Susceptibility to pneumococcal colonization

Identifying critical pneumococcal and mucosal markers

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

by

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May 2021

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Declaration

This thesis is the result of my own work and effort. In some instances, work was completed in conjunction with others. The attribution of work and responsibility is detailed in Table 0-1.

Chapter 3: Colonization rates, density and duration by classical microbiology are in part also presented in the degrees of Victoria Connor (MD) and Jenna Gritzfeld (PhD) [8, 9].

Table 0-1: Attribution of work related to this thesis

Activity	Responsibility
Recruitment, sample taking, pneumococcal challenge	Clinical team (Liverpool School of Tropical Medicine)
Inoculum preparation and assessment of colonization rates via microbiological methods	Sherin Pojar, Jenna Gritzfeld, Elena Mitsi, Esther German, Elissavet Nikolaou, Ashleigh Howards, Lisa Hitchins, Annie Blizard (Liverpool School of Tropical Medicine)
Assessment of colonization rates by DNA extraction and quantitative real-time polymerase chain reaction (qPCR)	Sherin Pojar, Victoria Connor, Elissavet Nikolaou, Lisa Hitchins, Ashleigh Howard, Esther German, James Court, Carla Solórzano-Gonzalez (Liverpool School of Tropical Medicine), Wouter A.A. de Steenhuijsen Piters, Debbie Bogaert (University Medical Center Utrecht)
Detection of asymptomatic virus carriage, microbiome detection and cluster identification	Wouter A.A. de Steenhuijsen Piters, Debbie Bogaert (University Medical Center Utrecht)
Cytokine detection	Simon Jochems (Liverpool School of Tropical Medicine)
Assessment of pneumococcal adherence by microscopy	Sherin Pojar (Liverpool School of Tropical Medicine), Caroline Weight (University College London)
Semi-quantitative analysis of pneumococcal chain length	Sherin Pojar, Ashleigh Howard (Liverpool School of Tropical Medicine)
Secondary cell culture	Sherin Pojar, Elissavet Nikolaou (Liverpool School of Tropical Medicine)
Primary nasal cell culture	Sherin Pojar, Jesús Reiné, Simon Jochems, Carla Solórzano-Gonzalez (Liverpool School of Tropical Medicine)
Pneumococcal adherence assay	Sherin Pojar, Elissavet Nikolaou (Liverpool School of Tropical Medicine)
Neutrophil surface killing assay	Sherin Pojar, Carla Solórzano-Gonzalez, Elissavet Nikolaou, Esther German, Elena Mitsi (Liverpool School of Tropical Medicine)
Detection of SPN6B in Saliva by DNA extraction and qPCR	Elissavet Nikolaou (Liverpool School of Tropical Medicine)
Assessment of agglutination by flow cytometry	Elena Mitsi, Jesus Reiné (Liverpool School of Tropical Medicine)
Mucosal cell typing by flow cytometry	Jesús Reiné, Simon Jochems (Liverpool School of Tropical Medicine)
Assessment of myeloperoxidase	Sherin Pojar, Jesús Reiné (Liverpool School of Tropical Medicine)
Assessment of anti-RrgB sIgA levels	Sherin Pojar (Liverpool School of Tropical Medicine), Ulrike Binsker (NYU School of Medicine)
Provision of chemically defined media components	Lucille Van Beek (Radboud UMC)
Assessment of MUC5AC, Pneumococcal growth assays in presence of mucins	Sherin Pojar, Jack Vojak (Liverpool School of Tropical Medicine)
Statistical comparison of experimental colonization density and duration	Tao Chen (Liverpool School of Tropical Medicine)

Acknowledgements

This thesis would not have been possible without the assistance, volunteering, patience and guidance of many people to who I owe my gratitude.

First and foremost, I would like to thank all the volunteers participating in the experimental human pneumococcal carriage (EHPC) studies, gifting the sample here analysed and allowing me and the EHPC team to conduct this research.

Furthermore, I would like to say thanks to the many funders of the EHPC studies amongst them: The Medical Research Council, The Bill and Melinda Gates Foundation, the National Institute for Health Research Comprehensive Local Research Network and Pfizer Inc. I would also like to pay gratitude to my collaborators Caroline Weight (UCL, UK), Prof. Jeffrey Weiser (NYU, USA), Ulrike Binsker (NYU, USA), Prof. Marien de Jonge (Radboud UMC, Netherlands), Lucille Van Beek (Radboud UMC, Netherlands) as well as Wouter A.A de Steenhuijsen Piters (Utrecht UMC, Netherlands) and Prof. Debbie Bogaert (Utrecht UMC, Netherlands), as well as to the statistical team at LSTM.

During the last 6 years of work in the EHPC laboratory team I have been blessed with many great colleagues who constantly offered guidance and assistance for which I am very grateful. I would also like to take the opportunity to say a special thank you to the clinical EHPC team who work tirelessly day in day out.

Always supportive and with advice at hand were my PhD supervisors: Prof. Daniela Ferreira, Dr. Simon Jochems and Dr. Jamie Rylance. Thank you for your guidance throughout the years.

Finally, I would like to thank my husband and my son for their unwaning patience and support in the most stressful days. I know it was hard work, but you kept me afloat.

Abbreviations

Abbreviations are explained at their first use. The following list is depicting some commonly used abbreviations.

CFU	colony forming unit
EHPC	experimental human pneumococcal carriage
ELISA	enzyme – linked immunosorbent assay
OD	optical density
PCV	pneumococcal conjugate vaccine
STGG	skim milk, tryptone, glucose, glycerol media
THY	Todd Hewitt broth with 0.5% yeast extract
qPCR	quantitative real-time polymerase chain reaction
Ig	immunoglobulin
sIg	secretory immunoglobulin
SPN	*Streptococcus pneumoniae*
DNA	deoxyribonucleic acid
MUC	human mucin type
WHO	World Health Organisation
CDM	chemically defined media
LAIV	live attenuated influenza vaccine
EC+	experimental colonization positive
EC-	experimental colonization negative
PBS	phosphate buffered saline
HBSS	Hank’s balanced salt solution
CO_2	carbon dioxide
NPE	reconstituted human nasopharyngeal epithelium
NEC	primary undifferentiated nasopharyngeal epithelial cells
Ct	cycle threshold
AU	arbitrary units
T-cells	T- lymphocytes
B-cells	B- lymphocytes
$HBSS^{+/+}$	HBSS containing calcium and magnesium

Papers and presentations arising from this work

Papers

Weight CM, Venturini C, **Pojar S**, Jochems SP, Reine J, Nikolaou E, Solorzano C, Noursadeghi M, Brown JS, Ferreira DM, Heyderman RS. Microinvasion by Streptococcus pneumoniae induces epithelial innate immunity during colonisation at the human mucosal surface. *Nat Commun* 2019; 10: 3060.

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Abstract

Susceptibility to pneumococcal colonization by Sherin Pojar

Introduction: Pneumococcal colonization is the pre-requisite for pneumococcal diseases such as pneumonia, otitis media and meningitis. Thus, its control is key. Pneumococcal conjugate vaccines protect against invasive pneumococcal diseases - in part through its effects on pneumococcal colonization prevalence - but present with limited serotype coverage. With the global pneumococcal burden not relenting, the need for more effective intervention strategies, as well as the possibility to test their effect on colonization is high. The experimental human pneumococcal carriage (EHPC) model – while currently limited in serotype coverage - allows the study of pneumococcal colonization control in its natural host.

Project aims: Here I aimed to 1.) identify microbial characteristics that associate with experimental human pneumococcal colonization efficiency thereby establishing a tool to pre-select pneumococcal strain candidates for EHPC studies prior to human challenge; 2.) identify mucosal markers of host susceptibility to human pneumococcal colonization and 3.) determine the effect of nasal mucins on human pneumococcal colonization.

Main findings: In a retrospective association study superior experimental colonization rates of pneumococcal strain SPN6B/BHN418 associated with high pneumococcal chain length in liquid culture. Implementation of the quantitative assessment of chain length as a tool for selecting pneumococcal strain candidates successfully highlighted a serotype 3 strain, which, when used for human challenge, achieved experimental colonization rates equal to those observed with SPN6B/BHN418.

An integral part of the host mucosal barrier, mucins were associated with increased pneumococcal growth and survival *in vitro*. *In vivo* baseline MUC5AC levels in nasal wash positively correlated with pneumococcal load following challenge in a subset of volunteers. Nasal MUC5AC also correlated with the formation of pneumococcal aggregates in nasal fluid, highlighting the ambiguous role of mucins in human pneumococcal colonization.

Implications: Pre-selection of successful pneumococcal strains for new challenge models is needed to save costs and reduce research waste, while allowing more rapid expansion of the EHPC model. Identification of factors and mechanisms influencing pneumococcal colonization of the human host further provides the basis for the improvement of current vaccines and intervention strategies against the pneumococcus.

Chapter 1 General introduction

1.1 Streptococcus pneumoniae

Streptococcus pneumoniae (also referred to as the pneumococcus) is a gram-positive, alpha haemolytic, non-motile, encapsulated coccus with more than 90 structurally and serologically distinct serotypes. While described often as a diplococcus due to its appearance in infected sputum, the pneumococcus – like other members of the genus – grows in chains of variable length [10]. When visualised on an agar plate pneumococcus appear as "draughtsman-like" with a depressed centre due to beginning autolysis. Autolysis occurs spontaneously during the stationary growth phase of the pneumococcus as a result of the activity of autolysin proteins (Lyt) which degrade and modify the pneumococcal cell wall [11]. Notably, autolysis deficient mutants are less virulent than their wildtype counterparts [12-14]. Pneumococcal autolysis is choline sensitive with high concentrations of choline disabling the ability of pneumococcus to undergo autolysis [15]. In contrast, deliberate activation of autolysin protein LytA can be achieved through exposure of the pneumococcus to bile salts (e.g. sodium deoxycholate) [16, 17] which serves as a classical test for pneumococcal identification. Pneumococcal identification is further based on the assessment of morphology and presence of alpha–haemolysis under aerobic conditions. Pneumococcal serotypes are distinguished by Quellung reaction and/or latex agglutination (Figure 1-1).

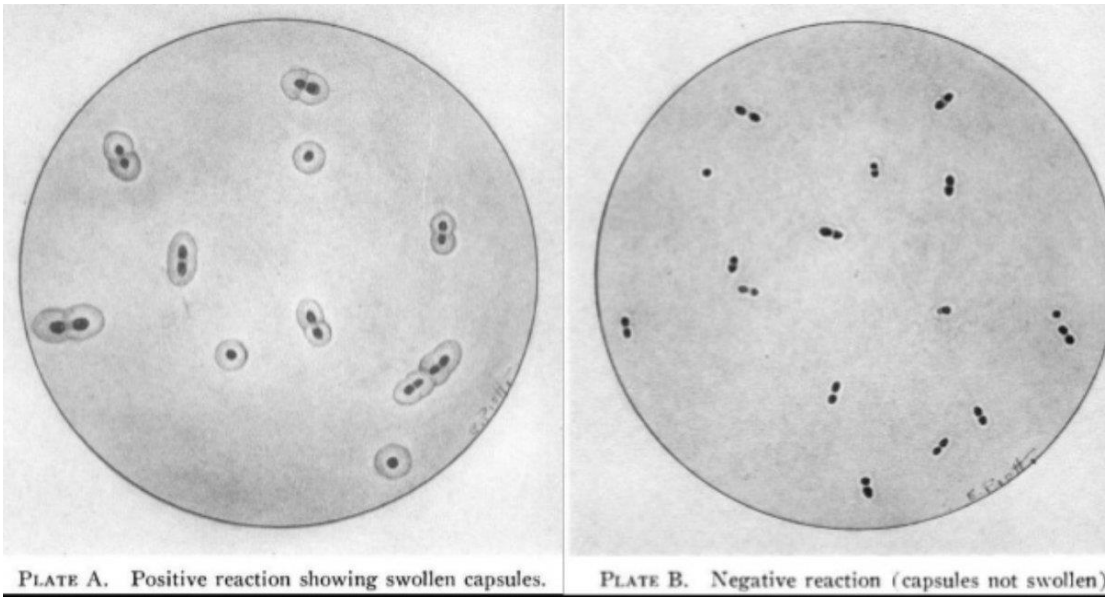


Figure 1-1: Schematic presentation of positive and negative Quellung reaction.

In addition to the swelling of the capsule agglutinating aggregates of pneumococci can be found in a positive reaction. Images republished with permission of the American Society for Clinical Investigation, from Beckler E and Macleod P. (1934): The Neufeld Method of Pneumococcus Type Determination as carried out in a Public Health Laboratory: A Study of 760 Typings, J Clin Invest.;13(6) © 1934 The American Society for Clinical Investigation [18]; permission conveyed through Copyright Clearance Center, Inc.

The basic pneumococcal structure is displayed in Figure 1-2. Three major pneumococcal surface layers can be distinguished: plasma membrane, cell wall and capsule [19]. The cell wall consists of peptidoglycans and teichoic acids. In addition, pneumococcal proteins can be found attached to the cell surface. Pneumococcal surface proteins play critical roles in pneumococcal-host interactions such as adhesion, colonization, invasion, immune evasion, and dissemination [20-22]. They can be subdivided into three major clusters: lipoproteins, choline binding protein family and proteins containing a cell wall sorting signal which starts with a LPxTG motif. In addition, filamentous surface appendages (pili) have been identified for some pneumococcal types [23].

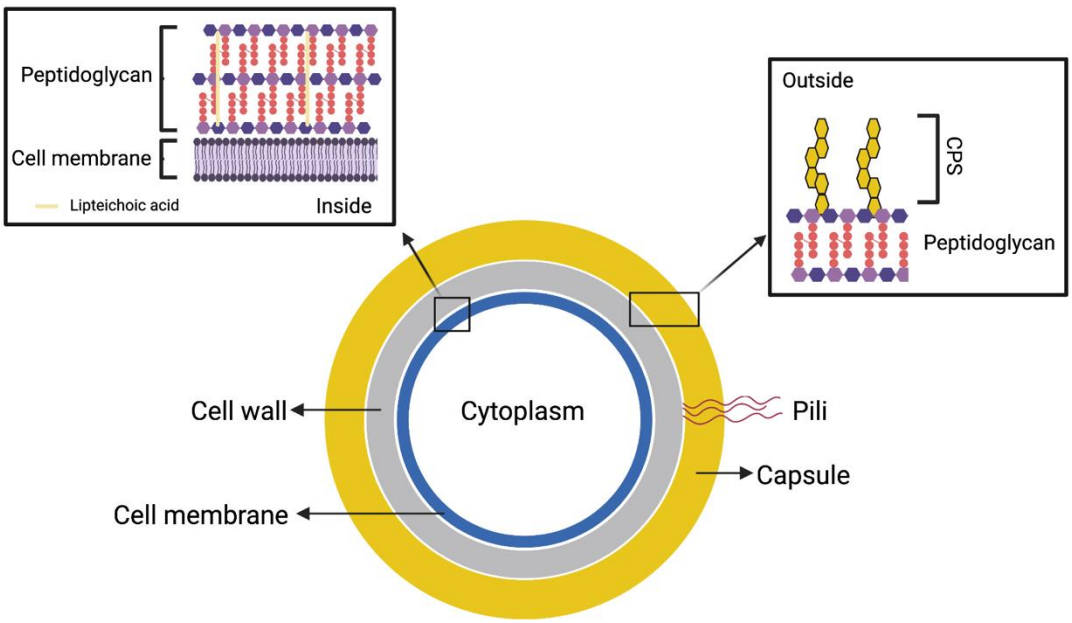


Figure 1-2: Schematic presentation of pneumococcal surface layers.

The pneumococcus distinguishes three major surface layers: cell membrane (blue), cell wall (grey) and capsule (yellow). The cell wall consists of peptidoglycans and teichoic acids. The pneumococcal capsule consists of polysaccharide chains (CPS) of varying complexity and is either covalently linked to peptidoglycan through a Wzy-dependent mechanism or embedded into the cell wall by transfer of a sugar to a lipid acceptor (synthase dependent mechanism). Figure created with BioRender.com, adapted (redrawn and remixed) under CC BY 4.0 licence from Brooks LRK, Mias GI. (2018): Streptococcus pneumoniae's Virulence and Host Immunity: Aging, Diagnostics, and Prevention, Front Immunol.; 9, Figure 3+4 © 2018 Brooks and Mias [5]

The capsule is the thickest layer, completely engulfing the inner structures. Whereas the chemical structure of the cell wall polysaccharide (CWPS) is common to all pneumococci, the capsular polysaccharide (CPS) is serotype-specific dictated by the composition of its polysaccharide chains [24]. The importance of identifying capsular composition first became apparent in the early 20th century. At that time pneumococcal disease was widely treated with serotype-specific antisera and identification of pneumococcal serotypes was at the forefront of pneumococcal research. To date pneumococci are distinguished based on their capsular composition using the Danish classification system which distinguishes more than 90 serotypes [25]. The classification is based on the chemical structure of the polysaccharides which varies in complexity from simple linear to complex branched structures (Table 1-1). Common sugar building blocks include galactose, glucose and rhamnose.

Table 1-1: Polysaccharide structure of selected pneumococcal serotypes

	Structure	**References**
SPN3	→3)-βD-Glc*p*A(1→4)βD-Glc*p*(1→	[25]
SPN6B	→ 2)-αD-Gal*p*(1→3)αD-Glc*p*(1→3)αL-Rha*p*(1→4)D-ribitol-5-PO_4→	[25]
SPN15B	→ 6)-ßD-Glc*p*NAc(1→3)ßD-Gal*p*(1→4)ßD-Glc*p*(1→ ↑1→4 αD-Gal*p*(1→2)ßD-Gal*p*-3-PO_4-R R = 80% H 20% choline	[26, 27]
SPN23F	glycerol-2-PO_4 ↓3 →4)βD-Glc*p*(1→4)βD-Gal*p*(1→4)L-Rha*p*(1→ ↑1→2 αL-Rha*p*	[28]

Gal,galactose; GalA, galacturonic acid; GalN, Galactosamine; GalNAc, N-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; GlcN, glucosamine; GlcNAc, N-acetylglucosamine; PO_4, phosphate; Rha, rhamnose; *p*, pyranose

The majority of capsular polysaccharides known to date have a net negative charge, with a few exceptions presenting a neutral charge (serotypes 7A, 7F, 14, 33F, 33A, 37) or being zwitterionic (serotype 1) [25, 29, 30].

In addition to differences in capsule composition (serotypes), the pneumococcus can undergo spontaneous reversible variations of its phenotype (opaque and transparent). These so-called phase variants are distinct in pathogenesis-associated characteristics such as the amount of polysaccharide capsule and cell wall teichoic acids [31-33]. They are generated by DNA inversions of three methyltransferase genes within the bacterial type I restriction-modification system [34] resulting in a reversible on/off switch in methylation of genomic DNA, distinct gene expression profiles and colony morphology variations. Phase variations appear to play an important role during colonization, invasion, and transmission [35-39].

1.2 Pneumococcal epidemiology

The pneumococcus has a complex relationship with its obligatory human host. Successful pneumococcal colonization of the human nasopharynx is vital for further pneumococcal transmission [40]. Colonization is also the first stage of pneumococcal infection as it preludes dissemination to the lower respiratory tract [41]. The pneumococcus can cause upper and lower respiratory tract infections (e.g. sinusitis, otitis media, pneumonia) and invasive diseases (e.g. sepsis, meningitis). It is the most common bacterial infection in both children and adults world-wide [42]. Despite this, colonization frequently occurs in healthy humans without causing disease. Colonization is immunogenic, inducing serum as well as mucosal IgG to pneumococcal polysaccharide and proteins [43]. Previous colonization episodes can protect against re-colonization with the same serotype and post-colonization human sera confers increased protection in a mouse model of pneumococcal pneumonia [43].

1.2.1 Pneumococcal transmission

The consensus is that human-to-human transmission of the pneumococcus is primarily occurring via inhalation of infected respiratory droplets, however, other routes of transmission are biologically plausible. Using an experimental colonization model of humans it was shown that hand to nose transmission of pneumococcus can occur [44] implicating indirect contact as a possible transmission route. The importance of close-contact type transmission routes is repeatedly demonstrated by the spread of the bacteria in close-contact and high-density settings such as prolonged mass gatherings (e.g. Hajj pilgrimage) as well as pneumococcal disease outbreaks in day care centres, military camps, prisons and nursing homes [45, 46].

1.2.2 Pneumococcal colonization rates

Colonization rates in the human population vary significantly depending on various factors such as age, geographical area and co-morbidities. Young children between 2-4 years old show the highest colonization rates with a range from approximately 27% in developed to 85% in developing countries [47]. The disparity may in part be explained by socio-economic differences. With increasing age colonization rates drop to about 10% in adults. In the elderly, colonization rates have been reported to be even lower [48]. Co-morbidities such as asthma increase the likelihood to be colonized by the pneumococcus [49, 50]. Young children are often colonized with multiple pneumococcal serotypes at once. Such co-colonization is associated with an overall higher pneumococcal density than those with single serotype colonization [51].

Interpretation of reported colonization rates is complicated by the large array of detection and sampling methods. The gold standard of pneumococcal detection is microbiological culture [52]. Nasopharyngeal swabs are commonly used as primary source for pneumococcal detection. Nevertheless, oropharyngeal swabs and saliva samples have recently been reported to show higher sensitivity when using molecular detection techniques such as multiplex qPCR [51, 53, 54]. The current recommendation of the World Health Organisation (WHO) for molecular identification of the pneumococcus is the detection of the pneumococcal autolysin A (*lytA*) gene. However, while *lytA* was previously considered exclusive to the pneumococcus, other closely related species belonging to the *streptococcus* genus have now been found to code for *lytA*-like enzymes which can confound molecular detection and account for false positive results [55-57].

1.2.3 Pneumococcal disease rates

The pneumococcus was first described as a cause for lobar pneumonia in 1882-3 [58]. To date it is known that the bacteria is further able to cause other diseases such as otitis media, bacteriaemia and meningitis with clinical data demonstrating an inverse relationship between severity and frequency of pneumococcal disease (Figure 1-3) [59].

