methodology. *Eur Respir J* 16:355-359.

96. Rogers, G. B., M. P. Carroll, D. J. Serisier, P. M. Hockey, G. Jones, V. Kehagia, G. J. Connett, and K. D. Bruce. 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. *J.Clin.Microbiol.* 44:2601-2604.

97. Magalhaes, J. G., I. Tattoli, and S. E. Girardin. 2007. The intestinal epithelial barrier: How to distinguish between the microbial flora and pathogens. *Seminars in Immunology* 19:106-115.

98. Crim, C., P. M. A. Calverley, J. A. Anderson, B. Celli, G. T. Ferguson, C. Jenkins, P. W. Jones, L. R. Willits, J. C. Yates, and J. Vestbo. 2009. Pneumonia risk in COPD patients receiving inhaled corticosteroids alone or in combination: TORCH study results. *Eur Respir J*09031936.

99. Ernst, P., A. V. Gonzalez, P. Brassard, and S. Suissa. 2007. Inhaled Corticosteroid Use in Chronic Obstructive Pulmonary Disease and the Risk of Hospitalization for Pneumonia. *Am.J.Respir.Crit.Care Med.* 176:162-166.

100. Tanaka S., M. Yoshida, Y. Murakami, T. Ogiwara, M. Shoji, Kobayashi S, Watanabe S, M. Machino, and S. Fujisawa. 2008. The relationship of Prevotella intermedia, Prevotella nigrescens and Prevotella melaninogenica in the supragingival plaque of children, caries and oral malodor. *J Clin Pediatr Dent.*

101. Naito, M., H. Hirakawa, A. Yamashita, N. Ohara, M. Shoji, H. Yukitake, K. Nakayama, H. Toh, F. Yoshimura, Kuhara S, M. Hattori, T. Hayashi, and K. Nakayama. 2008. Determination of the genome sequence of Porphyromonas gingivalis strain ATCC 33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in P. gingivalis. *DNA Res.*

102. Strauss, J., A. White, C. Ambrose, J. McDonald, and E. len-Vercoe. 2008. Phenotypic and genotypic analyses of clinical Fusobacterium nucleatum and Fusobacterium periodonticum isolates from the human gut. *Clinical Microbiology*.

103. Zaura, E., B. J. Keijser, S. M. Huse, and W. Crielaard. 2009. Defining the healthy "core microbiome" of oral microbial communities. *BMC Microbiology*.

104. Collins MD. and P. A. Lawson. 2000. The genus Abiotrophia (Kawamura et al.) is not monophyletic: proposal of Granulicatella gen. nov., Granulicatella adiacens comb. nov., Granulicatella elegans comb. nov. and Granulicatella balaenopterae comb. nov. *International Journal of Systematic and Evolutionary Microbiology*.

105. Woo PC, Fung AM, Lau SK, Chan BY, Chiu SK, Teng JL, Que TL, Yung RW, and K. Y. Yuen. 2003. Granulicatella adiacens and Abiotrophia defectiva bacteraemia characterized by 16S rRNA gene sequencing. *Journal of Medical Microbiology*.

106. Chernow, B., Johnson LF, Janowitz WR, and D. O. Castell. 1974. Pulmonary aspiration as a consequence of gastroesophageal reflux: a diagnostic approach. *Dig Dis Sci.*

107. Jack CI., Calverley PM., Donnelly RJ., J. Tran, G. Russell, Hind CR., and C. C. Evans. 1995. Simultaneous tracheal and oesophageal pH measurements in asthmatic patients with gastro-oesophageal reflux. *Thorax*.

108. Abusriwil, H. and R. A. Stockley. 2007. The Interaction of Host and Pathogen Factors in Chronic Obstructive Pulmonary Disease Exacerbations and Their Role in Tissue Damage. *Proc Am Thorac Soc* 4:611-617.

109. BRESSER, P. A. U. L., T. A. OUT, L. O. E. K. van ALPHEN, H. M. JANSEN, and R. E. N. E. LUTTER. 2000. Airway Inflammation in Nonobstructive and Obstructive Chronic Bronchitis with Chronic Haemophilus influenzae Airway Infection . Comparison with Noninfected Patients with Chronic Obstructive Pulmonary Disease. *Am.J.Respir.Crit.Care Med.* 162:947-952.

110. Wenzel SE, Schwartz LB, Langmack EL, Halliday JL, Trudeau JB, Gibbs RL, and H. W. Chu. 2009. Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am.J.Respir.Crit.Care Med.*

111. Pavord ID, Brightling CE, G. Woltmann, and A. J. Wardlaw. 1999. Non-eosinophilic corticosteroid unresponsive asthma. *Lancet* 353:2213-2214.

112. Burns JL, J. Emerson, Stapp JR, Yim DL, J. Krzewinski, L. Louden, Ramsey BW, and C. R. Clausen. 1998. Microbiology of sputum from patients at cystic fibrosis centers in the United States. *Clinical Infectious Disease*.

113. Wahab, A. A., I. A. Janahi, M. M. Marafia, and S. El-Shafie. 2004. Microbiological Identification in Cystic Fibrosis Patients with CFTR I1234V Mutation. *J Trop Pediatr* 50:229-233.

114. Sethi, S. and T. F. Murphy. 2008. Infection in the Pathogenesis and Course of Chronic Obstructive Pulmonary Disease. *N Engl J Med* 359:2355-2365.

115. Soler, N., S. Ewig, A. Torres, X. Filella, J. Gonzalez, and A. Zaubet. 1999. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *Eur Respir J* 14:1015-1022.

116. Zetterlund, A., P. H. LARSSON, C. M³ller-Suur, L. Palmberg, and K. Larsson. 1998. Budesonide but not terbutaline decreases phagocytosis in alveolar macrophages. *Respiratory Medicine* 92:162-166.

117. Wolfe, F., L. Caplan, and K. Michaud. 2006. Treatment for rheumatoid arthritis and the risk of hospitalization for pneumonia: associations with prednisone, disease-modifying antirheumatic drugs, and anti-tumor necrosis factor therapy. *Arthritis and Rheumatism*.

118. Wenzel, S. E., L. B. SCHWARTZ, E. L. LANGMACK, J. L. HALLIDAY, J. B. TRUDEAU, R. L. GIBBS, and H. W. Chu. 1999. Evidence That Severe Asthma Can Be Divided Pathologically into Two Inflammatory Subtypes with Distinct Physiologic and Clinical Characteristics. *Am.J.Respir.Crit.Care Med.* 160:1001-1008.

119. Simpson JL, R. Scott, Boyle MJ, and P. G. Gibson. 2006. Inflammatory subtypes in asthma: assessment and identification using induced sputum. *Respirology*.

120. Bacci, E., S. Cianchetti, M. Bartoli, F. L. Dente, A. Di Franco, B. Vagaggini, and P. Paggiaro. 2006. Low Sputum Eosinophils Predict the Lack of Response to Beclomethasone in Symptomatic Asthmatic Patients. *Chest* 129:565-572.

121. Green, R. H., C. E. Brightling, G. Woltmann, D. Parker, A. J. Wardlaw, and I. D. Pavord. 2002. Analysis of induced sputum in adults with asthma: identification of subgroup with isolated sputum neutrophilia and poor response to inhaled corticosteroids. *Thorax* 57:875-879.

122. Meijer RJ, Postma DS, Kauffman H, Arends LR, Koeter GH, and H. A. Kerstjens. 2002. Accuracy of eosinophils and eosinophil cationic protein to predict steroid improvement in asthma. *Clin Exp.Allergy* 32:1096-1103.

123. Gibson, P. G., J. L. Simpson, and N. Saltos. 2001. Heterogeneity of Airway Inflammation in Persistent Asthma : Evidence of Neutrophilic Inflammation and Increased Sputum Interleukin-8. *Chest* 119:1329-1336.