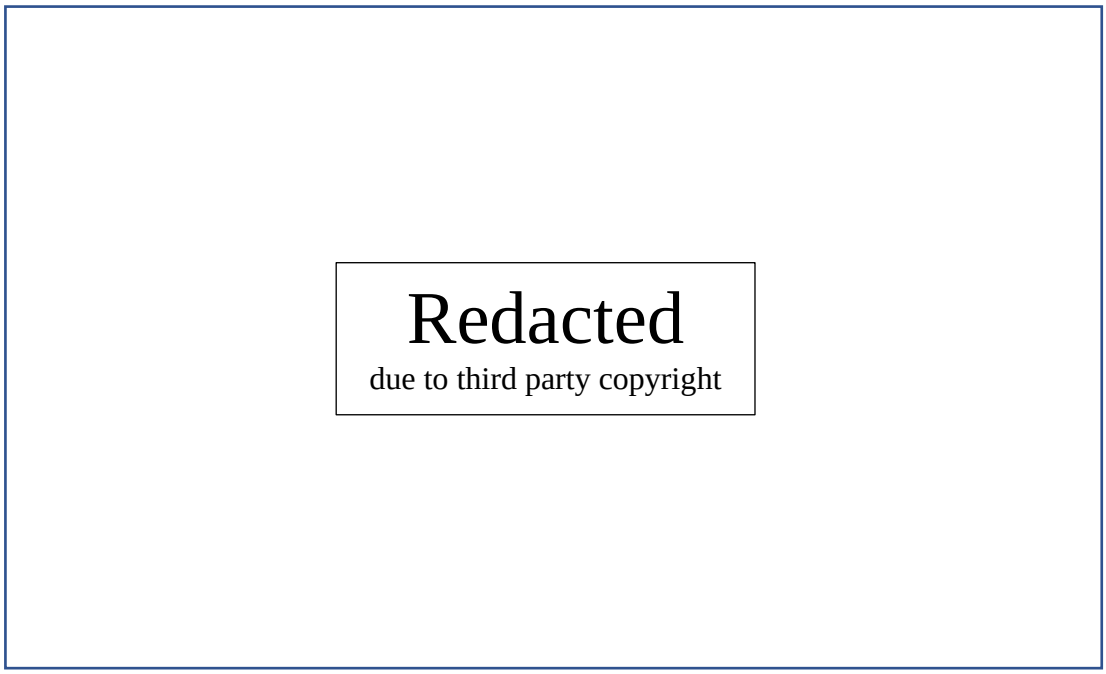


Figure 1-3: Schematic presentation of inverse relationship between burden and severity of pneumococcal disease.

Figure adapted with permission of Elsevier Ltd. from Levy C. et al. Pediatric Ambulatory and Hospital Networks for Surveillance and Clinical Epidemiology of Community-Acquired Infections, J Pediatr.; 194, Figure Spectrum of *Streptococcus pneumoniae* disease © 2018 Published by Elsevier Inc. [60]

Diagnosis of pneumococcal diseases is usually confirmed by isolation of the disease-causing organism from the body site. However, while etiologic determination is the gold standard for the diagnosis of pneumococcal disease, obtaining samples such as lung or inner ear fluid is too invasive to be routinely used. This in combination with a

lack of uniform diagnosis criteria [61] likely results in an underestimation of the true pneumococcal disease burden.

The impact of pneumococcal disease differs globally, with the highest burden located in developing countries [62]. Additionally, and similar to the pattern of colonization, pneumococcal disease rates correlate with age (Figure 1-4) with the highest burden seen in the young and in the elderly [63-66]. Gender, viral/bacterial co-infection, underlying co-morbidities as well as certain medications such as inhaled corticosteroids, antipsychotic drugs and proton pump inhibitors have been reported as important risk factors for pneumococcal disease [67-70]. In 2016, more than 1.1 million lower respiratory tract infection (LRTI) deaths alone have been attributed to the pneumococcus globally - more deaths than all other aetiologies combined [71].

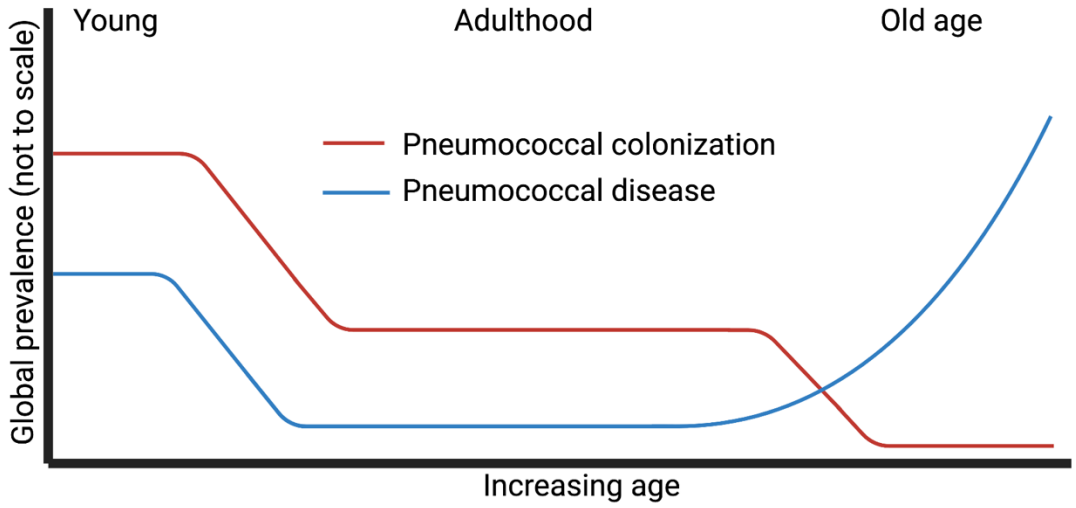


Figure 1-4: Schematic presentation of prevalence of global pneumococcal colonization and disease rates by age group.

Figure adapted with permission of the American Society of Microbiology (ASM) from Adler H et.al. (2017): Pneumococcal Capsular Polysaccharide Immunity in the Elderly. Clin Vaccine Immunol.; 24(6), Figure 2 © 2017 American Society for Microbiology [1]; permission conveyed through Copyright Clearance Center, Inc. Figure created with BioRender.com.

1.3 Treatment and prevention

Penicillin has been the treatment of choice for many pneumococcal infections for over half a century. However, with growing resistance of the pneumococcus to conventional antibiotics, advancements in treatment and prevention become increasingly important.

Notably, not all pneumococcal serotypes carry the same potential to colonize or cause disease [72, 73]. In fact, only a limited number of capsular serotypes cause the majority (70-80%) of invasive pneumococcal disease [72, 74]. However, global differences in serotype distribution as well as the adaptable nature of the pneumococcus complicate the development of effective disease interventions.

1.3.1 From pneumococcal whole cell to conjugate vaccines

Early pneumococcal vaccine products were based on whole-cell heat treated pneumococcus [75]. The first clinical trials of those products were reported in the 1910s and showed promising results [76, 77]. With the establishment of capsular antigens and their critical role as virulence factor, development of serotype specific vaccines followed suit and culminated in the licencing of a 23-valent pneumococcal polysaccharide vaccine (PPV23) in 1983. The formulation includes serotypes covering the majority of invasive pneumococcal disease (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F). Nevertheless, its efficacy is suboptimal, due to several limitations including: failure to mount an immune response in children under 2 years of age, lack of long-lasting immunity, lack of booster response and lack of herd immunity [78].

Studies also suggest that though PPV23 can lessen the severity of community acquired pneumonia (CAP) it cannot prevent disease to occur. The observation is likely attributed to the lack of stimulation of mucosal immunity by PPV23 [79]. A significant breakthrough to overcome the limitations of PPV was made with the licensing of the pneumococcal conjugate vaccine (PCV) in the year 2000. The vaccine contains purified capsular polysaccharides individually coupled with a nontoxic carrier protein. PCV induces T-lymphocyte (T-cell) dependent antibody response, immunological memory and mucosal immunity in young children [79, 80].

The first PCV induced immunity to 7 strains of pneumococcus that were responsible for most of the disease burden in young children in the United States of America at that time. Today a 13-valent version of the vaccine is part of the routine vaccination schedule for all children in the United Kingdom [81]. In addition, PCV is offered in many other countries world-wide (Figure 1-5).

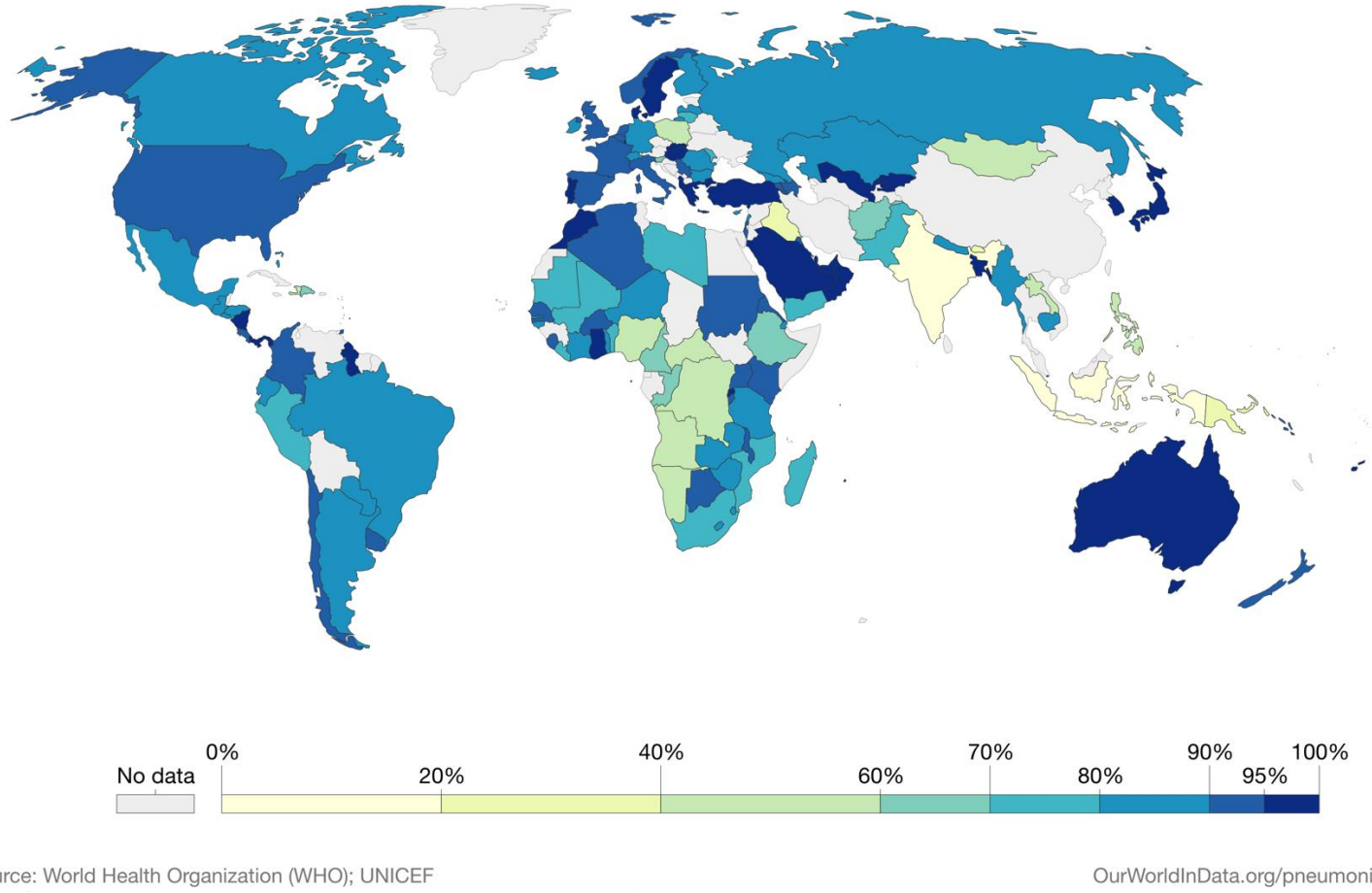


Figure 1-5: Percentage of infants vaccinated with PCV in 2019.

Figure adapted from Our World in data [82]

A meta-analysis of vaccine efficacy trials reported that the effectiveness of PCV-13 in children under five ranges from 67.2-96% against vaccine-type invasive pneumococcal disease (IPD) and 26.9% - 86% against otitis media [83]. However, according to the WHO a major drawback in evaluating the true efficacy of current pneumococcal vaccines is the use of clinical disease definitions as study endpoints. These definitions often lack specificity thus potentially leading to a substantial underestimation of vaccine efficacy [84].

Nasopharyngeal colonization is a prerequisite for pneumococcal disease, thus effects of pneumococcal vaccines against colonization are of high interest. Clinical trial data as well as observational studies clearly demonstrate a reduction of nasopharyngeal carriage of vaccine serotypes post-PCV [85]. Most importantly, reduction of colonization prevalence is not limited to those vaccinated. The herd-immunity effect of PCV may be caused by diminishing pneumococcal colonization density thereby reducing transmission of vaccine serotypes [86].

Despite the success of vaccination programs current pneumococcal conjugate vaccines have shown several limitations [87-90] which include:

- no cross-reactive protection amongst serotypes due to diversity of the pneumococcal capsule
- limited efficacy against certain serotypes such as serotype 3
- replacement of colonizing and disease-causing serotypes by those not included in current vaccines
- geographic variation in protection due to variable serotype distribution world-wide
- a high price limiting their availability to low- and middle-income countries.

1.3.2 Next generation pneumococcal vaccines

Given the challenges and limitations of current pneumococcal vaccines, the development of the next generation focuses on three major strategies:

1. development of new production methods to reduce costs of current pneumococcal conjugate vaccines
2. expansion of valency of already established vaccines
3. the development of new affordable vaccines able to offer broad serotype protection.

Adaptation of current conjugate vaccines through increasing their valency is well under way with PCV15 and PCV20 both expected to be licensed by 2021 [91]. However, increasing valency also increases overall vaccine costs. While novel production methods for PCVs, aimed at reducing costs, are being proposed (such as biosynthesis of targeted polysaccharides and carrier protein in *E-coli* combined with protein glycan coupling technology [92, 93]), alternative serotype independent approaches still present a promising alternative.

Whole cell-based vaccines were the earliest form of pneumococcal vaccines and have in present days been re-evaluated as a valid, serotype independent, vaccine alternative. The advantage of whole-cell vaccines over other forms of vaccinations is the induction of whole cell immunity against the pathogen similar to natural exposure, promising broad antigen protection as well as mucosal and systemic immunity [94]. They therefore likely offer protection against the range of pneumococcal disease as well as induction of herd immunity. In addition, whole-cell vaccines are cost-effective, easy to construct and administer and have a historic record of being effective against disease. There are several forms of whole cell pneumococcal vaccines possible.

Inactivated whole cell vaccines are organisms that have been grown in culture medium and inactivated by heat and/or chemicals, thereby effectively killing them and leaving them unable to reproduce. A whole cell pneumococcal vaccine containing chemically killed unencapsulated *S. pneumoniae* RM200 cells has been tested in Phase 1/2 clinical trials in 2014 (Table 1-2). Vaccination led to significant induction of protein specific IgG levels and serum transfer from vaccinated individuals protected mice from pneumococcal infection [95, 96]. Despite the apparent success of the first trials no further development of the vaccine has been reported so far.

An alternative to administering killed whole cell pneumococcus is to weaken the organism while leaving it able to replicate (live attenuated vaccines). Weakening could for example be achieved through direct genetic modification or multiple passaging under laboratory conditions resulting in a modified version of the disease-causing organism. Live attenuated vaccines have been used for decades against viral as well as bacterial diseases, however there is currently no live attenuated pneumococcal vaccine candidate in clinical development.

Another promising alternative to current pneumococcal vaccines is the approach of presenting highly immunogenic conserved bacterial proteins to the host (protein-based vaccines). Protein based vaccines promise to be less cost intensive and may offer broader protection than current polysaccharide based pneumococcal vaccines due to their lower sequence diversity amongst the pneumococcal species.

Today there are several delivery ways of protein-based vaccines possible:

- presenting purified pneumococcal proteins to the host (acellular vaccines)
- presenting pneumococcal protein on a weakened vector pathogen (vector vaccines)

- using pneumococcal protein as carrier in PCV vaccines (modified PCVs)
- delivering mRNA coding for pneumococcal protein into host cells (mRNA vaccines).

Despite extensive pre-clinical research of pneumococcal protein candidates within the last decades [43, 97-104] only a handful have thus far proceeded to clinical trials in humans and even fewer have entered Phase 2 clinical trials (Table 1-2). Amongst the success stories are multivalent protein vaccines Sanofi-Pasteur's tri-valent PPrV; Genocea's GEN-004 vaccine, Intercell's IC-47 and Immunobiology's PnuBio Vax. All four candidates are reported to have passed phase I safety and immunogenicity trials [105-108]. Genocea's Gen-004 vaccine is the only candidate that was additionally evaluated in a Phase II trial assessing its effect on pneumococcal colonization against a placebo using the EHPC model. Vaccination resulted in a non-significant reduction of colonization rates and median colonization densities following challenge, but did not affect colonization duration [105, 109].

Table 1-2: Overview of pneumococcal non-polysaccharide vaccine candidates

Vaccine type	Candidate	Antigens	Company	Related Trial numbers	Status	References
Multivalent protein	ASP3772	22PS + 2 proteins	Astellas Pharma	NCT03803202, NCT04525599	Phase 1/2 active	Not published
	PPrV	PhtD+PcpA+PlyD1	Sanofi	NCT01446926, NCT01764126	Phase 1 completed	[106]
	Gen-004	SP2108+SP0148+SP1912	Genocea Bioscience	NCT01995617, NCT02116998	Phase 2 completed	[105, 109]
	IC47	PcsB, StkP, PsaA	Intercell AG/Novartis	NCT00873431 NCT01444352	Phase 1 completed	[107]
	PnuBio Vax	pooled protein fractions from genetically modified TIGR4.B7.1	ImmunoBiology	NCT02572635	Phase 1 completed	[108]
Protein + PCV	10PP	PhtD, dPly, PCV10	GSK	NCT01262872, NCT01204658	Phase 2 completed	[110, 111]
Whole cell	WCV	Inactivated unencapsulated and genetically modified RM200	PATH	NCT01537185, NCT02097472, NCT02543892	Phase 1/2 completed	[95, 96]
Vector	Salmonella + PspA	recombinant attenuated Salmonella typhi expressing pneumococcal PspA	Arizona State U.	NCT01033409	Phase 1 completed	[112]

Progress in the licencing of serotype independent vaccine alternatives is slow. This may largely be attributed to the fact that any new vaccine against pneumococcal disease has to be trialled against the current gold standard vaccine (PCV-13) as administering a placebo would be unethical. Such non-inferiority trials present a major hurdle for many new vaccines to overcome as they often require large sample sizes and therefore are prohibitively expensive. In addition, with the roll out of pneumococcal vaccination programs worldwide, testing of new vaccines may be carried out in already vaccinated population groups (e.g children and elderly), further complicating the assessment of the true efficacy of new pneumococcal vaccines.

An additional hurdle to be addressed before licensure of serotype independent vaccines can occur is the need to define correlates of protection for these new vaccines [113]. The disruption of pneumococcal colonization by PCV and its subsequent impact on herd immunity highlights the importance of evaluating the effect of new pneumococcal vaccines on pneumococcal colonization. Such trials would be less cost-intensive compared to traditional trials as occurrence of colonization is higher than disease resulting in smaller sample sizes needed for clinical trials [114]. However, it is still unclear how pneumococcal vaccines should protect against colonization. Proxies of protection might include a reduction in carriage density or colonization rates. Immunological markers of mucosal immunity could also be used, but there is no current agreement as to what those markers would be.

1.4 Host defence against the pneumococcus

1.4.1 The mucosal barrier

Pneumococcal colonization requires the bacterium to penetrate the mucus barrier which overlies the host epithelium. Simultaneously, to persist in the nasopharynx the pneumococcus must avoid innate and adaptive host defences, replicate within the nutrient-poor environment of the nasopharynx and adapt to the challenge of intra and interspecies coexistence within this richly colonized niche. Despite the importance of pneumococcal colonization for disease development, the identification of host - as well as microbial factors capable of influencing this life stage of the pneumococcus is incomplete. Similarly, while the molecular processes of certain host-pneumococcal interactions are well defined, their relative importance during human colonization remain unclear.

In the nasopharynx the host mucosal barrier is comprising of the mucus lining on top of the cells (mucus), the epithelial layer (epithelium) as well as underlying innate as well as adaptive immune components (Figure 1-6). Taken together these compounds are believed to form a physical barrier with the ability to aid clearance.

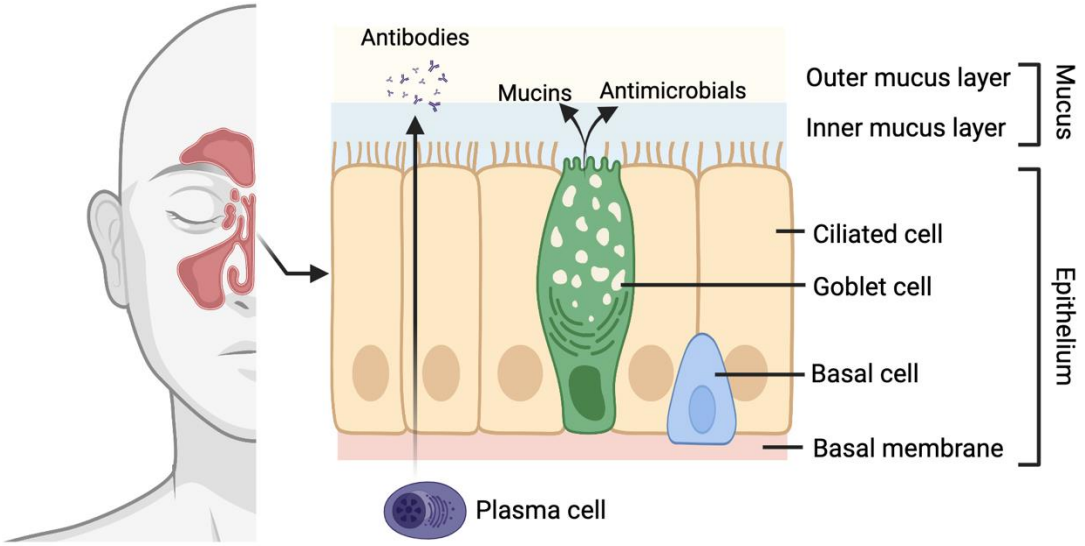


Figure 1-6: Schematic presentation of the mucosal barrier.

The mucosal barrier is shown comprising of two distinct mucus layers on top of the cells (outer and inner mucus layer), the epithelial layer consisting of goblet, ciliary and basal cells as well as the basal membrane. Mucus secreted by goblet cells contains defensive molecules such as antimicrobials and mucins. Antibodies produced by plasma B-cells are transported into the lumen via epithelial transcytosis. Figure created with BioRender.com and adapted with permission of John Wiley & Sons Ltd from Zhang N et al. (2016): Barrier function of the nasal mucosa in health and type-2 biased airway diseases, Allergy; 71(3), Figure 1 © 2015 John Wiley & Sons A/S [3]

1.4.1.1 Mucus

Mucus is present at the interface between many of the epithelial surfaces of the human body (including the nasopharyngeal epithelium) and their extracorporeal environment. Mucus - often referred to as mucosal fluid - is a complex network comprising of host and environmental particles. It acts as a matrix for the commensal flora while defending the body against the entry of harmful agents [115-117]. Analysis of human biopsies and animal tissue revealed that the mucus layer consists out of two very distinct sub-layers [118-120]. The outer layer, often referred to as the loose or non-adherent layer is largely soluble. The inner layer, also termed firm or adherent layer, is non-soluble and adhered to the epithelial cells below. The transition from outer to inner layer is described to be abrupt despite having similar composition. While the main function of the outer layer is believed to be the trapping and shedding of potentially dangerous agents, the inner layer contains hardly any bacteria [121].

1.4.1.1.1 Mucins

As structural part of the outer and inner mucus layer, mucins aid in the clearance of pathogens by forming a gel like layer which hinders microbiotic movement [4, 117]. To date, more than 17 human mucin genes have been assigned to the mucus family, with expression of mucins often varying between organs [122]. In respiratory tissue human mucin type (MUC) 5AC is the most abundant mucin of the mucus layer [123-125]. Mucins are produced by specialised mucus cells or by the epithelium itself [126-128]. Secretory mucins often form filamentous multimers or even more complex networks (Figure 1-7 C/D) [129, 130]. In contrast membrane bound mucins contain a C-terminal trans-membrane anchor and are believed to act as surface receptors and sensors [131, 132].

Mucins are proteins richly decorated with o-linked glycans. The carbohydrate structures on mucins comprise of three main regions: the linkage of N-acetyl-D-galactosamine (Gal-NAc) to peptides, a back bone region of repeated glycans and a peripheral terminal region, often mirroring those found in ABH or Lewis histo-blood group antigens decorated by either fucosylation, sulphation, methylation, sialyation or acetylation (Figure 1-7 A/B) [133-136]. Carbohydrate structures on mucins, determined by the expression of glycosyltransferases, are remarkably heterogenous and diverse with structural variations present in all three regions. Diversity and plasticity of glycosylation patterns is governed by genetics, tissue specific enzyme expression as well as host-environmental factors influencing transferase expression. Additionally, carbohydrate structures on individual mucin molecules are determined by stochastic events during posttranslational modification [137] and vary between individuals, tissues and developmental stage. Evidence has emerged that their plasticity also extend to mucosal infection and inflammation [138].

The function of mucins in bacterial colonization and disease is somewhat elusive. While in the past mucins were mainly seen as a structural and mechanical element of the mucus layer, in recent times a more ambiguous role with regard to bacterial colonization becomes apparent.

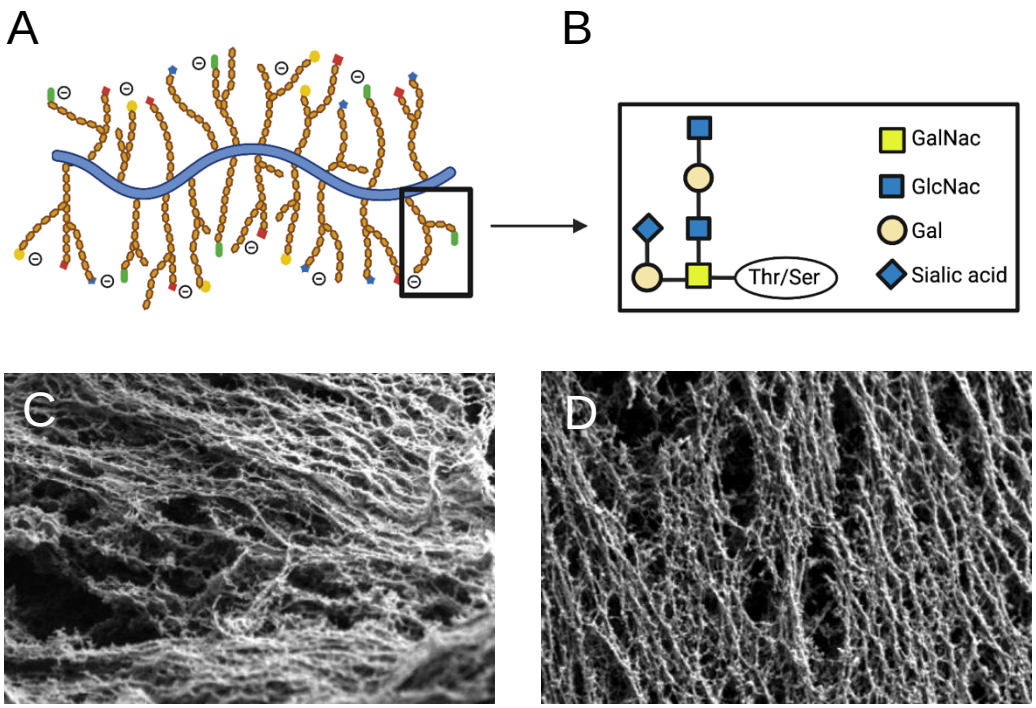


Figure 1-7: Illustration of mucin and mucin networks.

(A) Exemplary schematic presentation of human mucins. Mucin molecules share common building blocks of central protein structure decorated with o-linked glycans. An optional sialic acid end provides a negative charge. Figure adapted under CC BY 3.0 licence from Petrou G and Crouzier T. (2018): Mucins as multifunctional building blocks of biomaterials, Biomater. Sci.; 6(9), Figure 1A © The Royal Society of Chemistry 2018 [4] (B) Exemplary chemical glycosylation structure of human mucins. GalNa = N-acetylgalactosamine, GlcNa = N-acetylglucosamine, Gal = Galactose. Figure adapted (redrawn) under CC BY 4.0 licence from Pinzón M.S, Seeberger PH and Varón S.D. (2019): Mucins and pathogenic mucin-like molecules are immunomodulators during infection and targets for diagnostics and vaccines, Front Chem.; 7, Figure 1A © 2019 Pinzón Martín, Seeberger and Varón Silva [6] (A/B) Figures created with BioRender.com (C/D) Atomic force microscopic images of mucin network. Images republished under CC BY 3.0 licence from Critchfield A.S. et al. (2013): Cervical Mucus Properties Stratify Risk for Preterm Birth, PLoS One; 8(8), Figure 4 © 2013 Critchfield et al. [7]

1.4.1.1.2 Antimicrobial molecules

Antimicrobial peptides (AMPs) are widely distributed throughout the human body – including the respiratory tract - mediating important innate defence functions. To date over a hundred different AMPs have been identified [139]. AMPs are generated from larger precursor proteins and undergo posttranslational modifications such as glycosylation [140]. AMPs present in mucus include lactoferrin, defensins, lysozyme, peptide LL-37, secretory leukocyte protease inhibitor (SLPI) and other antimicrobial components [141]. Lysozyme is the most abundant antimicrobial peptide in the nasal fluid. It is widely accepted that most AMPs attack the bacterial membrane. However AMPs may also possess or enact their antimicrobial function in other ways such as through the modulation of the human immune response, neutralization of proinflammatory microbial products or the sequestration of molecules important for bacterial growth [139]. An overview of relevant respiratory AMPs, their mode of action and effects against pneumococcus is available in Table 1-3.

Table 1-3: Antimicrobial peptides in the human respiratory tract

Antimicrobial peptide	Mode of action	Produced by	Evidence of function against SPN	Counter mechanism by SPN	References
Lysozyme	Cell wall degradation	Monocytes, neutrophils, macrophages, glandular cells	Acceleration of autolysis *in vitro*	Modification of peptidoglycan structure	[142-145]
Lactoferrin	Iron sequestration, immune modulation, membrane destabilization, DNAse activity	Epithelial cells, neutrophils	Bactericidal against planktonic SPN, disaggregates SPN biofilms, inhibits acquisition of resistance genes	Binding of Lactoferrin by PspA	[144, 146-152]
Secretory leukocyte proteinase inhibitor (SLPI)	Membrane destabilization, immune modulation (inflammation, macrophages)	Neutrophils, macrophages, epithelial cells/ glandular cells	Biomarker of SPN pneumonia but not colonization		[153-156]
Human defensins	Neutralising secreted toxins, membrane permeabilization	Neutrophils and paneth cells (alpha-defensins), epithelial cells (beta-defensins)	Bactericidal effects of alpha defensins against SPN *in vitro*	Surface charge modification	[157]
LL-37	Disturbance of peptide-glycan synthesis membrane permeabilization, immune modulation	Innate immune cells, epithelial cells	Inhibition of pneumococcal growth *in vitro*	Cell surface modifications, efflux pump (MefE)	[158-161]

1.4.1.1.3 Antimicrobial activity of human mucus

Mucus is the mesh in which antimicrobial acting agents are working. In 1922 Alexander Fleming published an article describing his observation of the bactericidal activity of human mucus towards a bacteria referred to as *Micrococcus lysodeikticus* or nasopharyngeal coccus [162]. Mucus is comprised of 97% water and 3% solids amongst them mucins, cellular debris, salts, lipids and non-mucin proteins some of which possess anti-microbial, anti-protease and anti-oxidant activities [163, 164]. Though over the years, many components of the mucosal fluid have been identified, systematic analysis of the function of individual components towards its antimicrobial activity remains scarce. In 1999 Cole et al. demonstrated that while mucosal fluid is characterised by bacteriostatic or bactericidal activity against certain microbes, this anti-microbial property could - after heat-inactivation of the mucosal fluid - not be restored by addition of physiological levels of antimicrobials such as lactoferrin [141]. Observations made using the EHPC model corroborated their finding with regard to pneumococcal colonization [155]. In an EHPC study using human nasopharyngeal secretions of healthy adults who were experimentally challenged with pneumococcus, levels of lactoferrin, beta defensin 2 and antimicrobial factor SLPI were not different between colonized and un-colonized individuals 2 days after pneumococcal challenge. Taken together these results indicate that the intrinsic antimicrobial function of human mucus may be driven by other factors.

Mucin proteins may account for the missing link between human mucus and its antimicrobial activity. It has been shown that MUC6 shows direct bactericidal activity towards *H. pylori* growth [165], MUC5AC inhibits *Trichuris muris* survival [166] and Roy et al. discovered in 2014 the antibacterial activity of MUC5AC against

Staphylococcus aureus [167]. In addition, recent data indicate that mucins may also play an important role in inflammatory defence mechanisms [167, 168]. MUC5B knock-out mice showed accumulation of alveolar macrophages with reduced phagocytic function as well as reduced cytokine production [167]. Further, mice lacking MUC5B expression were unable to resolve lung inflammation after microbial exposure. A similar picture was seen in MUC1 knock-out mice where infection with pneumococcus resulted in increased lung inflammation and recruitment of monocytes and macrophages with reduced capacity to phagocytose pneumococcus [168]. Another function of mucins may be the enrichment of bacteriophages in the nasopharyngeal niche. As bacterial predators, bacteriophages can increase microbial diversity but can also establish symbiotic relationships with their bacterial and metazoan host. In fact, it has been shown that direct binding of bacteriophages to variable glycan residues displayed on human mucins leads to reduced attachment of bacteria to human lung cells *in vitro* [169].

1.4.1.2 The nasal epithelium

The nasopharyngeal epithelium presents the last physical barrier that prevents microbial invasion of the underlying tissue. It is classified as a pseudo-stratified respiratory epithelium consisting of goblet, ciliated and basal cells [170]. Ciliated and goblet cells are terminally differentiated. In contrast basal cells retain stem cell characteristics and are thus able to differentiate. Goblet cells contain membrane bound mucus containing granules which they secret into the nasal environment. Ciliated cells display motile cilia on the cell surface, which 'beat' in a coordinated wave, constantly shifting the mucus layer on top, thereby significantly contributing to mucociliary clearance.

1.4.2 Host immune system

Though not a direct part of the mucosal barrier below the epithelia layer awaits a battery of innate as well as adaptive immune components (Figure 1-8). The innate arm comprises of basophils, mast cells, non-conventional T-cells, phagocytes such as macrophages/monocytes and neutrophils, as well as the complement system. The adaptive arm comprises of B-lymphocytes (B-cells), T- cells and antigen presenting cells. These cells communicate with one another through cytokine production or cell-cell contact.

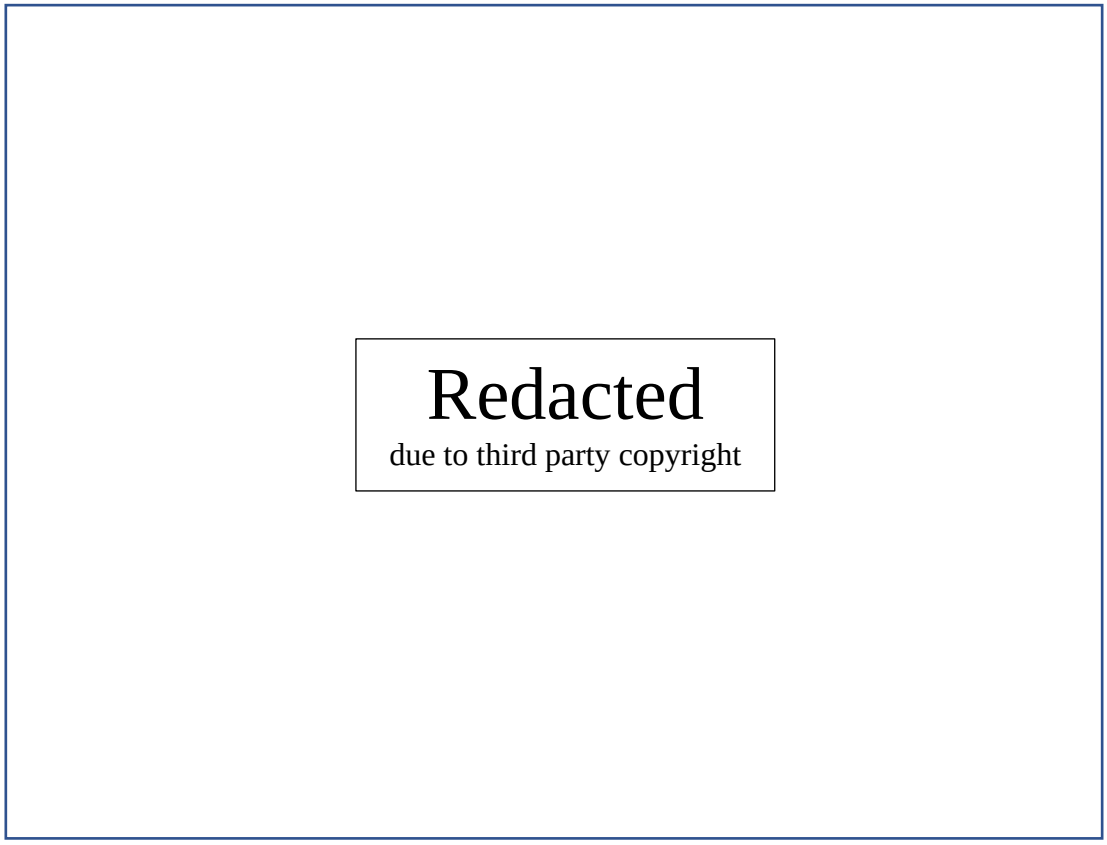


Figure 1-8: Schematic overview of the human immune components.

The human innate immune system takes part in the first line of defence against infection. Responses are mediated via cellular components such as granulocytes (neutrophils, eosinophils, and basophils), macrophages, dendritic cells, NK cells and monocytes as well as the soluble complement system. Adaptive immune responses are induced later during infection mediated via T-cells and B-cells and result in immunological memory and antigen specificity. NK T-cells and γδ T-cells present a cross link of innate and adaptive immunity. Figure sourced from Oliveira et al. (2015): Recent advances in characterization of nonviral vectors for delivery of nucleic acids: impact on their biological performance, Expert Opinion on Drug Delivery; 12(1), Figure 2 © 2015 Taylor & Francis Informa UK Ltd. [171]

1.4.2.1 Pattern recognition

Upon microbial infection, it is vital for the host to recognize the presence of a potentially harmful agent. This is achieved through pattern recognition receptors (PPRs) which recognise pathogen associated molecular patterns (PAMPs). PRRs can be secreted but can also be associated with cells either intracellularly or on their surface and include Toll-like receptors (TLRs), DNA sensors, scavenger receptors, cytosolic NOD-like receptors (NLRs) or C-type lectins. Much of what we know about the effect of PRR activation on pneumococcal colonization has been established in mouse models.

The nasal airway epithelium expresses all 10 of the known TLR proteins [172]. Expression varies depending on disease state and tissue site. Activation of TLRs in the upper respiratory tract can lead to mucin induction, production of antimicrobial substances and cytokines. The two most relevant TLRs in the upper airway epithelium are TLR2 and TLR4, both of which have been associated with pneumococcal host-defence. TLR-2 recognises lipoteichoic acid and lipoproteins [173, 174] and is involved in protection against colonization and disease [175]. TLR-4 is known for its recognition of lipopolysaccharide (LPS) [176] but has also been described to bind to the pneumococcal virulence factor pneumolysin [177]. TLR-4 knock-out models demonstrated increased pneumococcal colonization density [177] but showed no effect on clearance of colonization [178]. TLR-9 is the only known intracellular toll-like receptor described to play a role in susceptibility to and severity of pneumococcal pneumonia [179]. It recognises bacterial DNA - specifically unmethylated CpG motifs [180].

Scavenger receptors are predominantly expressed in myeloid cells such as macrophages and dendritic cells where they act as direct phagocytic receptors mediating non-opsonic phagocytosis of the pneumococcus, via binding of a large variety of ligands [181]. Two important scavenger receptors in pneumococcal defence are MARCO and the mannose receptor. In mice, MARCO is required for pneumococcal clearance from the nasopharynx [182] and absence of the receptor results in higher susceptibility to pneumococcal pneumonia [183]. Meanwhile recognition of carbohydrate containing ligands by the mannose receptor may serve to regulate inflammatory responses in the lung by preventing alveolar macrophages from releasing proinflammatory cytokines [184].

NOD-like receptors (NLRs) are intercellular PRRs recognising bacterial cell wall components. The functions of NLRs are still poorly understood. Activation of NLRs can lead to the induction of proinflammatory cytokines or the regulation of adaptive immune responses [185, 186]. These functions are achieved either via the formation of multiprotein complexes or directly through activation of a variety of intracellular signalling pathways. NOD2 activation via phagocytosis and lysozyme dependent digestion of pneumococcal derived peptidoglycan in macrophages has been reported to facilitate clearance of pneumococcal colonization [187].

1.4.2.1.1 Epithelial sensing at the respiratory mucosa

The integral role of the epithelium in the communications network that involves epithelial cells, pathogens, and host immune and inflammatory cells has become a research focus over the last decades [188-190]. To prevent excessive stimulation in response to frequent confrontation with mostly harmless commensals, mucosal epithelial cells tightly control localisation of PPRs [172]. Under homeostatic

conditions for example most TLRs in the gut epithelium are localised at the basolateral membrane [191]. A recent study demonstrated that micro-invasion of the nasal epithelium by different pneumococcal strains induces differential epithelial transcriptomic responses [192]. Further, data collected in mouse models suggests that those transcriptomic responses may be the result of transient epigenomic changes within epithelial cells induced by epithelial sensing upon interaction with the bacterium [193]. Nevertheless, to date, the importance of nasal epithelial sensing against the pneumococcus remains unclear.

1.4.2.2 Inflammation

Inflammation is a central part of innate immunity, defined as the local response to tissue injury that is marked by production of proinflammatory cytokines, infiltration of inflammatory cells including neutrophils and T-cells and increased blood flow coupled with capillary dilatation and vascular leakage [194].

Mucosal inflammation of the nasal passages may be caused by an array of agents including microorganisms such as bacteria and viruses. While the most common cause of acute nasal inflammation is viral infection [195], it is possible that, like the mucosal lining of the gastrointestinal tract, commensal colonization of the nasal mucosa results in a state of constant low-grade inflammation [196].

The possible triangular relationship between inflammation, viral infection and pneumococcal colonization is highlighted by a clinical association study, demonstrating significant association between rhinitis symptoms, pneumococcal carriage, and viral detection in pre-school children [197]. While the causality behind this relationship is yet to be determined, mouse models suggest that acute inflammation leads to higher rates of transmission of pneumococcus [198, 199],

possibly through an effect of inflammatory cytokines (e.g. TNFα, IL1β and others) on mucus secretion and mucin expression [200-203]. In addition, viral infection enhances pneumococcal adherence perhaps via upregulation of epithelial receptors or as a result of epithelial remodelling following insult and inflammation associated with virus infection [204-206].

1.4.2.3 Phagocytosis

Phagocytes bind to pathogens through their surface receptors, thereby initiating internalisation and ultimately destruction of the pathogen. Phagocytic uptake can be initiated through contact with a wide variety of phagocytic receptors with different ligand specificity. Direct recognition of bacteria via PPRs or indirect recognition through complement or immunoglobulin bound to the bacterial surface are two well described mechanisms of phagocytic uptake. Important phagocytes within the respiratory tract include monocytes, macrophages, and neutrophils.

Neutrophil mobilization is considered a hallmark of acute inflammation. However, neutrophils are present in the nasal lumen of healthy human adults and children in the absence of pneumococcal colonization, with numbers of nasal neutrophils increasing with age [207, 208]. The constant presence of neutrophils in nasal tissue perhaps supports the idea of the nasal mucosa existing in a state of low-grade inflammation.
Neutrophils protect against pneumococcal colonization through multiple mechanisms including phagocytosis of opsonised pneumococcus, degranulation, production of reactive oxygen species or release of neutrophil extracellular traps [207, 209, 210]. A previous study by Weinberger et al. demonstrated that highly prevalent serotypes

displayed higher resistance to non-opsonic neutrophil mediated killing compared to serotypes less frequently found in colonization [211].

Monocytes can phagocytose, present antigens, secrete chemokines and upon recruitment differentiate into macrophages and dendritic cells. Experimental challenge studies have shown that pneumococcal challenge leads to monocyte recruitment and their function was shown to associate with clearance of the bacteria [210].

Macrophages are the most common immune-cell type residing in the lower airways. In the upper respiratory tract of healthy individuals however, the number of macrophages was limited despite abundant numbers of classical monocytes being identified in human nasal biopsies [207, 212]. Like monocytes, macrophages produce inflammatory cytokines, reactive oxygen species, phagocytose, and present antigens to activated T-cells. Notably, binding of the pneumococcal virulence factor pneumolysin to the phagocytic mannose receptor has been shown to lead to lysosomal escape of pneumococcus in macrophages as well as reduced neutrophil infiltration [213].

1.4.2.4 Complement system

A heat-labile component of blood plasma the complement system consists of many circulating and membrane-bound proteins. Upon activation those proteins form an enzyme cascade that eventually results in the death of the pathogen. Traditionally the complement system was viewed as an effector arm of antibody response. Nowadays it is known that the complement system can be activated even in the absence of antibodies [214]. In fact, there are three pathways through which the complement system can be activated: the classical pathway, the alternative pathway and the lectin

pathway. All three pathways however converge in the activation of one molecule (C3) which eventually generates the same set of effector molecules. There are also three ways in which the complement system can enact its effector function: through opsonisation and subsequent phagocytosis, as a chemoattractant and through direct damage to the bacterial membrane.

The pneumococcal capsule has been shown to impair complement deposition on the bacterial surface in both the classical and alternative complement pathway, inhibit conversion of C3b on the bacterial surface and impair phagocytosis [215, 216]. Data collected in mice suggest that complement has no impact on colonization density but is most critical in stopping the progression of pneumococcal colonization towards infection [217]. The role of complement in disease is further corroborated by a link between complement deposition and pneumococcal invasiveness [218] as well as the increased risk of invasive pneumococcal disease in patients with complement deficiencies [219].

1.4.2.5 Adaptive immune responses

1.4.2.5.1 T-cells

T-cells belonging to the adaptive immune system include cytotoxic T-cells, helper T-cells, regulatory T-cells (T-reg) and memory T-cells. T-cells have mainly two important functions: 1.) kill infected cells (CD8) and 2.) regulate the immune response (CD4).