124. Legssyer, R. F., F. F. Huaux, J. F. Lebacq, M. F. Delos, P. Marbaix E FAU - Lebecque, P. F. Lebecque, D. F. Lison, P. Scholte BJ FAU - Wallemacq, P. F. Wallemacq, and T. Leal. 2006. Azithromycin reduces spontaneous and induced inflammation in DeltaF508 cystic fibrosis mice. *Respir.Res.*

125. Cigana, C., B. M. Assael, and P. Melotti. 2007. Azithromycin Selectively Reduces Tumor Necrosis Factor Alpha Levels in Cystic Fibrosis Airway Epithelial Cells. *Antimicrob.Agents Chemother.* 51:975-981.

126. Verleden, G. M., B. M. Vanaudenaerde, L. J. Dupont, and D. E. Van Raemdonck. 2006. Azithromycin Reduces Airway Neutrophilia and Interleukin-8 in Patients with Bronchiolitis Obliterans Syndrome. *Am.J.Respir.Crit.Care Med.* 174:566-570.

127. Piacentini, G. L., D. G. Peroni, A. Bodini, R. Pigozzi, S. Costella, A. Loiacono, and A. L. Boner. 2007. Azithromycin reduces bronchial hyperresponsiveness and neutrophilic airway inflammation in asthmatic children: A preliminary report. *Allergy and Asthma Proceedings* 28:194-198.

128. Mahler, D. A., S. Huang, M. Tabrizi, and G. M. Bell. 2004. Efficacy and Safety of a Monoclonal Antibody Recognizing Interleukin-8 in COPD*. *Chest* 126:926-934.

129. O'Connor BJ, Leaker BR Barnes PJ et al. Inhibition of LPS-induced neutrophilic inflammation in healthy volunteers [abstract]. 2009. Eur Respir J 2007; 30(suppl):1294.
Ref Type: Generic

130. Traves, S. L., S. V. Culpitt, R. E. K. Russell, P. J. Barnes, and L. E. Donnelly. 2002. Increased levels of the chemokines GRO{alpha} and MCP-1 in sputum samples from patients with COPD. *Thorax* 57:590-595.

131. Qiu, Y., J. Zhu, V. Bandi, K. K. Guntupalli, and P. K. Jeffery. 2007. Bronchial mucosal inflammation and upregulation of CXC chemoattractants and receptors in severe exacerbations of asthma. *Thorax* 62:475-482.

132. Qiu, Y., J. Zhu, V. Bandi, R. L. ATMAR, K. Hattotuwa, K. K. Guntupalli, and P. K. Jeffery. 2003. Biopsy Neutrophilia, Neutrophil Chemokine and Receptor Gene Expression in Severe Exacerbations of Chronic Obstructive Pulmonary Disease. *Am.J.Respir.Crit.Care Med.* 168:968-975.

133. Sutherland RE, Olsen JS, A. McKinstry, Villalta SA, and P. J. Wolters. 2008. Mast cell IL-6 improves survival from Klebsiella pneumonia and sepsis by enhancing neutrophil killing. *J Immunol* 181:5598-5605.

134. Martinon, F., A. Mayor, and J. Tschopp. 2009. The inflammasomes: guardians of the body. *Annual Review of Immunology* 27:229-65.

135. Persson CG, Erjefalt JS, L. Greiff, I. Erjefalt, M. Korsgren, M. Linden, F. Sundler, M. Andersson, and C. Svensson. 1998. Contribution of plasma-derived molecules to mucosal immune defence, disease and repair in the airways. *Scand.J.Immunol* 47:302-313.

136. Brims FJ, Chauhan AJ, B. Higgins, and J. K. Shute. 2009. Coagulation factors in the airways in moderate and severe asthma and the effect of inhaled steroids. *Thorax* 1468-3296 (Electronic).

137. Smith DL, Smith EG, Pitt TL, and D. E. Stableforth. 1998. Regional microbiology of the cystic fibrosis lung: a post-mortem study in adults. *J Infect* 37:41-43.