Much of what we know about the function of T-cells in pneumococcal colonization has been established in mouse models. In mice, CD4+ T-helper 17 cells have been shown to play a critical role in controlling pneumococcal density and duration,

promoting bacterial clearance through IL17 mediated recruitment and activation of neutrophils and macrophages [220, 221]. Regulatory T-cells have been found elevated in response to colonization in mice, [222] and increased levels of T-reg have been associated with a stable colonization state, promoting colonization through suppression of Th17 mediated recruitment and activation of phagocytes [220, 221, 223, 224].

The role and mechanisms of Th17 and Treg cell mediated immunity in humans is less clear. Children are the main reservoir of the pneumococcus demonstrating the highest levels of pneumococcal colonization. In congruence with data described in mice, a recent study found low levels of Th17 and high levels of Treg cells in children [225]. The study further demonstrated an induction of high Th17:Treg ratios and increased levels of IL17A in colonization negative children after stimulation of tonsillar tissue with pneumococcus. However, these findings are contradicted by another cross-sectional study sampling children and younger adults, showing lower Treg numbers in children, increased nasal IL17A levels and no induction of Treg cells in those colonized [208].

The role of non-conventional T-cells has recently shifted into focus in pneumococcal research. Non-conventional T-cells include natural killer T-cells (NK T-cell), mucosal-associated invariant T-cells (MAIT) and γδ T-cells. They display characteristics of both the innate and adaptive immune systems (NK T-cells), expression of T-cell receptor variants (γδ T-cells and invariant T-cells) and commonly reside in mucosal environments. Their protective effects may involve the activation of phagocytes (γδ T-cells) and epithelia cells, the control of intracellular infection (mucosal-associated invariant T-cells) or the contribution to clearance of intra - and extracellular pneumococcus via production of bacteriostatic and lytic molecules (γδ T-

cells) [226]. While some studies in mice suggest a potential role of these cells during pneumococcal infection [227-229] their involvement during human colonization and/or disease remains unclear.

1.4.2.5.2 B-cell and immunoglobulins

1.4.2.5.2.1 B-cell activation and germinal centre reaction

B-cells are antibody producing cells of the adaptive immune system. To produce immunoglobulins B-cells require activation. Their responses can be distinguished into two types: T-cell independent and T-cell dependent response [230]. Important features of T-cell dependent activation are the formation of germinal centres, diversification, and affinity maturation of B-cell responses as well as the formation of B-cell memory. The germinal centre reaction begins with the recognition and uptake of proteinaceous antigens by the B-cell receptor. Activated B-cells then in turn present relevant antigens on a major histocompatibility complex II molecule and migrate to the follicular B-cell/T-cell border. Through interaction of presented antigen with the T-cell receptor on antigen-experienced follicular T-cells, the B-cell receives a secondary activation signal resulting in proliferation into plasma and memory B-cells as well as somatic hypermutation whereby greater affinity antibodies are produced and selected. In contrast, B-cells can also become activated by crosslinking of the B-cell receptor due to binding of antigen with repetitive epitope units (e.g. polysaccharides). This T-cell independent activation leads to generation of plasma cells that rapidly secret low-affinity antibodies, which act as the first line defence against pathogens.

1.4.2.5.2.2 Primary and secondary immune responses

B-cell responses can be further divided into primary and secondary immune responses [231]. During primary responses the body faces a yet unknown antigen. Such response is usually delayed (lag phase) as B-cell clones expand and differentiate into plasma and memory B-cells and high affinity antibodies are selected. During primary immune responses the first measurable immunoglobulin is usually of the IgM type with IgG becoming measurable several weeks after initial antigen contact. Secondary immune responses occur when an already known antigen is encountered by the body. The hallmark of secondary responses is the lack of the lag phase observed during primary responses. Instead, the activation of memory B-cells results in fast and increased production of high affinity immunoglobulin.

1.4.2.5.2.3 Immunoglobulins – Classes and function

While immunoglobulins can differ in structure, they are build using common building blocks: two heavy and two light chains with variable and constant regions in the amino acid sequence, a hinge region within the heavy chains, inter and intra-chain disulphide bonds and oligosaccharides [232]. Immunoglobulins are divided into five classes based on sequence differences in the conserved region of the heavy chain. The immunoglobulin class that is secreted by a B-cell is the result of class switch recombination (CSR) [233, 234]. CSR is a process which allows the change of class of immunoglobulin produced by an activated B-cell and primarily occurs prior to germinal centre formation [235]. During this process proportions of the constant region within the heavy chain sequence are irreversibly deleted from the B-cell genome, while the variable region remains untouched. This allows the body to produce different classes of antibodies against the same target, enabling antibody specificity to remain

the same while the effector activity varies. Due to the location of the class exons and switch regions within the B-cell genome naïve B-cells express either IgM and/or IgD (decided by alternative splicing) and can switch into either IgA, IgG or IgE. Class switching occurs in response to cytokines and is thus dependent on the cytokines present in the B-cell environment. In humans, TGFβ has been shown to induce IgA and IL-4 skews production towards IgE and certain subclasses of IgG [236, 237]. The main function of immunoglobulins is the binding of pathogens. Finer effector function of immunoglobulins are dependent on class, but encompass agglutination, neutralization, complement binding and phagocytosis [238].

1.4.2.5.2.4 Immunoglobulins at the respiratory mucosa

Immunoglobulin (Ig) A is the most abundant immunoglobulin in mucosal secretions. IgA exists in monomeric as well as dimeric form (secretory IgA). Secretory IgA is the main immunoglobulin in mucosal secretions of the respiratory tract. IgA further consists of two subclasses, IgA1 and IgA2, with IgA1 accounting for approximately 90% of all IgA found in the upper respiratory tract [239]. IgA may primarily act through neutralisation (limitation of pathogen-cell interactions), receptor blockage and/or immune exclusion - the process of crosslinking (referred to as agglutination), entrapping and clearing pathogens [240].

IgG antibodies are the most versatile immunoglobulins as they can act as opsonin, agglutinins or interact with the complement cascade. Against microorganisms they are primarily believed to act by coordinated activity of complement and phagocytosis [241]. IgG is present in the lining fluid and secretions of the respiratory mucosa. However, their abundance in mucosal secretions of the upper respiratory tract is relatively small in comparison to IgA or total protein [242, 243]. IgG is monomeric

and is further divided into 4 subclasses (IgG 1-4). IgG subclasses mainly differ in their specificity to different stimuli (e.g. proteins, polysaccharides, allergens) as well as complement activation [244].

After production, immunoglobulins require transport across the epithelial membrane (transcytosis) in order to execute their function during pneumococcal colonization [245]. Transcytosis entails ligand-receptor binding, endocytosis, vesicular transfer, and exocytosis. Transport of IgA is mediated through binding of the immunoglobulin to the polymeric immunoglobulin receptor (pIgR). During the transport process pIgR is cleaved and the extracellular domain (secretory component) bound to IgA is released into the mucosal secretions on the apical site of the epithelium. Transport of IgG on the other hand is mediated through the neonatal Fc receptor and is pH dependent. Notably, while IgA transport only occurs in the basolateral-to-apical direction, transport of IgG is possible also in the apical-to-basolateral direction.

There is large amount of supporting evidence regarding the protective role of immunoglobulins against the pneumococcus. PCV vaccines, inducing serotype – specific IgG in vaccinated individuals, have been shown to prevent invasive disease and reduce pneumococcal colonization with vaccine serotypes [246-248]. Experimental colonization studies in adults revealed that colonization without disease is immunogenic – leading to increased levels of immunoglobulins against pneumococcal proteins and polysaccharides – and can protect against reacquisition of the initial challenge strain in a homologous human challenge model [43]. Epidemiological evidence further strengthens the role of serotype specific antibody-dependent immunity in the protection against pneumococcal colonization [249]. In addition, epidemiological data also implicates serotype-independent immunity in the

natural mechanisms of protection against the pneumococcus [250, 251]. Indeed, bioinformatic modelling demonstrated that anti-capsular and non-serotype specific immunity together reproduce realistic patterns of pneumococcal diversity and frequency when using strength and rate of acquisition as mathematical variables [252]. Despite this, the association of both protein as well as capsular specific immunoglobulin levels and protection against pneumococcal colonization, remains controversial [43, 253-259]. One study in particular showed that high levels of polysaccharide specific memory B-cells but not pre-existing levels of polysaccharide -specific IgG in serum or nasal fluid associated with protection against experimental pneumococcal challenge in adults [253]. The study further showed that while the number of polysaccharide-specific plasma cells increased after pneumococcal challenge in volunteers who did not develop carriage, this did not correlate with an increase in polysaccharide-specific IgG. The authors postulated that the agglutinating function of immunoglobulins - while preventing colonization to occur could also mask their increase – potentially explaining the lack of association between plasma cell and immunoglobulin levels. While agglutination of pneumococcus by polysaccharide-specific IgG has been shown to associate with protection in a vaccine context, no such association has yet been demonstrated during natural colonization [258].

1.5 Microbial mechanisms of host subversion

Humans are the natural habitat of the pneumococcus. Despite extensive defence mechanisms of the host, the pneumococcus is constantly overcoming these to successfully establish colonization of this niche. Colonization is one of three stages of the pneumococcus life cycle (Figure 1-9). From here the pneumococcus can either be transmitted to a new host or invade its current host.

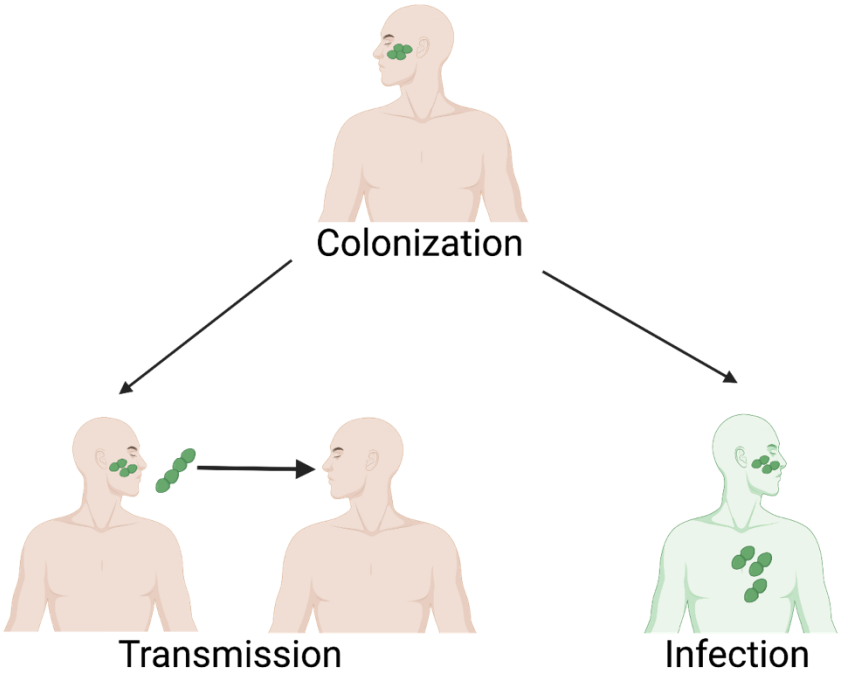


Figure 1-9: The three stages of the pneumococcal life cycle.
The pneumococcus is acquired by the human host, where it colonizes the nasopharynx. From there it either invades the human host (infection) or is shed and transmitted to a new host (transmission). Figure created with BioRender.com, adapted (redrawn with modifications) under CC BY 4.0 licence from Zivich et al. (2018): Streptococcus pneumoniae outbreaks and implications for transmission and control: a systematic review. Pneumonia; 10, Figure 4 and 5 © 2018, Zivich et al. [2]

1.5.1 Acquisition of the host

The main features that facilitate acquisition of the pneumococcus are adherence to the host epithelium, subversion of innate and adaptive host responses and avoidance of clearance by mucociliary flow. The first barrier that the pneumococcus encounters is the entrapment by nasal mucus. Here it needs to avoid a battery of defensive host strategies such as mucociliary clearance, immunoglobulins and antimicrobial peptides.

The **pneumococcal capsule** is the pneumococcus' first and probably most effective defence. With more than half of the known capsular polysaccharide types characterized, the most common feature amongst them is a net negative charge, leading to electrostatic repulsion of anionic molecules, including mucins [4, 260, 261]. In addition, the pneumococcal capsule masks the underlying surface-attached proteins from recognition by the hosts immune system, avoiding to a large degree effector functions of immunoglobulins, complement system and extracellular traps [5].

Activity of **neuraminidase A** (NanA) and **exoglycosidases** expose and cleave carbohydrates as well as sialic acid from host glycoproteins such as mucins [262]. This activity ultimately reduces the repellent nature and viscosity of mucus [5] and may allow the use of alternative carbon sources in an environment with limited supply of free sugars [262-267]. The use of mucins and other glycoproteins as nutrient source by the pneumococcus has been previously demonstrated *in vitro* [266, 268, 269]. Growth in mucin rich media led to increased *nanA* expression and mutants deficient in *nanA* were unable to grow in media containing mucin as only carbon source [269].

Expression of **IgA protease** is another mechanism of the pneumococcus to evade immune recognition. The IgA protease cleaves the immunoglobulin IgA1 thereby largely abrogating the protective effects of IgA against the pneumococcus [270-272].

The pneumococcus is capable of growing in **chains of varying length**. While short forms allow for complement evasion during invasive disease [273], large chains – perhaps due to their increased surface area - enhance the ability of the pneumococcus to adhere to the mucosal surface [274]. Knowledge of the molecular mechanisms driving chain formation remains incomplete. Absence of pneumococcal autolysins has been shown to promote longer chain formation and increase susceptibility to phagocytosis, while treatment of long chain forming cultures with autolysin B led to dispersion into shorter units [273, 275, 276].

The **pneumococcal pilus-1 protein** is expressed by 20-30% of pneumococcal strains [277]. It is expressed under the control of a transcriptional regulator and encodes three proteins (RrgA - C) and three sortases (SrtC1 - 3), the latter required for pilus assembly and linkage to the pneumococcal surface [278]. The presence of a transcriptional regulator allows the pneumococcus to vary the expression levels of pilus-1 proteins during its life cycle [279, 280]. The function of pilus-1 has not yet been completely elucidated, however its tip protein RrgA is a major adhesin to human cells [23, 281] and promotes invasion of the blood-brain barrier [282]. Pilus-1 expression furthermore stimulates host-inflammatory responses and provides a competitive edge in mixed intranasal challenge [23]. As such it is recognised as an important microbial factor influencing pneumococcal virulence.

The pneumococcal protein **pneumolysin** is one of the most important and well characterised virulence factors of the pneumococcus. It is a cholesterol-dependent cytolysin which mediates inflammatory responses and acts as pore-forming toxin towards the host cell membrane [283-285]. During the initial phase of colonization, an

important function of pneumolysin is the inhibition of ciliary beating thus hindering mucociliary clearance of the bacteria [286].

1.5.2. Adherence and invasion of host epithelial cells

Adherence and invasion of nasal mucosal cells is one of the most thorough studied host-pneumococcal interactions. It involves a diverse range of bacterial proteins as well as host factors. An extensive review of host-pneumococcal adherence interactions has been provided by Hammerschmidt et al. [287]. Adherence is initiated through binding of the pneumococcus to host cell-surface carbohydrates and is further mediated by a number of surface structures and proteins such as pneumococcal pilus protein, cell wall phosphorylcholine (ChoP), choline binding protein A (CbpA or PspC), pneumococcal lipoproteins (e.g. PsaA) and adhesins (e.g. PavA and enolase) [288-293]. Invasion is mediated via binding of ChoP to platelet-activating factor receptor (PAFR) as well as binding of CbpA to polymeric immunoglobulin receptor (PIGR) inducing endocytosis of the pneumococcus in epithelial as well as endothelial cells [292, 294, 295]. In addition, pneumolysin can damage the epithelium enabling invasion via paracellular transmigration through further interactions of enolase/GAPDH/CbpE – Hyl/Plasmin and subsequent breakdown of the extracellular matrix.

Interestingly, in response to an inflammatory environment the pneumococcus was found to increase many of its epithelial receptors, thereby promoting pneumococcal adherence [296].

While the pneumococcal **capsule** is promoting evasion of host clearance mechanisms during the initial phase of colonization, the capsule also sterically hinders attachment

of the bacteria to epithelial cells. However, during contact with and invasion of the epithelial cell the pneumococcus reduces capsular expression [37] thereby reducing steric hinderance of cell-wall attached bacterial proteins involved in the adherence process. In fact, it has been shown that transparent variants, showing reduced levels of capsular polysaccharide and increased exposure of cell-surface adhesins, are associated with increased adherence and colonization in humans and animal models [31, 32, 36, 37, 297, 298].

Cleavage of IgA-1 by the bacterial **IgA-1 protease** not only allows subversion of the host immune system, but also promotes bacterial attachment to epithelial cells as demonstrated in an *in vitro* study conducted by Weiser et al. [299]. The study further suggests that the adherence promoting effects may be due to 1.) neutralisation of electrostatic effects of the pneumococcal capsule through the binding of positively charged immunoglobulin fragments and 2.) unmasking of bacterial ChoP, thus allowing increased pneumococcal adherence.

Degradation of carbohydrate rich host proteins through the activity of **pneumococcal neuramidase NanA** and subsequent exoglycosidases is not only an advantage during the initial phase of colonization but may also promote pneumococcal adherence. Degradation of surface attached mucins may promote bacterial adhesion by exposing carbohydrate motives analogue to those believed to be used for initial attachment of the pneumococcus [117, 300-302]. In addition, release of terminal sialic acid acts as a signal to both the pneumococcus and the host, promoting colonization and invasion [303-305]. In the bacteria recognition and uptake of sialic acid results in activation of an intracellular signalling cascade, which leads to increased NanA expression as well as increased resistance to reactive oxygen species [306]. In the host cell, NanA itself

can activate the TGF-beta signalling cascade leading to the loss of endothelial tight junction, hence promoting invasion [305].

Notably, the synergism between pneumococcal illness and respiratory infection such as influenza [155, 206] may in part be attributed to the additional expression of neuramidases by respiratory viruses. The so enhanced effects of NanA during pneumococcal colonization may allow for increased pneumococcal colonization as well as the progression to disease [267, 307]. In addition, inflammation caused by viruses as well as the pneumococcus itself can upregulate the host receptor PAFR amplifying ChoP-PAFR interactions and mediating invasion [308].

1.5.3. Exit from the colonized host and survival in the environment

Exit from the colonized host (shedding) as well as subsequent survival in the environment are important processes during transmission of the pneumococcus. Nevertheless, until very recently all what was known of pneumococcal transmission was based on epidemiological data. This general lack of mechanistic knowledge was caused by a shortage of suitable animal and human models. While in depth mechanistic insight still remains largely elusive, it is now known that host inflammatory processes are one of the main features used by the pneumococcus to exit the host [309].

While the strong proinflammatory effect of **pneumolysin** can hasten bacterial clearance [310] it also enables and in fact is required for pneumococcal transmission [199]. Inoculation with *ply*-knockouts in mouse models reduced inflammation as well as transmission both of which could be complemented by intranasal administration of purified wild-type pneumolysin.

Given the important role of **inflammation** during host exit by the pneumococcus, the influence of **viral infection** on this stage of the pneumococcal life cycle may not surprise. Data collected in animal models show that levels of pneumococcal shedding correlate with upper respiratory tract inflammation in response to influenza infection [198]. In addition, respiratory viruses can increase mucin expression through inflammatory mechanisms, thereby increasing bacterial load and transmission as shown in mice [311, 312]. Studies of pneumococcal shedding in humans are rare, however, higher prevalence of rhinorrhoea, upper respiratory viral infection and pneumococcal colonization in early childhood [197] may indicate a similar synergism in humans.

Shedding is highly dependent on serotype as well as capsular expression. Pneumococcus with thinner capsule is shed poorly and shows increased attachment to mucins [313]. **Variation of capsular expression** therefore is an important factor in both transmission and colonization.

To fully complete the life stage of pneumococcal transmission the pneumococcus has to survive in the external environment before being reacquired by a new host. A number of recent studies have shown that the pneumococcus can survive on common surfaces such as soft toys as well as on human skin due to their ability to form biofilms thereby enhancing their chance of survival [314]. In human secretions such as saliva the pneumococcus can survive for more than 24 hours independent of capsular expression [315]. Under nutrient poor conditions, the pneumococcal capsule as well as the effects of pneumococcal proteins such as pneumolysin have been shown to increase survival, perhaps by providing or increasing the abundance of nutrients [199, 316].

1.6 Experimental models of pneumococcal colonization

Pneumococcal research is most needed in those most vulnerable to colonization and disease: children under five years of age. Over the last decades, the number of systematic studies in this population group has increased but results often are observational rather than experimental. Various experimental models allow for the interrogation of mechanisms and markers of pneumococcal colonization; these are described below.

1.6.1 Cell culture models

The human respiratory tract is a complex multi-cellular organ with anatomical regions of differing physiological and cellular complexity. In the past immortalised cell lines have been vital for the study of human diseases. Detroit 562 cells, a human pharyngeal carcinoma cell line, are one of the most abundantly used *in vitro* models in upper respiratory research. They offer several advantages, such as cost effectiveness, easy use, unlimited supply of material and reproducibility of results. In addition, their use bypasses ethical concerns associated with the use of animal and human tissue. However, cell lines such as Detroit 562 may not adequately represent *in vivo* physiology as they are derived from cancerous tissues.

Primary cells are an alternative to the use of immortalised cell lines. Two types of nasopharyngeal primary cell models are presently available. Submerged primary cells form single layer cultures of undifferentiated nasopharyngeal cells (NEC). In contrast, reconstituted nasopharyngeal epithelium (NPE) are fully differentiated multi-layer and multi-cellular primary air-liquid interface cell cultures and thus are considered to more

closely resemble *in vivo* physiology [317-321]. However, the use of primary cells can be hampered by an unreliable supply as well as changes of phenotype and/or gene expression with increasing time in culture [322, 323], thus limiting their use for respiratory research.

Detroit 562 cells and to a lesser extent primary undifferentiated nasopharyngeal cells have been used in numerous studies to evaluate pneumococcal adherence and invasion of clinical pneumococcal isolates or genetically modified strains [192, 265, 324-327]. While the former may serve as an *in vitro* marker of host-pneumococcal interactions *in vivo*, the latter is often used to identify the function of individual microbial factors and genes. Another possible readout of the Detroit 562 cell model is the assessment of host factors and mechanisms involved in bacterial-host interactions. An example of such an approach is the evaluation of epithelial transcriptomic responses after pneumococcal stimulation [192, 193].

In contrast, the model of reconstituted nasopharyngeal epithelium is rarely used in pneumococcal research of the upper respiratory tract. Due to their more complex structure and higher resemblance of *in vivo* conditions likely applications of NPEs include the pre-clinical evaluation of vaccine delivery systems and studies of pneumococcal-host interactions in specific disease populations.

1.6.2 Animal models

Animal models have been extensively used in the field of pneumonia, otitis media and pneumococcal colonization research [328, 329]. They are used for clarifying mechanisms of pathogenesis, testing novel drugs and vaccine candidates, and characterizing the role of bacterial and host factors.

Murine models are the most widely used animal models in pneumococcal colonization and disease research [328, 329]. Mice can be colonized by multiple pneumococcal serotypes [330] and show a several weeks long persistence of pneumococcal colonization [178, 331], representative of what is believed to occur in humans. Mice are further susceptible to invasive infection [332] subject to strain type, bacteria dose and inoculum volume. However, mice are not the natural host of the pneumococcus. In contrast to humans, laboratory-bred mice are naïve to the pneumococcus and may present with a limited or non-existent microbiome. These differences between organisms may result in distinct immunological responses upon pneumococcal challenge ultimately limiting the direct translation of results obtained from mouse models to humans. Other animal models used in pneumococcal colonization research are rats [333] and chinchillas [334-336].

1.6.3 Human models

Experimental bacterial infection and/or colonization models have been successfully applied to humans [337]. Over the last decade an experimental human pneumococcal carriage (EHPC) model has been developed in which humans are challenged intranasally with saline containing pneumococcus [338], allowing the systematic study of pneumococcal colonization biology. The model is currently used for vaccine development, examining pneumococcal biology and exploring mucosal immunity and host susceptibility in a controlled and safe manner [43, 155, 248, 253, 339-341]. However, limitations of the model include its restriction to studies involving human adults as well as a limited number of pneumococcal serotypes available for human challenge.

1.7 Aim and objectives

The purpose of this thesis is:

- to identify microbial characteristic that enable more accurate estimation of experimental pneumococcal colonization efficiency of EHPC candidate strains prior to human challenge (Chapter 3)
- identify mucosal markers of host susceptibility to human pneumococcal colonization (Chapter 4)
- to determine the effect of nasal mucins on human pneumococcal colonization (Chapter 5)

This is achieved through several objectives listed below.

Chapter 3: Expansion of the EHPC model

Research question: Can microbial factors predict experimental pneumococcal colonization efficiency?

Objective 1: Compile and compare colonization data of 4 dose-ranging studies creating a colonization profile for each pneumococcal strain used in the EHPC model.

Objective 2: Associate three microbial characteristics and colonization success profiles of strains studied in Objective 1.

Objective 3: Preselect a pneumococcal strain of serotype 3 based on outcomes of Objective 2 and establish experimental colonization profile in the EHPC model to assess feasibility of the chosen preselection method.

Chapter 4: Establishing markers of host susceptibility

Research question: **Can mucosal factors influence host susceptibility to human pneumococcal colonization?**

Objective 1: Associate mucosal levels of sIgA targeting the pneumococcal pilus-1 protein RrgB with experimental human pneumococcal colonization outcome.

Objective 2: Associate *ex vivo* levels of pneumococcal agglutination capacity of nasal fluid with experimental human pneumococcal colonization outcome. Identify mucosal factors linked with pneumococcal agglutination.

Objective 3: Associate *in vitro* levels of pneumococcal adherence with experimental human pneumococcal colonization outcome.

Chapter 5: Effect of nasal mucins on experimental human pneumococcal colonization

Research question: **Does nasal MUC5AC protect against or facilitate pneumococcal colonization in humans?**

Objective 1: Associate nasal MUC5AC levels with experimental pneumococcal colonization outcome.

Objective 2: Identify mucosal factors linked with nasal MUC5AC levels.

Objective 3: Establish effects of mucins on pneumococcal growth and survival *in vitro*.

Chapter 2 General Methods

Volunteer recruitment

Healthy non-smoking volunteers were consented to participate in EHPC studies. Ethical approval for experimental human pneumococcal colonization was obtained from the National Health Service Research Ethics Committee (study numbers: 16/NW/0031, 15/NW/0931,14/NW/1460, 15/NW/0146, 11/NW/0592, 18/NW/0481, 17/NW/0029, 19/NW/0238). After consent and assessment of eligibility, volunteers were invited for a screening safety assessment. Those with abnormal findings after clinical examination and blood analysis were excluded from participation. Naturally colonized volunteers at baseline were not routinely excluded (except for trials 11/NW/0592, 17/NW/0029 and 19/NW/0238). General EHPC exclusion (e.g. regular contact with at risk individuals, pregnancy, allergy to penicillin/amoxicillin, history of pneumococcal illness) and screening safety assessment criteria were used to minimise the risk to participants as well as those at higher risk of pneumococcal disease. In addition, volunteers with self-reported or confirmed previous pneumococcal vaccination were excluded from participation (except for 16/NW/0031). More details on inclusion and exclusion criteria for specific studies can be found in the respective publications [9, 207, 342, 343]. Study protocols (except for 11/NW/0592) allowed for early termination of recruitment to that protocol in the case that early (interim) analysis suggested futility.

Bacterial stocks

Clinical strains used for challenge comprised four pneumococcal serotypes (SPN6B, SPN23F, SPN15B and SPN3) and will be further referred to as serotype/clonal name. Strains were gifted to the EHPC group by Prof. Peter Hermans, Radboud University Medical Center (SPN6B), Prof. Jeffrey Weiser, NYU School of Medicine (23F/P833

and 23F/P1121), Dr David Cleary, University of Southampton (15B/SH8286) or made available by Pfizer Inc. (SPN3/PFESP231, SPN3/PFESP306 and SPN3/PFESP505). 23F/P1121 was originally isolated from the nasopharynx of subjects enrolled in a USA pneumococcal challenge study [254]. Strains used for *in vitro* experiments were gifted by David Cleary, University of Southampton (Southampton collection), made available by Pfizer Inc. (Pfizer collection) or part of the EHPC collection of natural colonization.

Bacterial stock preparation

All pneumococcal stocks were grown as described elsewhere [338] with minor modifications. Briefly, stocks were plated onto Columbia blood agar supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) overnight and colonies transferred into liquid medium. So prepared liquid cultures were placed into a tissue incubator (37°C, 5% CO_2, >90% relative humidity) with slightly opened cap without agitation. Optical density was assessed throughout the growth process using FLUOstar Omega microplate reader (BMG Labtech) and Omega software (V 5.1 R2, BMG Labtech). Once bacteria reached an OD of 0.3-0.4, cultures were centrifuged at maximum speed (3350 x g) for 15 - 30 minutes (dependent on serotype), after which media was refreshed and 20% glycerol added. Aliquots of bacteria were prepared at 1 ml and bacteria frozen at -80°C until further use. Bacterial concentration was assessed using classical culture methods as described before [338].

Pneumococcal challenge

Inoculum preparation and pneumococcal challenge were performed as previously described [338]. Where studies were designed as dose ranging trials, volunteers were

randomly assigned to receive a particular dose as per study protocol with doses typically ranging from $1x10^4$ CFU/naris to $32x10^4$ CFU/naris (based on early work by Prof. Jeffrey Weiser, University of Pennsylvania, USA). Otherwise, volunteers were challenged with a dose of $8x10^4$ CFU/nostril.

Detection of pneumococcal colonization

Experimental colonization status was determined by serial nasal washes. Colonization was defined as detection of the pneumococcal challenge strain by culture and/or molecular methods at any sampling time point following challenge. Briefly, for classical microbiology detection nasal wash pellet was plated onto Columbia blood agar plates supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) and 80 µl of 1 mg/mL gentamycin spread across the agar (G1264-250mg, Sigma-Aldrich). Plates were inspected following 24 hours incubation at 37°C, 5% CO_2. Alpha haemolytic, draughtsman-like colonies were sub-cultured for optochin sensitivity and where necessary bile solubility. Latex agglutination and/or Quellung reaction was used to confirm pneumococcal serotype. Pneumococcal density was determined as previously described with minor modifications [338]. Briefly, 100 µl of skim-milk, tryptone, glucose and glycerol (STGG) medium was added to the nasal wash bacterial pellet and the total volume of pellet plus STGG was determined. The resuspended pellet was then serially diluted and plated on Columbia blood agar supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) and 80 µl of 1 mg/mL gentamycin spread across the agar (G1264-250mg, Sigma-Aldrich). The bacterial pellet was further diluted with 800 µl STGG and frozen at -80°C. Columbia blood agar plates were incubated overnight at 37°C, 5% CO_2. The next day CFU per µl were

determined and multiplied by the total pellet volume. This value was then divided by the amount of nasal wash returned by the volunteer to calculate CFU/ml of nasal wash.

For molecular detection bacterial genomic DNA was extracted from EHPC nasal samples. Aliquots of bacterial suspensions were pelleted at 20,238xg for 10 minutes and resuspended in 257 µl of lysis buffer with 43 µl of protease (Agowa Mag mini DNA extraction kit; LGC Genomics), 100 µl of sterilized zirconia/silica beads (diameter of 0.1 mm; Biospec Products) and 300 µl of phenol (Phenol BioUltra; Sigma-Aldrich). The sample was mechanically disrupted by bead beating in TissueLyser twice at 50 Hz for 3 minutes. After 10 minutes centrifugation at 9,391xg, the aqueous phase was transferred to a sterile tube. Binding buffer was added at twice the volume of the aqueous phase plus 10 µl of magnetic beads, after which the sample was incubated in a mixing machine (~265 rpm) for 30 minutes at room temperature. The magnetic beads were sequentially washed with 200 µl of wash buffer 1 and wash buffer 2, and eluted with 63 µl of elution buffer, according to the manufacturer's instructions. For each performed extraction run parallel extraction of kit's buffers was performed.

Extracted DNA was used for quantitative polymerase chain reaction (qPCR) of pneumococcal genes (*lytA, piaB* or 6AB specific *cpsA)*. The primers and probe sequences used are shown in Table 2-1.

Table 2-1: Primers and probes for qPCR

Target	Sequence	Reference
6AB *cpsA* forward	5'-AAGTTTGCACTAGAGTATGGGAAGGT-3'	[344]
6AB *cpsA* reverse	5'-ACATTATGTCCATGTCTTCGATACAAG-3'	
6AB *cpsA* probe	5'-(FAM)-TGTTCTGCCCTGAGCAACTGG-(BHQ1)-3'	

lytA forward *lytA* reverse *lytA* probe	5′-ACGCAATCTAGCAGATGAAGCA-3′ 5′-TCGTGCGTTTTAATTCCAGCT-3′ 5′-(FAM)-TGCCGAAAACGCTTGATACAGGGAG-(BHQ1)-3′	[345]
piaB forward *piaB* reverse *piaB* probe	5′-CATTGGTGGCTTAGTAAGTGCAA-3′ 5′-TACTAACACAAGTTCCTGATAAGGCAAGT-3′ 5′-(TAMRA)-TGTAAGCGGAAAAGCAGGCCTTACCC-(BHQ1)-3′	[53]

Unless otherwise specified qPCRs were run as single plex reactions targeting one pneumococcal gene per reaction. Each qPCR reaction consisted of 12.5 µl 1 × TaqMan Universal PCR Master Mix (Life Technologies), forward and reverse primers (0.225 µl *cpsA*; 0.225 µl *lytA*; 0.2 µl *piaB*), probe (0.125 µl *cpsA*, 0.125 µl *lytA*; 0.175 µl *piaB*) and 2.5 µl of the extracted DNA. For qPCRs run on the Mx3005P system (Agilent Technologies) 25 µl total reaction volume was used. For qPCRs run on QuantStudioTM 5 system (Applied Biosystems) 20 µl total reaction volume was used. Reactions were adjusted to the full reaction volume using molecular graded water. Thermal cycling conditions were: 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. A qPCR negative control (master mix only) and a minimum of two extractions of each sample were amplified. A standard curve of a ten-fold dilution series of genomic pneumococcal DNA extracted with the QIAamp DNA Mini Kit (Qiagen) and quantified with a spectrophotometer (Nanodrop ND-1000; Thermo Fisher Scientific) was used. To convert the amount of DNA to number of copies the equation below was used - using the length of one TIGR4 genome – whereby 10 ng of DNA (amount of DNA in highest concentrated standard) equals 4.14 x 10^6 DNA copies.

$$Number\ of\ copies = \frac{Amount\ of\ DNA\ (ng)\ x\ Avogadro's\ number}{Weight\ of\ one\ genome\ copy\ (g/mol)\ x\ 10^9}$$

Cell culture

Immortalised nasopharyngeal cells Detroit 562 (ATCC® CCL138™) were cultured according to ATCC guidelines. Primary nasal cells were collected as previously described [207]. Cells were dislodged from curette in RPMI media containing 10% foetal bovine serum (FBS) and 1% Penicillin-Neomycin-Streptomycin (Life technologies) by repeated pipetting and spun down at 440 x g for 5 minutes. Cells were cultured in a submerged state (NEC) for a maximum of 27 days. Establishment of differentiated nasopharyngeal epithelium (NPE) via the initiation of air-liquid interface was performed as described elsewhere [346]. NPE cultures were maintained for a further 30 days.

Pneumococcal adherence assay

Adherence assays were performed based on a well-established method outlined by Glennie et al. [155]. Cells were seeded in a 24 well plate at a minimum of $1x10^5$ (NEC) or $6x10^5$ (Detroit 562) cells per well and grown until confluency or for a maximum of 7 days. Where NPE cultures were used the assay was performed after establishing air liquid interface. On the day of the assay, cells were washed 3 times with HBSS containing calcium and magnesium ($HBSS^{+/+}$). A pneumococcal stock aliquot grown a described previously (Section: 'Bacterial Stock preparation') was thawed and diluted to a concentration of $5x10^6$ CFU/ml in EMEM media (M5650, Sigma-Aldrich) supplemented with 1% heat-inactivated foetal bovine serum (FBS, Fisher Scientific) and 2 mM L-Glutamine (Sigma-Aldrich). $1x10^6$ CFU of bacteria were added to the cells, centrifuged at 200 g for 1 minute and placed into a tissue cell incubator (37°C, 5% CO_2, >90% relative humidity) for 3 hours. After 3 hours the bacteria containing liquid was removed and cells were washed 3 times with ice-cold $HBSS^{+/+}$. To dislodge

cells from the culture plate 0.25% Trypsin (Fisher Scientific) was added for 10 minutes at 37°C. Cells were collected in a microcentrifuge tube before washing the culture plate thoroughly and collecting any additional liquid. To permeabilise cells 1% Saponin was added to the collected liquid for 10 minutes at room temperature. Thereafter liquid was vortexed and bacteria were quantified by plating serial dilutions onto Columbia blood agar supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) followed by incubation in a tissue incubator (37°C, 5% CO_2, >90% relative humidity) over night. Results were expressed as CFU and corrected for dose. Adherence is defined as adherent and internalized pneumococcus.

MUC5AC enzyme linked immunosorbent assay (ELISA)

ELISA for MUC5AC was performed based upon a method described elsewhere with minor modifications [317]. Pre-diluted mucin standard (Mucin from porcine stomach Type II, Sigma-Aldrich) and samples were added to a 96-well ELISA plate and incubated for 90 minutes at 37°C. Plates were washed 3 times with phosphate buffered saline (PBS) with 0.05%Tween-20 (PBS-T). Washing was repeated after each step up to the application of substrate solution. Plates were blocked with 1% bovine serum albumin (BSA) in PBS-T for 1 hour at 37°C. Bound MUC5AC was detected using monoclonal anti-human MUC5AC primary antibody (45M1, 1:200, Thermo Scientific) for 1 hour at 37°C, followed by incubation with Biotin-conjugated goat anti-mouse IgG (1:5000, Vector Laboratories) for 1 hour at 37°C and incubation with Streptavidin-HRP conjugate (1:5000, Life technologies) for 30 minutes at room temperature. For detection 100 µl of tetramethylbenzidine substrate solution (TMB) was added. Reaction was stopped with 100 µl of 2N H_2SO_4. Optical densities were

measured at 450 nm using FLUOstar Omega microplate reader (BMG Labtech). All samples were analysed in duplicates. Results are expressed as arbitrary units (AU) and calculated based on the standard curve.

Statistical analysis

Graph and statistical analysis were performed using GraphPad prism version 8.0 (GraphPad software Inc, USA), SAS/STAT® Version 9.4 (SAS Institute Inc, USA) and RStudio (V1.1.463 and V3.6.2). Data were log transformed (log_{10}) where appropriate. All P values are two-tailed with significance level set at $P < 0.05$.

Chapter 3 Expansion of the EHPC model: How to predict experimental colonization efficiency

3.1 Introduction

Colonization of the nasopharynx is the first and obligatory step in pneumococcal pathogenesis and the reservoir for transmission in the population [40]. Colonization rates vary considerably between pneumococcal serotypes as well as between community groups.

Host factors known to affect susceptibility to colonization include age, geographic area, socio-economic status, and innate and adaptive immune responses [155, 220, 253, 347-350]. Microbial factors influencing pneumococcal colonization patterns are less well understood. Pneumococcal capsule plays a large role in determining colonization as well as transmission efficiency [73, 261, 313, 330]. Further, factors potentially influencing successful colonization are microbial properties contributing to epithelial adherence, phase variation in capsular expression and interaction with the resident microflora [40].

The EHPC model is a well-established and controlled infection model with precise bacterial dose, timing of colonization onset and duration known [43, 155, 210, 253, 258, 351]. The model allows the study of human-pathogen interactions, host immunity and colonization dynamics as well as testing of new pneumococcal vaccines using colonization as endpoint. Given these, the model would benefit from a broad range of pneumococcal strains available for human challenge. However, establishing new strains within the model has proven to be challenging in the past, largely due to the current inability to determine experimental colonization rates of pneumococcal candidate strains prior to human challenge.

Here I sought to identify microbial characteristics which allow an estimation of experimental pneumococcal colonization rates prior to human challenge. To this effect I compiled and compared clinical derived colonization data of four different pneumococcal strains used previously in the EHPC model. I then analysed key microbiological properties which vary by capsular type and have been previously associated with pneumococcal colonization prevalence in other research settings: non-opsonic neutrophil mediated killing [211], adherence to human nasal epithelial cells [352] and pneumococcal chain length [274]. Proof of concept was sought in the development of a pneumococcal serotype 3 EHPC model using the established prediction tool to select candidate strains for human challenge.

3.2 Methods

Volunteer recruitment and pneumococcal challenge

Volunteer recruitment and pneumococcal challenge were carried out as outlined in Chapter 2.

Pneumococcal growth curve

Bacterial stocks were prepared and subjected to optical density measurement (as described in detail in Chapter 2) over time using either Todd-Hewitt Broth with 2% Yeast Extract (THY) or Vegitone as growth media.

Detection of pneumococcal colonization in nasal wash

Detection of pneumococcal colonization in nasal wash was performed as outlined in Chapter 2. For molecular detection 300 µl of nasal wash pellet was extracted and subjected to single plex qPCR detection of the pneumococcal gene *lytA* (as detailed in Chapter 2) using QuantStudioTM 5 system (Applied Biosystems) or Mx3005P system (Agilent Technologies). Cross experiment threshold was set manually at 3500 on Mx3005P system (Agilent Technologies) or 0.35 on QuantStudioTM 5 system (Applied Biosystems). Samples were considered positive if a minimum of two reactions yielded a cycle threshold (CT) < 40 cycles.

For samples that showed positive detection of *lytA* by molecular methods but were negative for colonization by classical microbiology confirmation of colonization was sought via detection of a second pneumococcal gene (*piaB* for volunteers challenged with SPN15/SH8286, 23F/P1121, 23F/P833 and 6AB specific *cpsA* for volunteers challenged with SPN6B/BHN418) using the QuantStudioTM 5 system (Applied

Biosystems). Cross experiment threshold was calculated using relative threshold algorithm and controlled manually by an inter-run calibrator (positive control). Samples were considered positive if a minimum of two reactions yielded a CT < 40 cycles.

Home sampling and detection of pneumococcus in saliva

Home sampling of saliva was performed as described elsewhere [353]. For molecular detection of pneumococcus in saliva, samples were thawed for 30 minutes at room temperature and vigorously vortexed for 20 seconds. 200 µl of raw saliva was aliquoted for DNA extraction. In addition, culture-enrichment from the raw saliva sample was performed. Briefly, 10 µl of raw saliva was diluted with 90 µl of saline and cultured on Columbia blood agar supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) and 80 µl of 1 mg/mL gentamycin spread across the agar (G1264-250mg, Sigma-Aldrich). Plates were incubated overnight at 37 ºC and 5% CO_2. After incubation, all bacterial growth was harvested into 2 mL STGG and vigorously vortexed until homogenised. 200 µl of culture-enriched saliva was aliquoted for DNA extraction.

DNA extraction and qPCR were performed as outlined in Chapter 2 with minor modifications. Samples were subjected to multiplex qPCR for *lytA* (0.15 µl primers; 0.075 µl probe) and 6AB specific *cpsA* (0.1 µl primers and 0.05 µl probe) using Mx3005P system (Agilent Technologies). Cross experiment threshold was calculated using inter-run calibrators and multiple experiment analysis. Each sample was analysed in triplicates and samples were considered positive if two or all triplicates yielded a CT value of < 40 cycles for both genes.

Flow cytometry

Nasal cells were collected using curettage as described elsewhere [207] and dislodged from the curette by repeated pipetting. Cells were spun down at 440 x g for 5 minutes and stained with LIVE/DEAD® Fixable Aqua Dead Cell Stain (Invitrogen) according to the manufacturer's instructions. Cells were then stained with an antibody cocktail containing Epcam-PE, HLADR-PECy7, CD66b-FITC, CD19-BV650 (all Bio Legend), CD3-APCCy7, CD14-PercpCy5.5 (all BD Biosciences) and CD45-PACOrange (Thermo Fisher) was added to the cells. Following a further 15 minutes incubation on ice, PBS was added and the cells were filtered using a 70 μm filter. The filtered cell solution was spun down at 440 x g for 5 minutes, resuspended in PBS containing 0.5% heat-inactivated foetal bovine serum and 5 mM EDTA and acquired on a flow cytometer (LSRII; BD). Flow cytometry data was analysed using Flowjo V. 10 (Tree Star Inc). The gating strategy for this experiment has been described previously [353]. Samples with less than five hundred immune cell events and/or 250 epithelial cell events were excluded from further analysis. Cell numbers were normalised for epithelial cell count.

Human myeloperoxidase ELISA

Levels of myeloperoxidase (MPO) were determined using the Human Myeloperoxidase DuoSet ELISA Kit (R&D Systems) as per manufacturer's instructions. Briefly, 96-well ELISA plates were coated with 4 μg/ml capture antibody in PBS at room temperature overnight. Plates were washed 3 times with PBS (Sigma-Aldrich) containing 0.05% Tween-20 (Sigma-Aldrich). Washing was repeated after each step up to the application of substrate solution. Wells were blocked with 1% BSA in PBS for 1 hour at room temperature. Samples and standards were diluted in 1%

BSA-PBS, 100 µl transferred onto the pre-coated plates and incubated at room temperature for 2 hours. Detection was performed by incubating plates with detection antibody at 50 ng/ml for 2 hours at room temperature, followed by 20 minutes incubation with Streptavidin-HRP (1:200) (Fisher Scientific) at room temperature. Signal was developed using TMB substrate solution (Fisher Scientific) and stopped by adding 2N H_2SO_4 in a 1:1 ratio. Optical density reading was performed at 450 nm and corrected for optical imperfection (540 nm). All samples were run in duplicate. Results are expressed as µg/ml and calculated using MPO standard curve.