138. Gutierrez, J. P., K. Grimwood, D. S. Armstrong, J. B. Carlin, R. Carzino, A. Olinsky, C. F. Robertson, and P. D. Phelan. 2001. Interlobar differences in bronchoalveolar lavage fluid from children with cystic fibrosis. *Eur Respir J* 17:281-286.

139. Gok, U., Y. Bulut, E. Keles, S. Yalcin, and M. Z. Doymaz. 2001. Bacteriological and PCR analysis of clinical material aspirated from otitis media with effusions. *International Journal of Pediatric Otorhinolaryngology* 60:49-54.

140. Ozyilmaz E, Akan OA, M. Gulhan, K. Ahmed, and T. Nagatake. 2005. Major bacteria of community-acquired respiratory tract infections in Turkey. *Jpn J Infect.Dis.*

141. Shen, H., H. Yoshida, F. Yan, W. Li, F. Xu, H. Huang, H. Jono, and J. D. Li. 2008. Synergistic induction of MUC5AC mucin by nontypeable Haemophilus influenzae and Streptococcus pneumoniae. *Biochemical and Biophysical Research Communications* 365:795-800.

142. Harrison, F. 2007. Microbial ecology of the cystic fibrosis lung. *Microbiology* 153:917-923.

143. Pauwels, R., G. Verschraegen, and S. M. van der. 1980. IgE antibodies to bacteria in patients with bronchial asthma. *Allergy* 35:665-669.

144. Margaret Dunkley, Robert Clancy Ben Green Peter Howarth. Serum Non-typeable *Haemophilus influenzae*-specific IgE in asthma and COPD. Abstract presented Australasian Society for Immunology 2008 . 1-12-2008.
Ref Type: Generic

145. Clemans, D. L., R. J. Bauer, J. A. Hanson, M. V. Hobbs, J. W. Geme, III, C. F. Marrs, and J. R. Gilsdorf. 2000. Induction of Proinflammatory Cytokines from

Human Respiratory Epithelial Cells after Stimulation by Nontypeable Haemophilus influenzae. *Infect.Immun.* 68:4430-4440.

146. Ahren, I. L., E. Eriksson, A. Egesten, and K. Riesbeck. 2003. Nontypeable Haemophilus influenzae Activates Human Eosinophils through {beta}-Glucan Receptors. *Am.J.Respir.Cell Mol.Biol.* 29:598-605.

147. Shiomori, T., S. Yoshida, H. Miyamoto, and K. Makishima. 2000. Relationship of nasal carriage of Staphylococcus aureus to pathogenesis of perennial allergic rhinitis. *J.Allergy Clin.Immunol.* 105:449-454.

148. Balaban, N. and A. Rasooly. 2000. Staphylococcal enterotoxins. *International Journal of Food Microbiology* 61:1-10.

149. Choi, Y., Lafferty JA, Clements JR, Todd JK, Gelfand EW, J. Kappler, P. Marrack, and B. L. Kotzin. 1990. Selective expansion of T cells expressing V beta 2 in toxic shock syndrome. *J Exp Med*.

150. Wei S - Charmley, P. Charmley, Robinson MA, and P. Concannon. 1994. The extent of the human germline T-cell receptor V beta gene segment repertoire. *Immunogenetics*.

151. Proft, T. and J. D. Fraser. 2007. Streptococcal superantigens. *Chem.Immunol.Allergy* 93:1-23.

152. Jarraud, S., M. A. Peyrat, A. Lim, A. Tristan, M. Bes, C. Mougel, J. Etienne, F. Vandenesch, M. Bonneville, and G. Lina. 2001. egc, A Highly Prevalent Operon of Enterotoxin Gene, Forms a Putative Nursery of Superantigens in Staphylococcus aureus. *J Immunol* 166:669-677.

153. Rowen, L., Koop BF, and L. Hood. 1996. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. *Science* 272:1755-1762.

154. Chatila, T. and R. S. Geha. 1993. Signal transduction by microbial superantigens via MHC class II molecules. *Immunol Rev.* 131:43-59.