Confocal microscopy

Mucosal cells collected by curettage as described elsewhere [207] were placed directly into 4% PFA for 1 hour. Cells were dislodged and spun onto microscope slides at 450 rpm (Cytospin4, Thermo Scientific) for 5 minutes at room temperature. Thereafter cells were left to dry for a minimum of 30 minutes. Cells were permeabilized using 1% Triton for 10 minutes and blocked with blocking buffer (3% goat serum, 3% BSA in $HBSS^{+/+}$) for 1 hour at room temperature. This was followed by incubation with primary antibodies in blocking buffer for 1 hour at room temperature. Thereafter cells were washed 3 times in distilled water and incubated with secondary antibodies in blocking buffer for 1 hour at room temperature. Antibodies used were: WGA - Rhodamine (1:500, Vector Laboratories), group 6 antiserum (1:100, Oxford Biosystems), mouse CD45 -Alexa Fluor 633 (1:50 Santa Cruz Biotechnologies) and goat anti-rabbit IgG – Alexa Fluor 488 (1:1000, Thermo Scientific). Thereafter cells were washed 3 times in distilled water and mounted using Vectashield Hard set mounting media with DAPI (Vector laboratories). The entire cytospin for each sample was manually viewed by microscopy for detection of pneumococcus and the total sum

of detected pneumococcus for each participant reported. Images were captured using an inverted LSM 880 (Zeiss) and analysed using the LSM Image Browser (Zeiss). Z stacks were recorded at 1 µm intervals at either ×40 oil or ×63 oil objectives. For analysis the operator was blinded to the colonization status of the volunteer.

Cell culture and pneumococcal adherence assay

Cell culture of NEC and Detroit 562 cells was performed, and cells were used for pneumococcal adherence assay as outlined in Chapter 2.

Quantitative assessment of chain length

Quantitative assessment of chain length was performed based on a method developed and validated by Basset et al. [354], whereby chain length was expressed as ratio of optical density (OD) to colony forming units (CFU). Bacteria were grown in Vegitone media as described in Chapter 2. Briefly, bacteria were cultured overnight on Columbia blood agar and transferred the next day into liquid broth at an OD_{600nm} of 0.1-0.15. Optical densities were blank corrected using a media only measurement. Liquid cultures were placed with slightly opened cap without agitation into a tissue incubator (37°C, 5% CO_2, >90% relative humidity) and left to grow while monitoring the OD periodically. Once bacteria reached an OD_{600nm} of 0.3 to 0.4, optical density was recorded (OD_{end}) and bacterial dilutions were plated onto Columbia blood agar. Measurements of OD and plating of bacteria was performed in duplicate. Columbia blood agar plates were incubated in a tissue incubator (37°C, 5% CO_2, >90% relative humidity) over night. CFU were counted the next day and bacterial concentration per ml was calculated. Chain length ratio was calculated by dividing OD_{end} by CFU/ml.

Neutrophil surface killing assay

Resistance to non-opsonic neutrophil mediated killing was measured as previously described [211] with minor modifications. Briefly, an aliquot of pneumococcal stock (grown as described in Chapter 2) was thawed and diluted to 5×10^3 CFU/mL in saline, plated onto Columbia blood agar supplemented with 5% horse blood (PB0122A, Oxoid/Thermo Scientific) in 10 replicates and allowed to dry at room temperature. Neutrophils were freshly isolated from peripheral blood of young healthy volunteers (LSTM Tissue Bank) as described elsewhere [253, 355] and diluted to 2×10^6 cells/ml. Neutrophils were then overlaid onto the bacterial spots and allowed to dry. Columbia blood agar plates were incubated in a tissue incubator (37°C, 5% CO_2, >90% relative humidity) over night and quantified the next day. Results were expressed as CFU and corrected for dose.

Nucleotide sequence accession numbers

Strains used for human challenge were sequenced in a reference laboratory. Nucleotide sequence of the challenge strain SPN6B/BHN418 can be accessed in DDBJ/EMBL/GenBank under accession number ASHP00000000.1. Nucleotide sequence of the challenge strains SPN23F/P833, SPN23F/P1121, and SPN15B/SH8286 can be accessed in the European Nucleotide Archive (ENA) using the following accession numbers: for SPN23F/P833 - ERS743506, for SPN23F/P1121 - ERS1072059 and for SPN15B/SH8286 - ERS2632437.

Statistical analysis

Graphical and statistical analysis were performed as outlined in Chapter 2.

3.3 Results

3.3.1 Establishing pneumococcal colonization efficiency in the EHPC model

3.3.1.1 EHPC study design

A total of 190 volunteers were recruited to four dose-ranging studies (study number 11/NW/0592 and 15/NW/0931) and intranasally challenged with pneumococcus. Briefly, sixty volunteers were challenged with pneumococcal strain SPN6B/BHN418, 60 with SPN23F/P833, 16 with serotype SPN23F/P1121 and 54 with SPN15B/SH8286. Doses administered ranged from 10,000 CFU/nostril to 320,000 CFU/nostril. Age and gender distribution were comparable between the studies (Table 3-1). There were no reported severe adverse events secondary to pneumococcal challenge in these studies.

Table 3-1: Demographics

Pneumococcal strain	Median Age - years (range)	Female - no. (%)
SPN6B/BHN418	22 (18-58)	32/60 (53%)
SPN15B/SH8286	21 (18-49)	31/54 (57%)
SPN23F/P1121	29 (25-47)	11/16 (69%)
SPN23F/P833	21 (18-45)	33/60 (55%)

Colonization was determined analysing nasal wash samples by classical microbiology (growth of pneumococcal strain on blood agar plate) and molecular detection of pneumococcal genes *lytA* (autolysin A) and *piaB* (permease gene of the pia ABC transporter). To account for previous reports highlighting the suboptimal performance of *piaB* qPCR for the detection of serotype 6B strains [356] the detection limit of *piaB* qPCR was analysed using standard curve comparisons of EHPC strains. In contrast to a previous report [356], *piaB* was amplified in samples containing pure SPN6B/BHN418 suggesting presence of the *piaB* gene in this strain. However, results showed that *piaB* was only detectable at higher concentrations of SPN6B/BHN418 when compared to other pneumococcal serotypes (Figure 3-1). The observed discrepancy may be caused by single nucleotide polymorphisms (SNPs) in the target sequence of qPCR primers. SNPs have previously been shown to reduce the number of molecules that can be detected at a given concentration when compared to perfect match primers [357]. Given the inferior detection of *piaB* for SPN6B/BHN418, molecular detection of experimental colonization for this strain was based on presence of pneumococcal genes *lytA* and 6AB-specific *cpsA.*

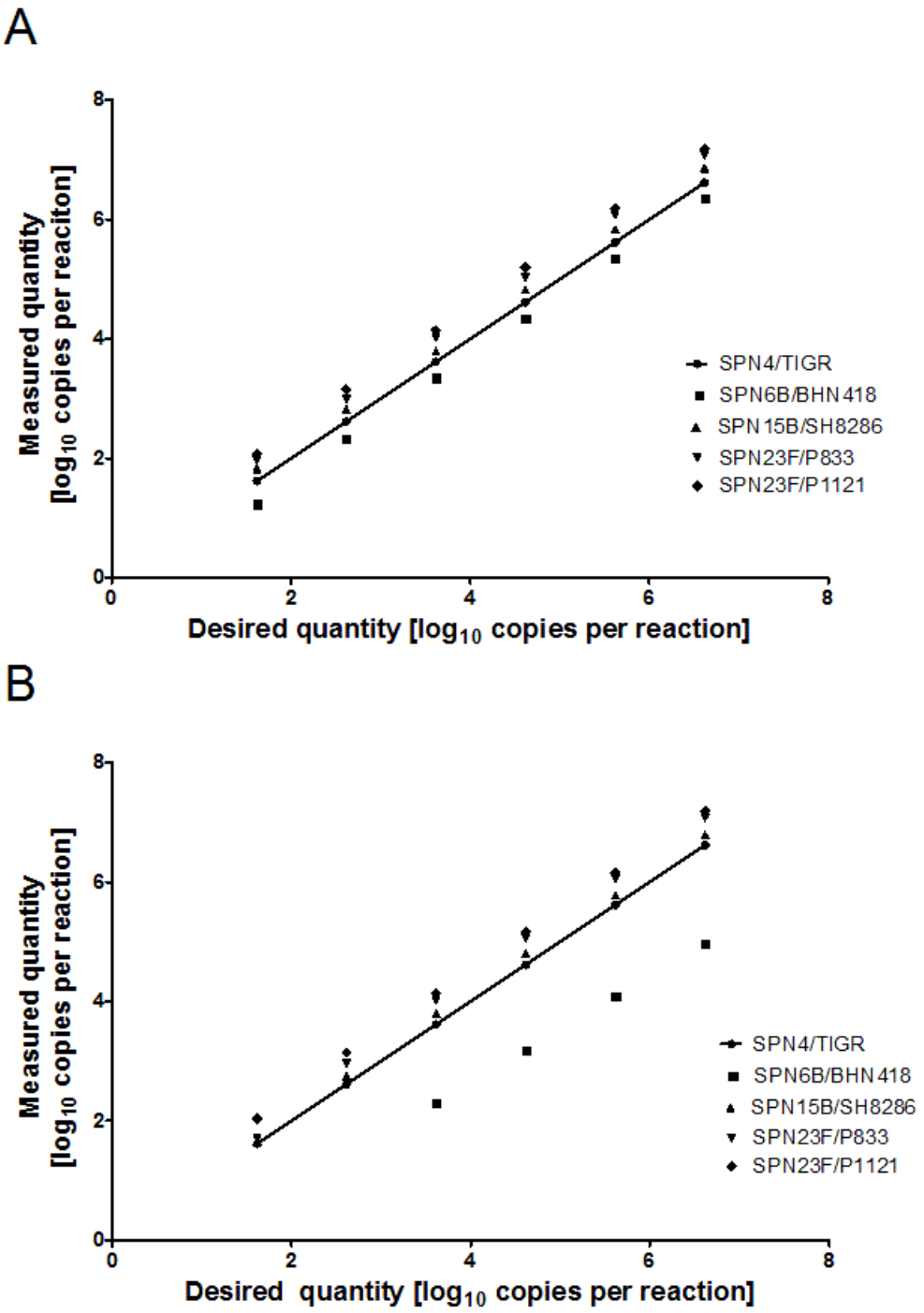


Figure 3-1: Detection of *piaB* in SPN6B/BHN418 containing samples.

Standard curves using qPCR detection of (A) *lytA* and (B) *piaB* of four pneumococcal strains. In samples containing strain SPN6B/BHN418 *piaB* could only be detected at higher concentrations when compared to other pneumococcal strains. Standard curves were prepared measuring DNA concentration of a pure extract of the relevant pneumococcal strain using nanodrop and diluted in a 1:10 ratio. Concentration was calculated using the weight of one genome copy of TIGR4 (SPN4) as reference.

3.3.1.2 Growth characteristics of pneumococcal strains used for EHPC challenge

Growth curves of pneumococcal strains SPN6B/BHN418, SPN15B/SH8286, SPN23F/P1121 and SPN23F/P833 were analysed in Todd-Hewitt Broth with 0.5% Yeast Extract (THY, standard laboratory media for pneumococcal growth) and Vegitone infusion broth (Vegitone, used for the preparation of pneumococcal stocks for human challenge) for up to 7 hours (Figure 3-2). Strains showed overall similar growth behaviour amongst each other as well as under different culture conditions with a lag phase of up to 3 hours and an exponential phase of about 2-3 hours. Notable differences between culture media were observed with regard to stationary and declining phase. In THY the stationary phase was non-existent for all strains but SPN6B/BHN418, as evident by a sharp decline after culture optimum was reached. In Vegitone stationary phase was observed for up to 2 hours (or the end of the experiment). Peak densities varied considerably between strains, experiment and culture conditions. Notably, in Vegitone one growth experiment showed overall poor growth behaviour, with a prolonged lag-phase and decreased peak densities for all four tested strains, highlighting the experimental variability observed with this media.

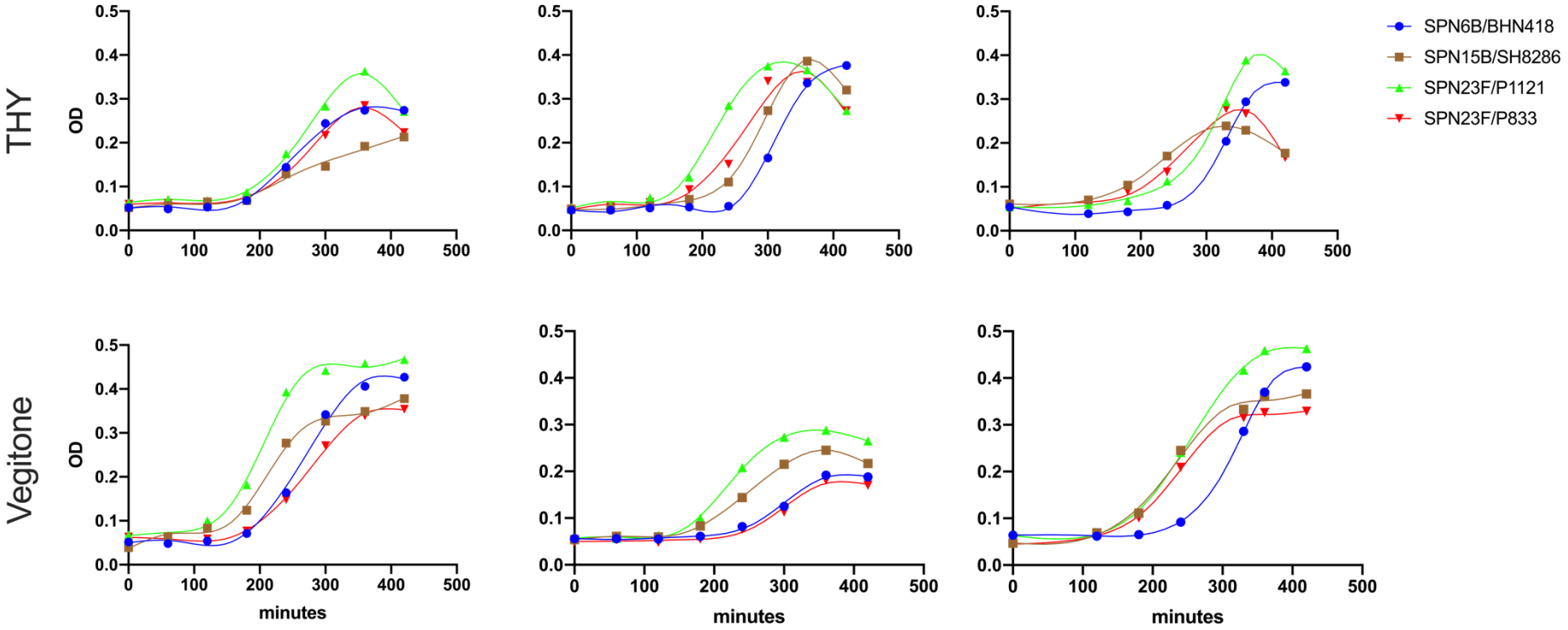


Figure 3-2: Growth curves of pneumococcal strains tested in the EHPC model.

Optical density (OD) of strains SPN6B/BHN418 (blue), SPN15B/SH8286 (brown), SPN23F/P1121 (green) and SPN23F/P833 (red) during pneumococcal incubation in Todd-Hewitt Broth with 0.5% Yeast Extract (THY) or Vegitone infusion broth (Vegitone) at 37°C using FLUOstar Omega microplate reader without wavelength correction. Each graph presents one experimental replicate.

3.3.1.3 Dose-response relationship of pneumococcal strains upon human challenge

The pneumococcal colonization rate of the challenge strains was assessed for each dose group as the percentage of volunteers who showed growth of the challenge strain on Columbia blood agar from nasal wash at any time point post-challenge. The dose-response relationship upon human challenge differed between the tested pneumococcal strains as shown in Figure 3-3. Notably, strains SPN23F/P833, SPN23F/P1121, and SPN15B/SH8286 showed declining colonization rates for doses higher than $8x10^4$ CFU/nostril. This was not the case for strain SPN6B/BHN418 which achieved maximum colonization rates at doses higher than $8x10^4$ CFU/nostril.

Maximum colonization rates (highest rates achieved at any given dose) as determined by classical microbiology (CLASSIC) were 60% for SPN6B/BHN418 (6/10, 95% Cl: 29-90%, Doses: $4x10^4$, $16x10^4$ and $32x10^4$ CFU/nostril), 31% for SPN15B/SH8286 (11/35, 95% Cl: 16-46%, Dose: $8x10^4$ CFU/nostril), 16% for SPN23F/P1121 (1/6, 95% Cl: 0-46%, Doses: $2x10^4$ and $8x10^4$ CFU/nostril) and 10% for SPN23F/P833 (1/10, 95% Cl: 0-28%, Doses: $2x10^4$, $4x10^4$ and $8x10^4$ CFU/nostril).

Colonization rates were also assessed using molecular methods. Details of colonization rates per dose, strain and method of assessment can be found in Table 3-2. Determination of colonization by molecular methods did not change maximum colonization rates detected with the exception of strain SPN23F/P833, which by molecular methods showed maximum colonization rates of 20% (2/10, Cl: 0-44%, Dose: $2x10^4$ CFU/nostril). When combining both methodologies (detection of colonization by either classical or molecular method, COMB) maximum colonization

rates increased for 3 out 4 strains as follows: 70% for SPN6B/BHN418 (7/10, 95% Cl: 41-98%, Dose: $16x10^4$ CFU/nostril), 37% for SPN15B/SH8286 (13/35, 95% Cl: 21-53%, Dose: $8x10^4$ CFU/nostril), 16% for SPN23F/P1121 (1/6, 95% Cl: 0-46%, Doses: $2x10^4$ and $8x10^4$ CFU/nostril) and 20% for SPN23F/P833 (2/10, 95% Cl: 0-44%, Dose: $2x10^4$ CFU/nostril.

Table 3-2: Colonization rates per dose compared by pneumococcal strain and assessment method

		Colonization positive [a]/n total (%)					
Dose	Strain	CLASSIC		MOLECULAR		COMBINED	
10000	6B/BHN418	3/10	30%	3/10	30%	3/10	30%
10000	23F/P833	0/10	0%	0/10	0%	0/10	0%
20000	6B/BHN418	1/10	10%	1/10	10%	1/10	10%
20000	23F/P833	1/10	10%	2/10	20%	2/10	20%
20000	23F/P1121	1/6	16%	1/6	16%	1/6	16%
20000	15B/SH8286	3/10	30%	3/10	30%	3/10	30%
40000	6B/BHN418	6/10	60%	6/10	60%	6/10	60%
40000	23F/P833	1/10	10%	1/10	10%	1/10	10%
80000	6B/BHN418	5/10	50%	5/10	50%	5/10	50%
80000	23F/P833	1/10	10%	1/10	10%	1/10	10%
80000	23F/P1121	1/6	16%	1/6	16%	1/6	16%
80000	15B/SH8286	11/35	31%	11/35	31%	13/35	37%
160000	6B/BHN418	6/10	60%	6/10	60%	7/10	70%
160000	23F/P833	0/10	0%	1/10	10%	1/10	10%
160000	23F/P1121	0/6	0%	0/6	0%	0/6	0%
160000	15B/SH8286	1/9	11%	2/9	22%	2/9	22%
320000	6B/BHN418	6/10	60%	6/10	60%	6/10	60%
320000	23F/P833	0/10	0%	0/10	0%	0/10	0%

[a] Colonization was determined by classical microbiology (growth of the specified pneumococcal strain from nasal wash on Columbia blood agar, CLASSIC), by molecular methods (detection of *lytA/piaB* or *lytA*/6AB-*cspA* genomic DNA in nasal wash via qPCR, MOLECULAR) or by combining classical microbiology and molecular methods (COMBINED). Grey highlights existing differences in colonization rates between assessment methods. Colonization rate = percentage of challenged volunteers who were positive for experimental pneumococcal colonization. Volunteers were considered positive if the challenge strain was detected at any time point following challenge as assessed by the defined method of detection.

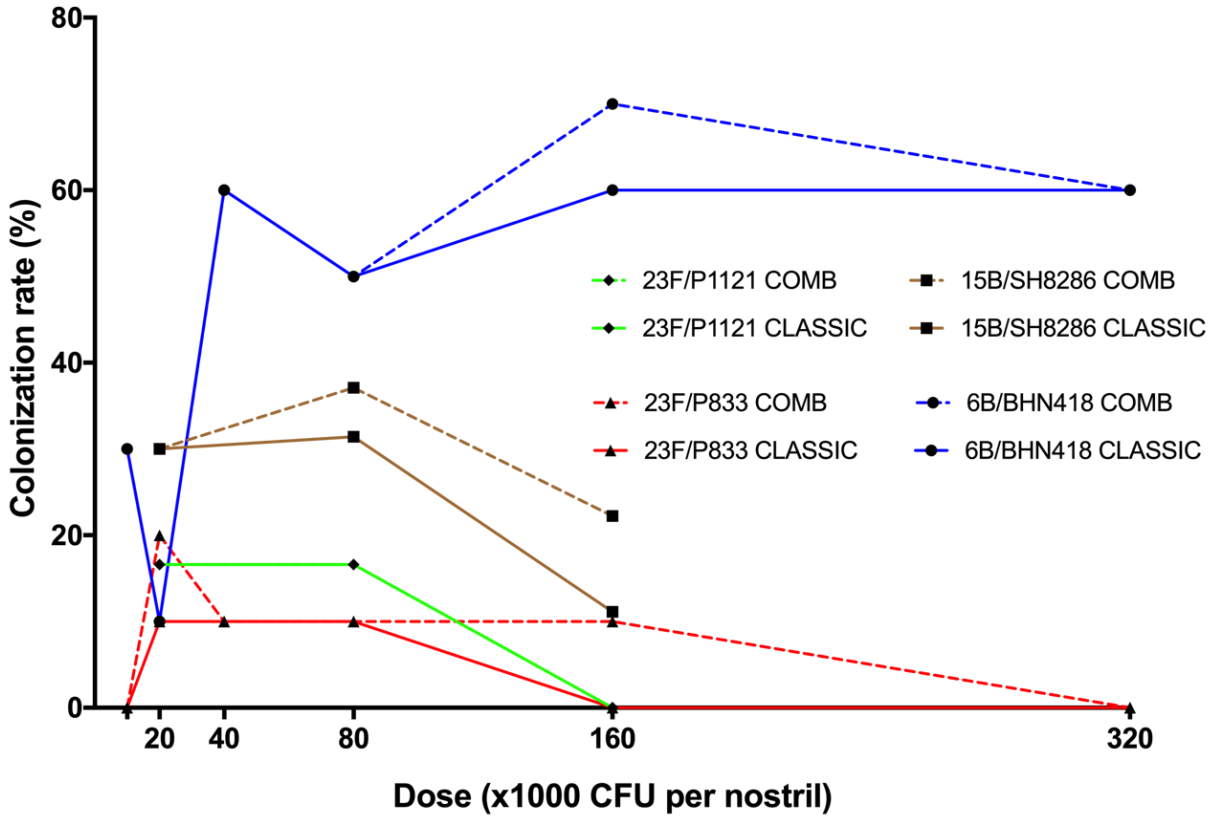


Figure 3-3: Dose-response relationship of pneumococcal strains upon human challenge.
Volunteers were intranasally challenged with either SPN6B/BHN418 (circle), SPN15B/SH8286 (square), SPN23F/P1121 (triangle apex up) or SPN23F/P833 (triangle apex down). Colonization was determined by classical microbiology (growth of the specified pneumococcal strain on Columbia blood agar, solid line, CLASSIC) or by combining classical microbiology and detection of *lytA/piaB* or *lytA*/6AB-*cspA* genomic DNA (dotted line, COMB) in nasal wash. Each point represents the percentage of colonization acquisition achieved with the specified pneumococcal strain when volunteers were exposed with 1 x 10^4, 2 x 10^4, 4 x 10^4, 8 x 10^4, 16 x 10^4 and 32 x 10^4 pneumococci per nostril. Volunteers were considered positive if the challenge strain was detected at any time point following challenge as assessed by the specified method of detection

3.3.1.4 Pneumococcal strain SPN6B/BHN418 shows highest colonization rate upon human challenge

Average colonization rates of each strain were assessed as the percentage of all challenged volunteers who showed growth of the challenge strain on Columbia blood agar from nasal wash at any time point post-challenge (Table 3-3). Colonization rates differed significantly between the tested pneumococcal strains (Chi-square test, P<0.0001). Most efficient colonization was observed with SPN6B/BHN418 (27/60, 45%, 95% Cl: 32-57%). A trend towards less efficient colonization was observed with SPN15B/SH8286 (15/54, 28%, 95% Cl: 15-39%) when compared to SPN6B/BHN418 (P=0.0570). Colonization efficiency of SPN23F/P1121 (2/16, 13%, 95% Cl: 0-28%) and SPN23F/P833 (3/60, 5%, 95% Cl: 0-10%) were comparable (P=0.2823) but were significantly lower than that of SPN6B/BHN418 (P=0.0174 and P<0.0001, respectively).

Determination of colonization by molecular methods did increase detection of colonization in two out of four pneumococcal strains (Table 3-3) but did not change pattern of colonization observed. When combining both methodologies (detection of colonization by either classical or molecular method) average colonization rates increased for 3 out 4 strains and remained significantly different between the strains (Table 3-3, Chi-square test, P<0.0001). Most efficient colonization was observed with SPN6B/BHN418 (28/60, 46%, 95% Cl: 34-59%). Less efficient colonization was observed with SPN15B/SH8286 (18/54, 33%, 95% Cl: 20-45%, P = 0.1474). Colonization efficiency of SPN23F/P1121 (2/16, 13%, 95% Cl: 0-28%) and SPN23F/P833 (5/60, 8%, 0-15%) were comparable (P=0.6086) but were significantly lower than that of SPN6B/BHN418 (P=0.0130 and P<0.0001, respectively).

Table 3-3: Pneumococcal strain SPN6B/BHN418 shows highest colonization rates upon human challenge

	Classic [a]		Molecular [b]		Combined [c]	
Pneumococcal strain	Colonized individuals	Colonization rate (%)	Colonized individuals	Colonization rate (%)	Colonized individuals	Colonization rate (%)
SPN6B/BHN418	27/60	45	27/60	45	28/60	46
SPN15B/SH8286	15/54	28	16/54	30	18/54	33
SPN23F/P1121	2/16	13	2/16	13	2/16	13
SPN23F/P833	3/60	5	5/60	8	5/60	8

Colonization rate = percentage of challenged volunteers who were positive for experimental pneumococcal colonization, independent of the dose received. Volunteers were considered positive if the challenge strain was detected at any time point following challenge as assessed by the specified method of detection.[a] Detection of pneumococcal growth from nasal wash on Columbia blood agar plates. [b] Detection of pneumococcal DNA (*lytA/piaB* or *lytA*/6AB-*cpsA*) in nasal wash [c] Colonization as defined by the combination of classical and molecular methods.

3.3.1.5 Colonization density and duration upon experimental human challenge are comparable between pneumococcal strains

Pneumococcal colonization densities (Figure 3-4) and duration of colonization (Table 3-4) as determined by classical microbiology were assessed over a two-week study period and compared using a general linear mixed model. Densities and durations of colonization episodes of SPN23F/P1121 and SPN23F/P833 were excluded from the analysis due to the low number of colonized individuals (n=2 and n=3, respectively). Colonization densities were comparable between SPN15B/SH8286 and SPN6B/BHN418 after adjusting for day and dose (P=0.4924). Similarly, the duration of colonization defined as the last day at which colonization could be detected was comparable between those challenged with SPN6B/BHN418 and SPN15B/SH8286 after adjusting for dose (P=0.7009).

Table 3-4: Duration of experimental human pneumococcal colonization.

	Duration of colonization [a]		
Pneumococcal strain	**2 days**	**7 days**	**14 days**
SPN6B/BHN418	2/27 (7.4%)	7/27 (25.9%)	18/27 (66.6%)
SPN15B/SH8286	3/15 (20%)	3/15 (20%)	9/15 (60%)
SPN23F/P1121	0/2 (0%)	0/2 (0%)	2/2 (100%)
SPN23F/P833	1/3 (33.3%)	0/3 (0%)	2/3 (66.6%)

[a] Duration defined as number of volunteers who were colonized for 2, 7 or 14 days. Volunteers were considered colonization positive if the challenge strain was grown from nasal wash on Columbia blood agar at any time point following challenge.

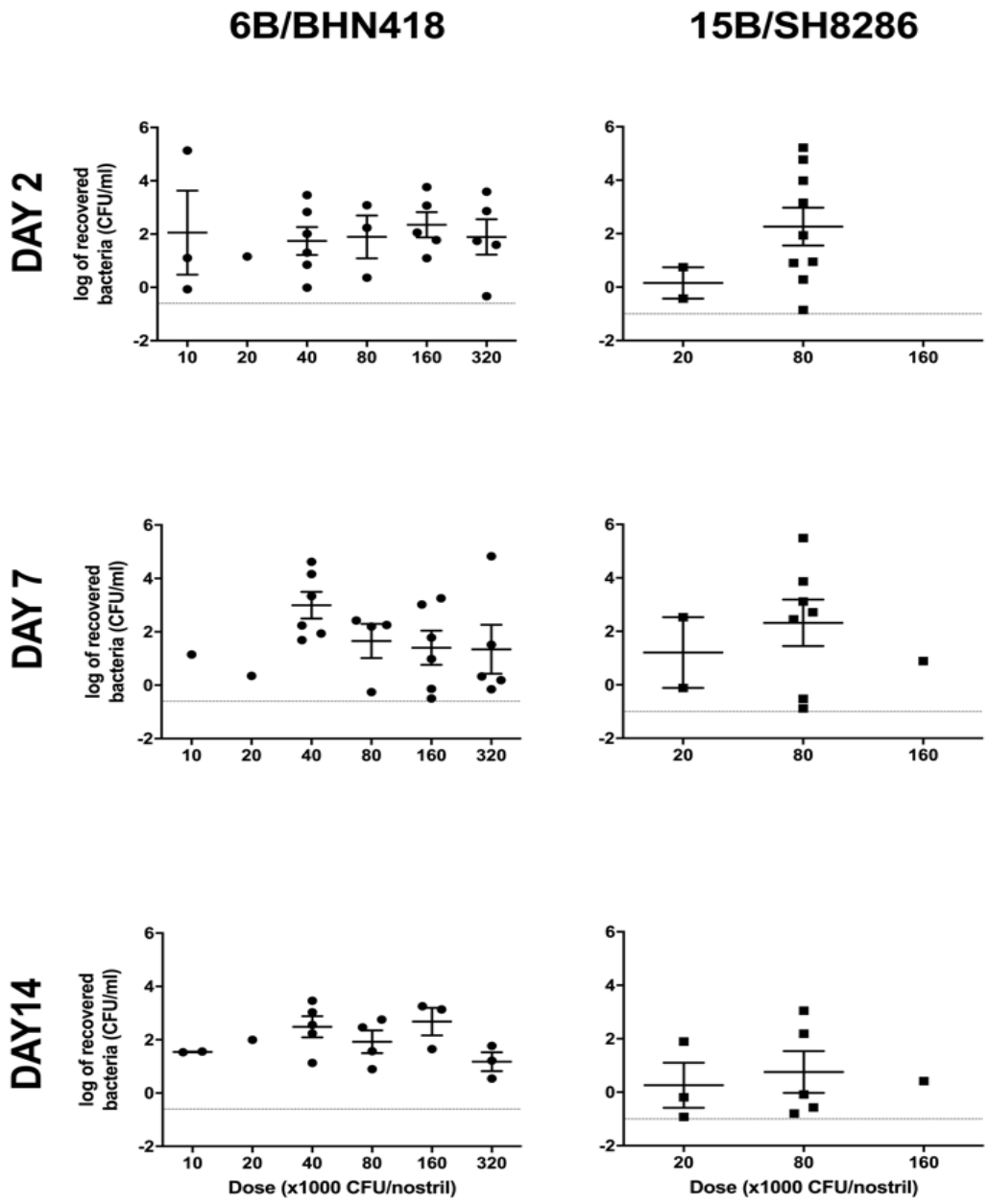


Figure 3-4: Experimental pneumococcal colonization densities are comparable between SPN6B/BHN418 and SPN15B/SH8286.

Nasal washes were taken at 2-, 7-, and 14-days post-challenge to determine pneumococcal density in volunteers colonized with either SPN6B/BHN418 and SPN15B/SH8286. Fresh nasal wash was serially diluted and pneumococcal colonies were quantified on Columbia blood agar. Density of those colonized, is reported as the log of colony forming units per ml of nasal wash (CFU/ml) returned. Bars represent the mean ± SEM. Volunteers were considered colonization positive if the challenge strain was grown from nasal wash on Columbia blood agar at any time point following challenge.

3.3.2 Pneumococcal characteristics and colonization efficiency

3.3.2.1 Resistance to non-opsonic neutrophil mediated killing

Neutrophils are an important part of the innate host-defence response. They can kill harmful microbes via phagocytosis and subsequent digestion, degranulation or via extracellular traps (NETs). In this chapter I will focus on the role of non-opsonic neutrophil mediated killing of the pneumococcus (not dependent on antibodies or complement deposition on the bacterial surface) in the establishment of pneumococcal colonization.

3.3.2.1.1 Experimental approach

Early pneumococcal clearance profiles were established by determining the presence of the pneumococcal challenge strain in saliva during the first 48 hours after challenge with SPN6B/BHN418. In order to reduce the impact of sampling on colonization outcome volunteers were asked to self-collect non-invasive samples at home at certain intervals after challenge, store them in the cold and return them for analysis to the research facility at day 2 after challenge. To ensure sample integrity sample temperature as well as sample time point was recorded. A total of 63 volunteers participated (study number 14/NW/146, 18/NW/0481 and 15/NW/0931), of which two were excluded from analysis due to non-compliance with home sampling protocols.

Details of experimental design and sampling collection time points can be found in Figure 3-5. Next, the role of host neutrophil activity in the control of pneumococcal colonization was established. Pneumococcal clearance profiles were associated with

activity and level of neutrophils in the nasopharynx prior to and during the first 48 hours post challenge.

After establishing the role of neutrophil activity against the pneumococcus, *in vitro* pneumococcal resistance to non-opsonic neutrophil mediated killing was measured and associated with experimental pneumococcal colonization rates as assessed during human challenge (study number 11/NW/0592 and 15/NW/0931).

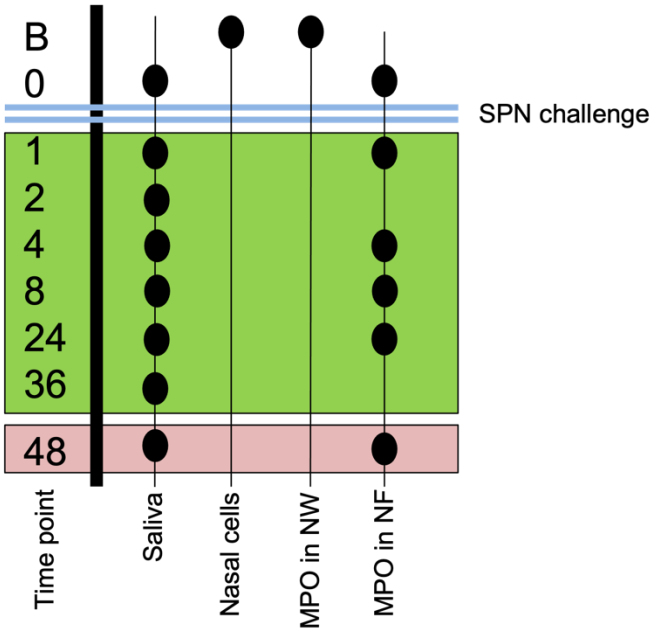


Figure 3-5: Schematic representation of sample collection design.

Nasal cells and nasal wash samples were collected at screening visit (Time point = B) before pneumococcal exposure. On the day of pneumococcal challenge with SPN6B/BHN418 saliva and nasal fluid (NF) samples were collected in clinic before exposure (Time point = 0 hours). Volunteers were asked to self-collect saliva at 1, 2, 4, 8, 24, 36, and 48 hours and NF samples at 4, 8, 24 and 48 hours post exposure. Nasal wash and nasal fluid samples were analysed for levels of myeloperoxidase (MPO). Saliva samples were analysed for the presence of pneumococcal DNA.

3.3.2.1.2 Identification of two distinct early pneumococcal clearance profiles

Pneumococcal clearance from the nasopharynx was assessed as the presence of pneumococcal DNA of the challenge strain (SPN6B/BHN418) in saliva in the first 48 hours after nasal challenge. From the 61 volunteers who were analysed, 40 volunteers were colonization negative, whereas 21 were colonization positive (as determined by pneumococcal detection in nasal wash culture at any time point following challenge).

At 1 hour, 24 hours, 36 hours and 48 hours post-challenge, pneumococcal detection in saliva was significantly different between those colonization positive and those colonization negative (Fisher's, 1 hour: $P = 0.0005$; 24 hours: $P = 0.0019$; 36 hours: $P = 0.0112$; 48 hours: $P < 0.0001$; Figure 3-6 A). At 1 hour post challenge, pneumococcus could be detected in saliva in 40% of colonization negative volunteers. In those colonized, pneumococcus could not be detected (0%) at this time point. After 24, 36 and 48 hours, pneumococcus was readily detectable in saliva of those colonized (24 hours: 38.1%; 36 hours: 42.8% 48 hours: 66.6%), but not in colonization negative volunteers (24h: 5%, 36h: 12.5%; 48h: 10%). This suggests that colonization is gradually established over the first 24 hours after challenge.

In colonization negative volunteers, two distinct profiles of pneumococcal removal could be identified, based on the presence or absence of pneumococcal DNA 1 hour after challenge (Figure 3-6 B). I will refer to these two groups as "saliva clearers" (presence of pneumococcal DNA in saliva at 1 hour post challenge) and "nasal clearers" (absence of pneumococcal DNA in saliva at 1 hour post challenge).

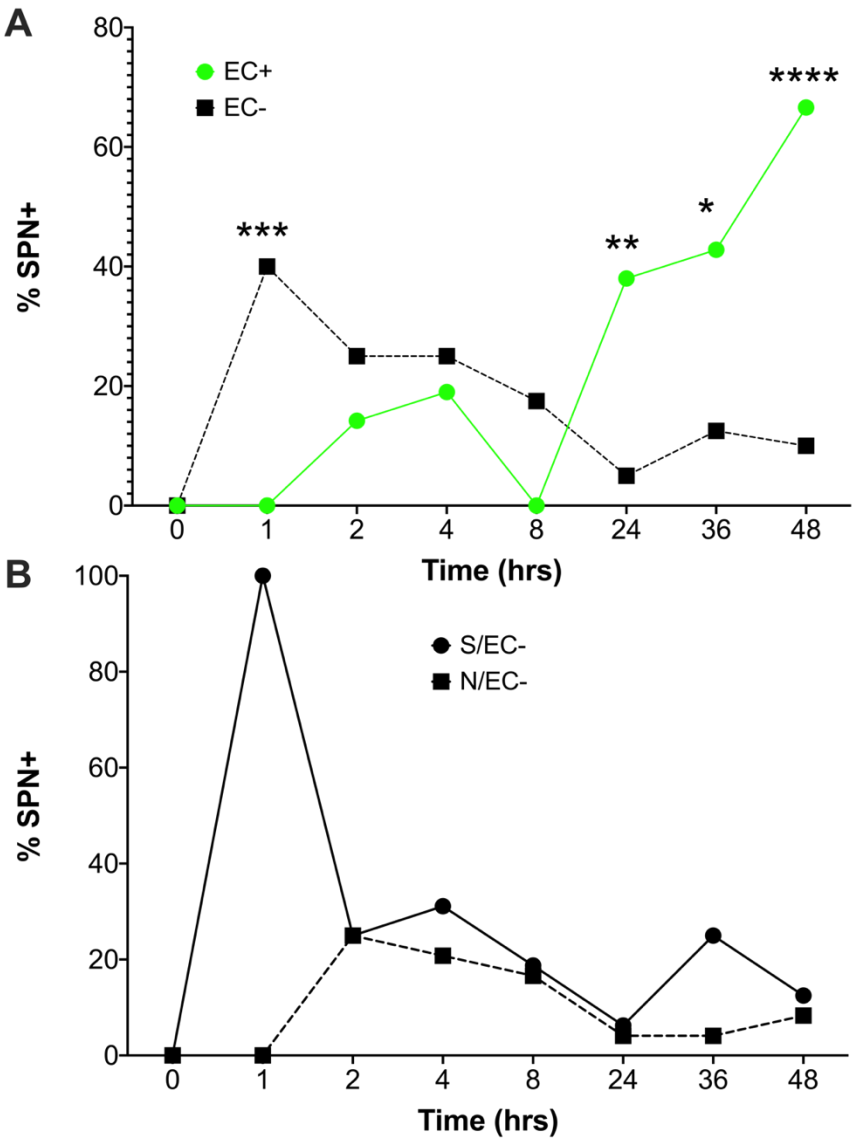


Figure 3-6: Kinetics of pneumococcal clearance in saliva.

Percentage of volunteers that are positive for pneumococcal detection in saliva. Saliva samples were collected before (T0) and 1, 2, 4, 8, 12, 24, 36, and 48 hours after pneumococcal challenge. (A) All volunteers stratified into those colonized (EC+) and those not colonized (EC-). (B) Volunteers not colonized stratified based on presence (S/EC-) or absence (N/EC-) of pneumococcal DNA in saliva at 1 hour post challenge. Detection of pneumococcal DNA in saliva was determined by *lytA*/*cpsA* (specific for SPN6AB) qPCR. Volunteers were young healthy non-smoking individuals, who were challenged with SPN6B/BHN418. Colonization status as determined by the growth of the pneumococcal challenge strain on Columbia blood agar from nasal wash at any time point following challenge. Significance levels determined using Fisher's exact test. *$P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

3.3.2.1.3 Early neutrophil activity

The abundance of immune cells in the nasopharynx was measured and compared between early clearance profiles (Figure 3-7 A). Levels of activated neutrophils were identified as $CD66b^{Hi}$ granulocytes [358].

Difference between clearance profiles were apparent for the absolute number of granulocytes (Kruskal-Wallis, P = 0.04) as well as $CD66b^{Hi}$ granulocytes (Kruskal-Wallis, P = 0.05). Granulocytes and $CD66b^{Hi}$ granulocytes were increased significantly in nasal clearers compared to colonization positive volunteers (Dunn's, P = 0.01 and P = 0.02, respectively). There was no significant difference in levels of any other measured cell type (B cells, T cells, epithelial cells and monocytes) between the three groups indicating that $CD66b^{Hi}$ granulocytes are involved in the early removal of the pneumococcus from the nasopharynx.

To assess the relevance of neutrophil activity during early pneumococcal removal myeloperoxidase (MPO) levels in nasal fluid were measured for the first 48 hours after challenge and results compared between clearance profiles (Figure 3-7 B). Nasal clearers showed the highest levels of MPO at challenge compared to saliva clearers (Mann-Whitney, P=0.014) and colonization positive volunteers (Mann-Whitney, P=0.055). In nasal clearers and colonization positive volunteers MPO levels remained stable over time. In saliva clearers, levels of MPO increased after challenge and reached a significant peak at 24 hours (Dunn's, P=0.01), indicating an induction of early neutrophil response following challenge.

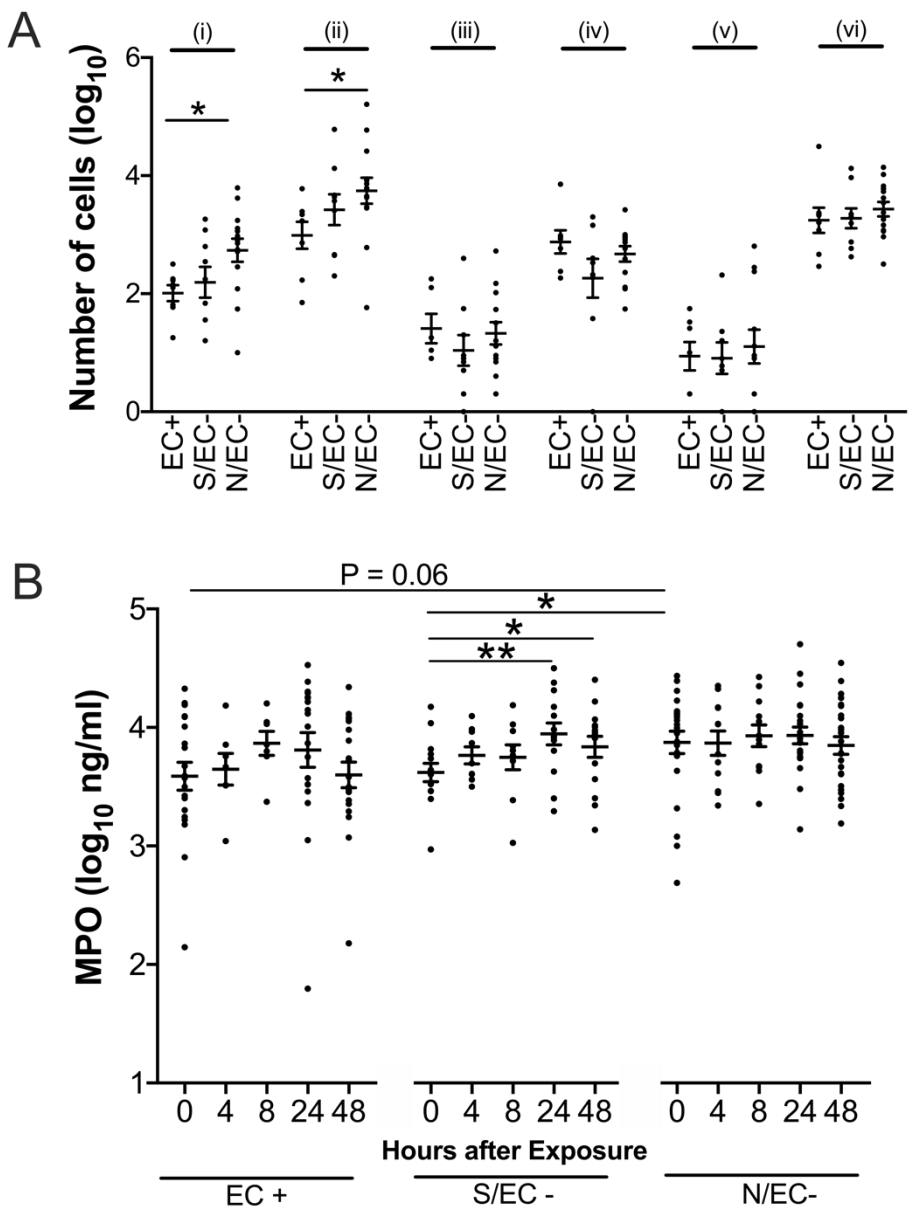


Figure 3-7: Association of neutrophil activity with early pneumococcal profiles.