155. O'Brien, G. J., G. Riddell, J. S. Elborn, M. Ennis, and G. Skibinski. 2006. Staphylococcus aureus enterotoxins induce IL-8 secretion by human nasal epithelial cells. *Respir.Res.* 7:115.

156. Herz, U., R. Ruckert, K. Wollenhaupt, T. Tschernig, U. Neuhaus-Steinmetz, R. Pabst, and H. Renz. 1999. Airway exposure to bacterial superantigen (SEB) induces lymphocyte-dependent airway inflammation associated with increased airway responsiveness--a model for non-allergic asthma. *Eur.J.Immunol.* 29:1021-1031.

157. Bueno, C., G. Criado, McCormick JK, and J. Madrenas. 2007. T cell signalling induced by bacterial superantigens. *Chem.Immunol.Allergy* 93:161-180.

158. Herrmann, T. and H. R. MacDonald. 1993. The CD8 T cell response to staphylococcal enterotoxins. *Seminars in Immunology* 5:33-39.

159. Herrmann, T., Baschieri S, Lees RK, and H. R. MacDonald. 1992. In vivo responses of CD4+ and CD8+ cells to bacterial superantigens. *Eur J Immunology* 22:1935-1938.

160. MacDonald, H. R., S. Baschieri, and R. K. Lees. 1991. Clonal expansion precedes anergy and death of V beta 8+ peripheral T cells responding to staphylococcal enterotoxin B in vivo. *Eur J Immunol* 21:1963-1966.

161. Herman, A., Kappler JW, P. Marrack, and A. M. Pullen. 1991. Superantigens: mechanism of T-cell stimulation and role in immune responses. *Annual Review of Immunology* 9:745-772.

162. Guo, X., W. Li, W. Deng, P. Ni, S. Yu, Y. Li, and H. Zhang. 2002. [Restricted usage of repertoire of T-cell receptor V beta in Chinese asthmatics]. *Zhonghua Jie.He.He.Hu Xi.Za Zhi.* 25:78-80.

163. Murphy TF. and S. Sethi. 1992. Bacterial infection in chronic obstructive pulmonary disease. *Am Rev Respir Dis* 146:1067-1083.

164. Bateman, J. R., D. Pavia, N. F. Sheahan, J. E. Agnew, and S. W. Clarke. 1983. Impaired tracheobronchial clearance in patients with mild stable asthma. *Thorax* 38:463-467.

165. O'Riordan TG, J. Zwang, and G. C. Smaldone. 1992. Mucociliary clearance in adult asthma. *Am Rev Respir Dis* 146:598-603.

166. Del Donno, M., D. Bittesnich, A. Chetta, D. Olivieri, and M. T. Lopez-Vidriero. 2000. The effect of inflammation on mucociliary clearance in asthma: an overview. *Chest* 118:1142-1149.

167. Allegra, L Fabbri LM Picotti G et al. Bronchial epithelium and asthma. European Respiratory Journal *2(suppl)*, 460-468. 1989.
Ref Type: Generic

168. Mezey RJ, Cohn MA, Fernandez RJ, Januszkiewicz AJ, and A. Wanner. 1978. Mucociliary transport in allergic patients with antigen-induced bronchospasm. *Am Rev Respir Dis* 118:677-684.

169. Wilson, R., D. Roberts, and P. Cole. 1985. Effect of bacterial products on human ciliary function in vitro. *Thorax* 40:125-131.

170. Adler KB, Hendley DD, and G. S. Davis. 1986. Bacteria associated with obstructive pulmonary disease elaborate extracellular products that stimulate mucin secretion by explants of guinea pig airways. *The American Journal of Pathology* 125:501-514.

171. Wilkinson TM, Hurst JR, Perera WR, M. Wilks, Donaldson GC, and J. A. Wedzicha. 2006. Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest* 129:317-324.

172. Sajjan, U., Q. Wang, Y. Zhao, D. C. Gruenert, and M. B. Hershenson. 2008. Rhinovirus Disrupts the Barrier Function of Polarized Airway Epithelial Cells. *Am.J.Respir.Crit.Care Med.* 178:1271-1281.

173. Tristram, D. A., W. Hicks, Jr., and R. Hard. 1998. Respiratory Syncytial Virus and Human Bronchial Epithelium. *Arch Otolaryngol Head Neck Surg* 124:777-783.

174. Davison, V. E. and B. A. Sanford. 1981. Adherence of staphylococcus aureus to influenza A virus-infected Madin-Darby canine kidney cell cultures. *Infect.Immun.* 32:118-126.

175. Avadhanula, V., C. A. Rodriguez, J. P. DeVincenzo, Y. Wang, R. J. Webby, G. C. Ulett, and E. E. Adderson. 2006. Respiratory Viruses Augment the Adhesion of Bacterial Pathogens to Respiratory Epithelium in a Viral Species- and Cell Type-Dependent Manner. *J.Virol.* 80:1629-1636.

176. Ishizuka S, M. Yamaya, T. Suzuki, H. Takahashi, Ida S, T. Sasaki, D. Inoue, K. Sekizawa, H. Nishimura, and H. Sasaki. 2003. Effects of rhinovirus infection on the adherence of Streptococcus pneumoniae to cultured human airway epithelial cells. *J Infect.Dis.* 188:1928-1939.

177. Eder, W., W. Klimecki, L. Yu, E. von Mutius, J. Riedler, C. Braun-Fahrlander, D. Nowak, and F. D. Martinez. 2004. Toll-like receptor 2 as a major gene for asthma in children of European farmers. *Journal of Allergy and Clinical Immunology* 113:482-488.

178. Gantier, M. P., S. Tong, M. A. Behlke, D. Xu, S. Phipps, P. S. Foster, and B. R. G. Williams. 2008. TLR7 Is Involved in Sequence-Specific Sensing of Single-Stranded RNAs in Human Macrophages. *J Immunol* 180:2117-2124.

179. Moller-Larsen, S., M. Nyegaard, A. Haagerup, J. Vestbo, T. A. Kruse, and A. D. Borglum. 2008. Association analysis identifies TLR7 and TLR8 as novel risk genes in asthma and related disorders. *Thorax* 63:1064-1069.

180. Wu, Q., R. J. Martin, S. LaFasto, B. J. Efaw, J. G. Rino, R. J. Harbeck, and H. W. Chu. 2008. Toll-like Receptor 2 Down-regulation in Established Mouse Allergic

Lungs Contributes to Decreased Mycoplasma Clearance. *Am.J.Respir.Crit.Care Med.* 177:720-729.

181. Fitzpatrick, A. M., F. Holguin, W. G. Teague, and L. A. Brown. 2008. Alveolar macrophage phagocytosis is impaired in children with poorly controlled asthma. *Journal of Allergy and Clinical Immunology* 121:1372-1378.

182. Meyer, K. C. 2005. Aging. *Proc Am Thorac Soc* 2:433-439.

183. HO, J. C., K. N. CHAN, W. H. HU, W. K. LAM, L. I. N. G. ZHENG, G. L. TIPOE, J. U. N. E. SUN, R. A. Y. M. LEUNG, and K. W. TSANG. 2001. The Effect of Aging on Nasal Mucociliary Clearance, Beat Frequency, and Ultrastructure of Respiratory Cilia. *Am.J.Respir.Crit.Care Med.* 163:983-988.

184. Puchelle E, Zahm JM, and A. Bertrand. 1979. Influence of age on bronchial mucociliary transport. *Scand.J.Resp.Disease*.

185. Cole, P. J. 1986. Inflammation: a two-edged sword--the model of bronchiectasis. *Eur J Respir Dis Suppl* 147:6-15.

186. Robinson, D. S., D. A. Campbell, S. R. Durham, J. Pfeffer, P. J. Barnes, and K. F. Chung. 2003. Systematic assessment of difficult-to-treat asthma. *Eur Respir J* 22:478-483.

187. Harmanci, E., M. Kebapci, M. Metintas, and R. Ozkan. 2002. High-Resolution Computed Tomography Findings Are Correlated with Disease Severity in Asthma. *Respiration* 69:420-426.

188. Michel, O., R. Ginanni, B. Le Bon, J. Content, J. Duchateau, and R. Sergysels. 1992. Inflammatory response to acute inhalation of endotoxin in asthmatic patients. *Am Rev Respir Dis* 146:352-357.

189. Michel, O., J. Duchateau, and R. Sergysels. 1989. Effect of inhaled endotoxin on bronchial reactivity in asthmatic and normal subjects. *J Appl Physiol* 66:1059-1064.

190. Schaumann, F., M. Muller, A. Braun, B. Luettig, D. B. Peden, J. M. Hohlfeld, and N. Krug. 2008. Endotoxin Augments Myeloid Dendritic Cell Influx into the Airways in Patients with Allergic Asthma. *Am.J.Respir.Crit.Care Med.* 177:1307-1313.

191. Vernooy, J. H. J., M. A. Dentener, R. J. van Suylen, W. A. Buurman, and E. F. M. Wouters. 2002. Long-Term Intratracheal Lipopolysaccharide Exposure in Mice Results in Chronic Lung Inflammation and Persistent Pathology. *Am.J.Respir.Cell Mol.Biol.* 26:152-159.

192. Wickens, K., N. Pearce, J. Crane, and R. Beasley. 1999. Antibiotic use in early childhood and the development of asthma. *Clin Exp.Allergy* 29:766-771.

193. Alm, B., L. Erdes, P. Mollborg, R. Pettersson, S. G. Norvenius, N. Aberg, and G. Wennergren. 2008. Neonatal Antibiotic Treatment Is a Risk Factor for Early Wheezing. *Pediatrics* 121:697-702.

194. Cullinan, P., J. Harris, P. Mills, S. Moffat, C. White, J. Figg, A. Moon, and A. J. Newman Taylor. 2004. Early prescriptions of antibiotics and the risk of allergic disease in adults: a cohort study. *Thorax* 59:11-15.

195. Preston, J. A., A. T. Essilfie, J. C. Horvat, M. A. Wade, K. W. Beagley, P. G. Gibson, P. S. Foster, and P. M. Hansbro. 2007. Inhibition of allergic airways disease by immunomodulatory therapy with whole killed Streptococcus pneumoniae. *Vaccine* 25:8154-8162.

196. Thorburn AN FAU - Hansbro, P., P. Hansbro PM FAU - Gibson, and P. G. Gibson. 2009. Pneumococcal vaccines for allergic airways diseases. *Expert Opin.Biol.Ther.*

197. Foxwell AR, Cripps AW Dear KB. *Haemophilus influenzae* oral whole cell vaccination for preventing acute exacerbations of chronic bronchitis. In: *The Cochrane Library*, issue 2. Chichester, UK. 2003.
Ref Type: Generic

198. Braido, F., F. Tarantini, V. Ghiglione, G. Melioli, and G. W. Canonica. 2007. Bacterial lysate in the prevention of acute exacerbation of COPD and in respiratory recurrent infections. *Int.J Chron.Obstruct.Pulmon.Dis.* 2:335-345.

199. Steurer-Stey, C., L. M. Bachmann, J. Steurer, and M. R. Tramer. 2004. Oral Purified Bacterial Extracts in Chronic Bronchitis and COPD*. *Chest* 126:1645-1655.

200. Health Protection Agency. Standard operating procedure 8. Investigation of sputum. Issue 5.1. Standards Unit, Evaluations and Standards Laboratory, Specialist and Reference Microbiology Division, Health Protection Agency, London, United Kingdom. 2003.
Ref Type: Generic

201. Dunbar, J., S. White, and L. Forney. 1997. Genetic Diversity through the Looking Glass: Effect of Enrichment Bias. *Appl.Environ.Microbiol.* 63:1326-1331.

202. Hill, A. T., E. J. Campbell, S. L. Hill, D. L. Bayley, and R. A. Stockley. 2000. Association between airway bacterial load and markers of airway inflammation in patients with stable chronic bronchitis. *The American Journal of Medicine* 109:288-295.