(A) Abundance of (i) CD66b^{Hi} granulocytes (ii) granulocytes (iii) B-cells (iv) T-cells (v) monocytes and (vi) epithelial cells in the nasopharyngeal niche prior to pneumococcal challenge. (B) Kinetics of myeloperoxidase (MPO) levels in nasal fluid after pneumococcal challenge. MPO was assessed before (T0) and up to 48 hours after pneumococcal challenge. All analysed samples were obtained from young healthy non-smoking individuals. Colonization status as determined by growth of pneumococcal challenge strain on Columbia blood agar from nasal wash at any time point following challenge (EC- = colonization negative, EC+ = colonization positive). Clearance profile as determined by detection of pneumococcal DNA in saliva (S/EC- = saliva clearers, defined by presence of pneumococcal DNA in saliva at 1 hour after challenge, N/EC - = nasal clearers, defined by the absence of pneumococcal DNA in saliva at 1 hour after challenge). Bars represent the mean ± SEM. Significance determined using Mann Whitney t-test (between groups) and Kruskal Wallis with uncorrected Dunn's post-hoc test (within groups) *P < 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

In agreement with MPO levels in nasal fluid, nasal clearers showed a trend for higher levels of MPO at baseline in nasal wash compared to colonization positive volunteers (Dunn's, P = 0.05, Figure 3-8 A). MPO levels further correlated significantly with the number of granulocytes (Figure 3-8 B) and CD66bHi granulocytes at baseline (Figure 3-8 C). In summary, the data highlight the active role of neutrophils during early pneumococcal colonization control.

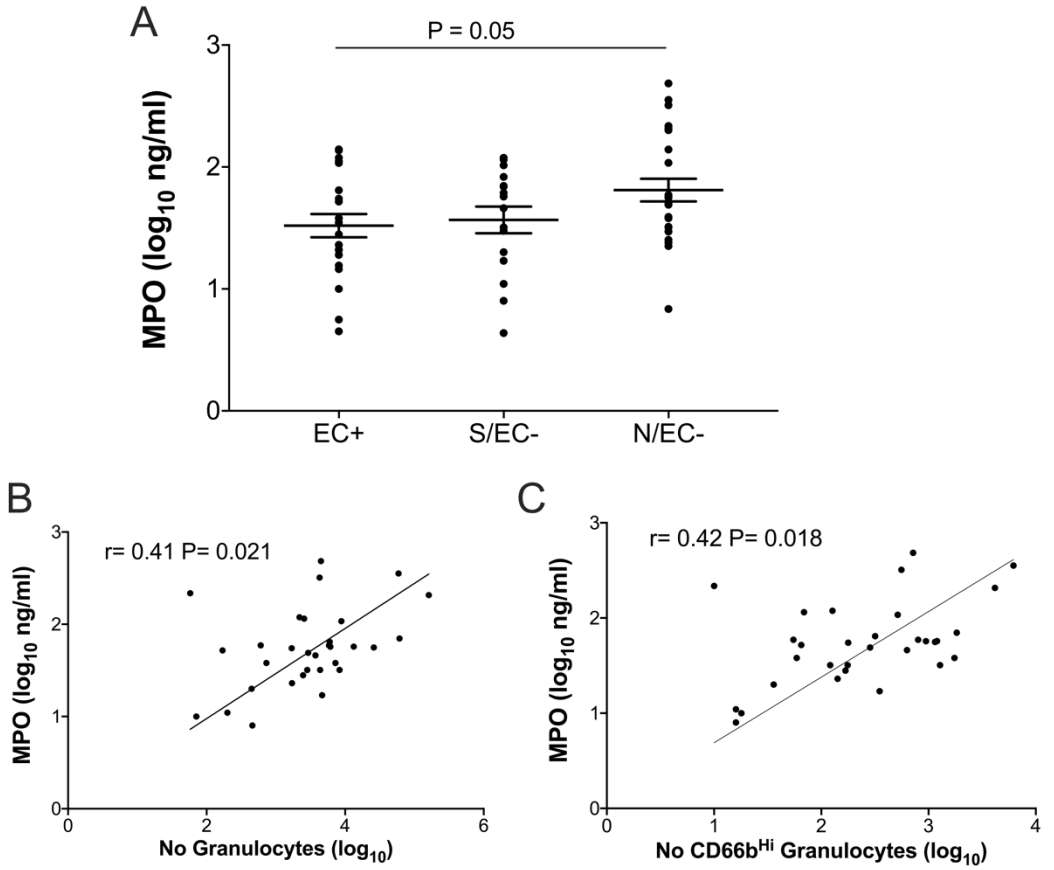


Figure 3-8: MPO levels in nasal wash correlate with number of neutrophils in the nose.
Correlation of (B) the number of granulocytes and (C) CD66b^{Hi} granulocytes in the nasopharyngeal niche with (A) levels of MPO in nasal wash prior to pneumococcal challenge. All analysed samples were obtained from young healthy non-smoking individuals. Colonization status as determined by growth of pneumococcal challenge strain on Columbia blood agar from nasal wash samples at any time point following challenge (EC- = colonization negative, EC+ = colonization positive). Clearance profile as determined by detection of pneumococcal DNA in saliva (S/EC- = saliva clearers, defined by the presence of pneumococcal DNA in saliva at 1 hour after challenge, N/EC- = nasal clearers, defined by the absence of pneumococcal DNA in saliva at 1 hour after challenge). Bars represent the mean ± SEM. Significance determined using Kruskal Wallis with uncorrected Dunn's post-hoc test and Spearman correlation. Significance level $P < 0.05$

3.3.2.1.4 Superior experimental colonization rate of pneumococcal strain SPN6B/BHN418 is not associated with resistance to non-opsonic neutrophil mediated killing

Investigating the superior colonization rates of SPN6B/BHN418, I evaluated the ability of pneumococcal strains used for human challenge to survive non-opsonic neutrophil mediated killing (NMK). Notably, non-opsonic NMK was not dependent on media used for bacterial preparation (Figure 3-9 A).

Evaluation of non-opsonic NMK showed significant differences between the tested strains (ANOVA, P = 0.0003, Figure 3-9 B). In contrast to the human challenge data, I observed the lowest level of NMK with SPN6B/BHN418 (38.2% ± 4.8) and SPN15B/SH8286 (39.3% ± 6.4). Significantly higher levels of survival could be observed with SPN23F/P833 (59.3% ± 4.1, Fisher's LSD, P=0.0041) and SPN23F/P1121 (66.0 ± 4.9%, Fisher's LSD, P=0.0004) when compared to SPN6B/BHN418. The data demonstrate superior resistance to non-opsonic NMK for SPN23F strains.

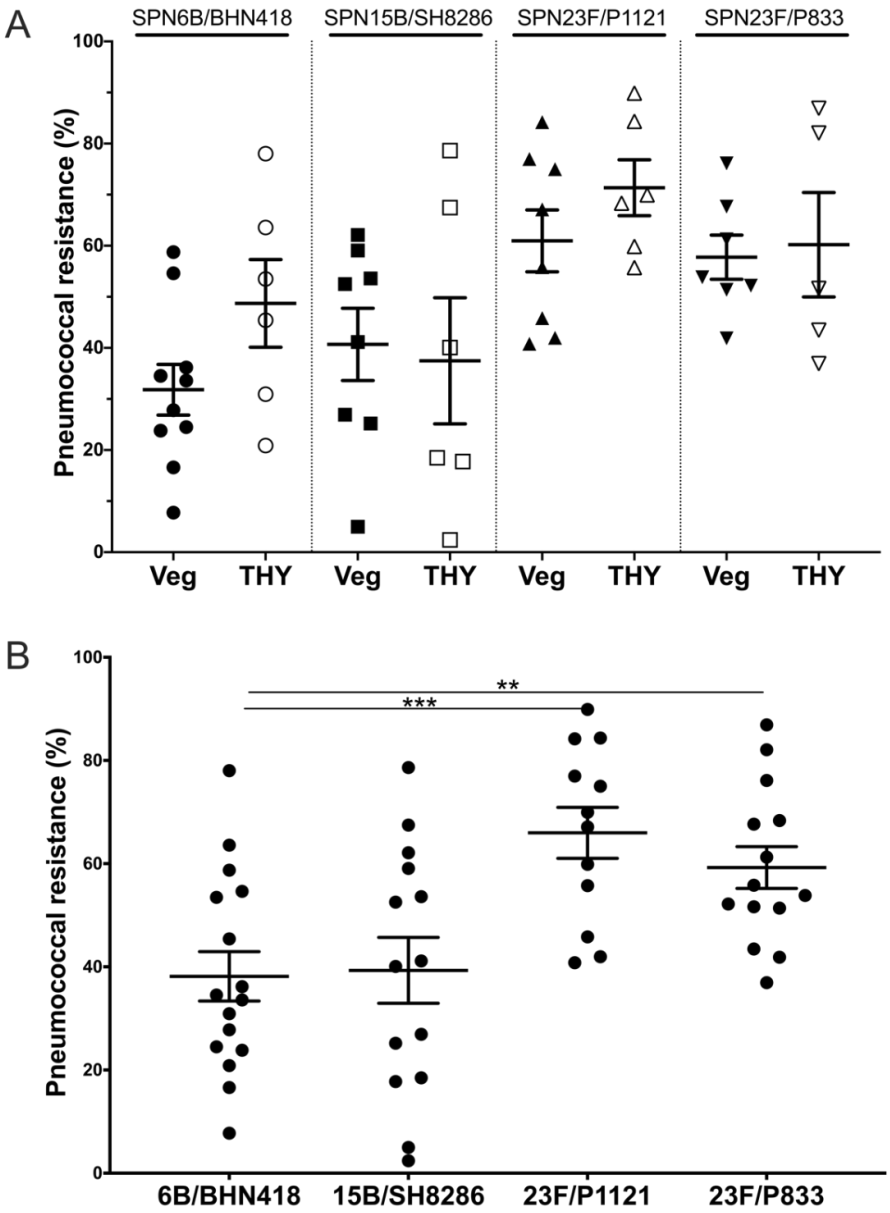


Figure 3-9: Superior experimental colonization rate of pneumococcal strain SPN6B/BHN418 upon human challenge is not associated with resistance to non-opsonic neutrophil mediated killing
(A) Influence of media type (Veg = Vegitone, THY = Todd Hewitt broth with 0.5% yeast extract) on non-opsonic neutrophil mediated killing (NMK) of pneumococcal strains SPN6B/BHN418 (circle), SPN15B/SH8286 (square), SPN23F/P1121 (triangle apex up) and SPN23F/P833 (triangle apex down). (B) NMK of pneumococcal strains used in human challenge. Bacteria were grown in either Vegitone or THY. Neutrophils were obtained from peripheral blood of young healthy volunteers. Bars represent the mean ± SEM. Significance determined using (A) student's t-test (B) one-way ANOVA with uncorrected Fisher's LSD post-hoc test. *P < 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

3.3.2.2 Pneumococcal adherence to nasal epithelial cells

3.3.2.2.1 Experimental approach

The association of pneumococcal adherence and experimental colonization rates using the EHPC model was studied. Due to the general lack of evidence of the occurrence of pneumococcal adherence during human colonization, visual evidence of *in vivo* adherence of pneumococcal strain SPN6B/BHN418 to the human nasopharyngeal epithelium was obtained (Figure 3-10, study number 14/NW/1460). After establishing the occurrence of pneumococcal adherence during colonization in humans, *in vitro* adherence of different pneumococcal strains was measured using undifferentiated primary nasal cells (study number 14/NW/1460, 17/NW/0029 and LSTM tissue bank) as well as nasopharyngeal cell line Detroit 562. Pneumococcal adherence levels were then associated with experimental pneumococcal colonization rates of the respective pneumococcal strain as assessed during human challenge (study number 11/NW/0592 and 15/NW/0931). Nasal cell and human challenge cohorts were matched for age distribution and health status of volunteers.

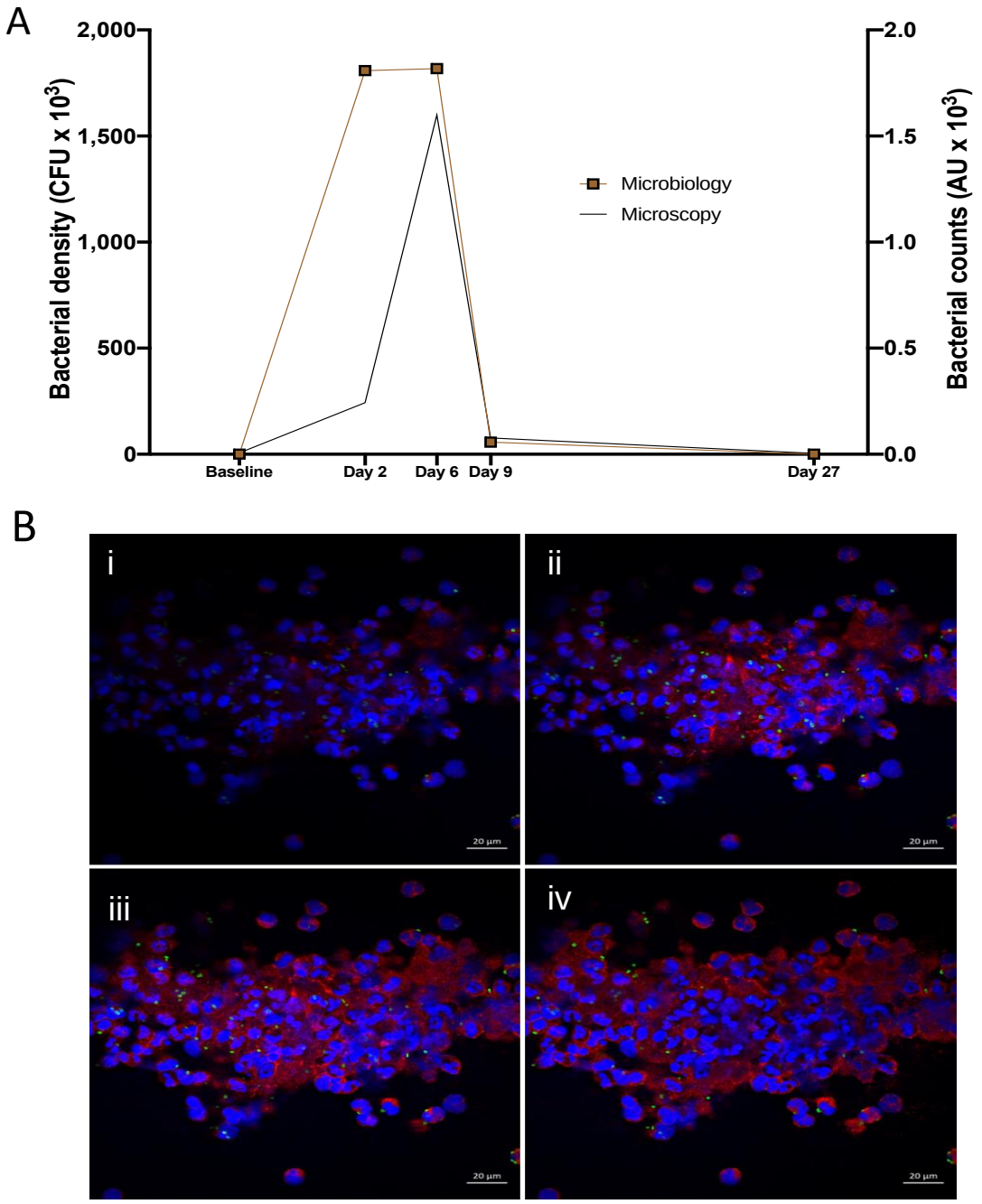


Figure 3-10: Pneumococcal adherence at the human nasal mucosa.

(A) Comparison of bacterial counts using detection by classical microbiology in nasal wash (left axes) and microscopy of nasal cells (right axes). The total bacterial sum across individuals is shown. (B) Cells were stained for surface carbohydrates using wheat germ agglutinin (WGA, red), the nuclei were stained using 4',6-diamidino-2-phenylindole (DAPI, blue) and the bacteria were marked with specific serotype antiserum (green). XY images of 1 µm slices through a layer of cells from bottom (stack i) to top (stack iv), with bacteria associated. (A/B) Volunteers were exposed to SPN6B/BHN418. Microscopic images were captured and analysed by Caroline M. Weight using Zeiss LSM 880 and Zeiss LSM Image Browser.

3.3.2.2.2 Superior experimental colonization rate of pneumococcal strain SPN6B/BHN418 is not associated with pneumococcal adherence

The ability of the pneumococcus to adhere (epithelial adherence) to undifferentiated primary nasal cells (NEC) and a nasopharyngeal cell line (Detroit 562) was assessed. Notably, adherence was not dependent on media used for bacterial preparation (Figure 3-11 A) but was dependent on cell type used (Figure 3-11 B).

Evaluation of epithelial adherence with both cell types showed significant differences between the strains (ANOVA, $P<0.0001$, Figure 3-11 C). The highest level of epithelial adherence (mean ± SEM for NEC and Detroit 562, respectively) was observed with SPN15B/SH8286 ($1.7 \times 10^6 \pm 3.7 \times 10^5$ CFU and $3.9 \times 10^5 \pm 4.9 \times 10^4$ CFU), while SPN6B/BHN418 ($2.0 \times 10^5 \pm 2.7 \times 10^4$ CFU and $1.6 \times 10^5 \pm 4.6 \times 10^4$ CFU) showed significantly lower levels of adherence ($P < 0.0001$, respectively). Epithelial adherence of SPN23F/P833 ($3.6 \times 10^4 \pm 1.5 \times 10^4$ CFU and $3.5 \times 10^4 \pm 4.6 \times 10^3$ CFU) and SPN23F/P1121 ($3.1 \times 10^4 \pm 1.3 \times 10^4$ CFU and $3.1 \times 10^4 \pm 6.6 \times 10^3$ CFU) were significantly lower when compared to SPN6B/BHN418 ($P<0.0001$ and $P=0.0003$ for SPN23F/P833, $P = 0.0002$ and $P<0.0001$ for SPN23F/P1121). The data demonstrate superiority of pneumococcal strain SPN15B/SH8286 in the ability to adhere to nasopharyngeal cells.

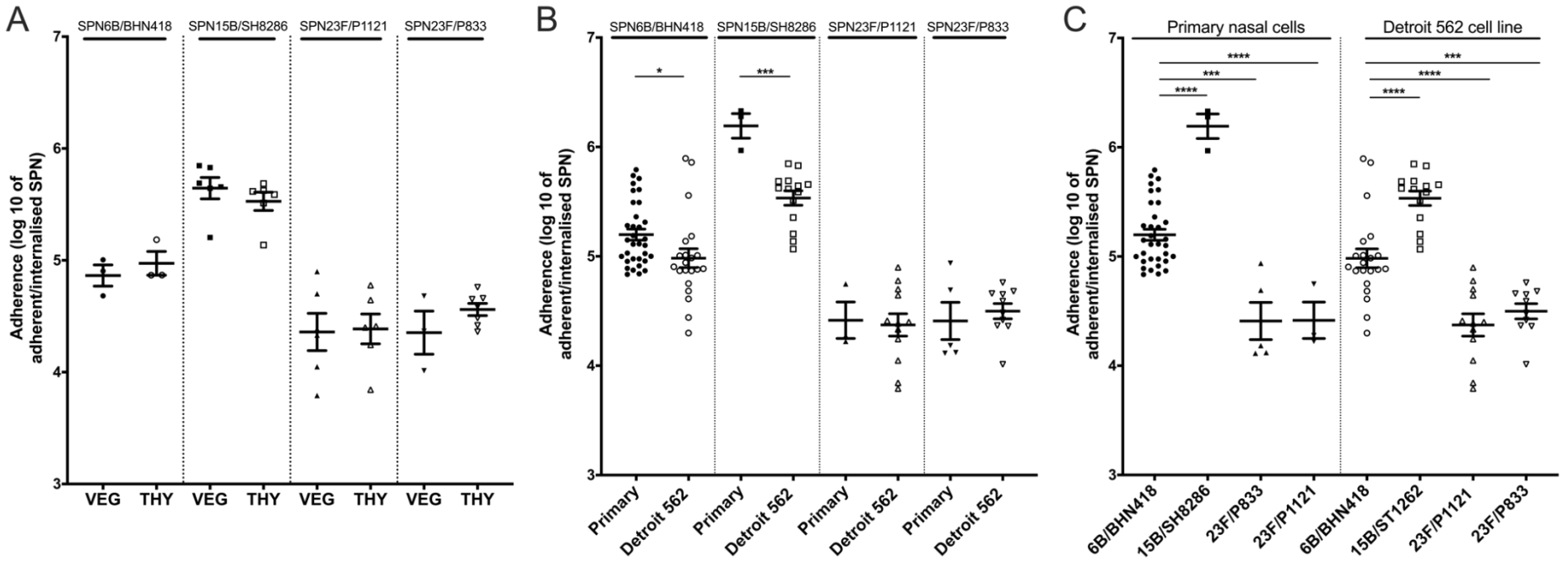


Figure 3-11: Superior experimental colonization rate of pneumococcal strain SPN6B/BHN418 upon human challenge is not associated with pneumococcal adherence. Pneumococcal strains SPN6B/BHN418 (circle), SPN15B/SH8286 (square), SPN23F/P1121 (triangle apex up) and SPN23F/P833 (triangle apex down) were evaluated for epithelial adherence. Cells were seeded at a concentration of $6x10^5$ (Detroit 562) or $1x10^5$ (NECs) and grown until confluent or for a maximum of 7 days, after which they were co-cultured with $1x10^6$ CFU of pneumococcus for 3 hours. Primary nasal cells were either collected prior to or one month after pneumococcal challenge. All cells were cultured in the presence of penicillin/neomycin/streptomycin prior to experiment. (A) Influence of media type (Veg = Vegitone, THY = Todd Hewitt broth with 0.5% yeast extract) used for pneumococcal stock preparation on pneumococcal adherence to Detroit 562 cells. (B) Influence of cell type on adherence levels of different pneumococcal strains. (A/B) Significance determined using student's t-test. (C) Adherence of pneumococcal strains to primary nasal cells and Detroit 562 cells. Significance was determined using one-way ANOVA with uncorrected Fisher's LSD post-hoc test. (B/C) Bacteria were grown in either Vegitone or THY. (All) Bars represent the mean ± SEM. *$P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

3.3.2.3 Pneumococcal chain length

3.3.2.3.1 Experimental approach

The pneumococcus exists in chains of different sizes. Here, I measured the ratio of optical density (OD) to colony forming units (CFU/ml) in liquid broth first described as a marker of pneumococcal chain length by Basset et al., [354] whereby small OD/CFU ratios associate with small pneumococcal chain length. I further investigated whether chain length (defined as OD/CFU ratio) is isolate and/or serotype specific using pneumococcal strains obtained from colonized individuals (gifted by David Clearly, University of Southampton). Second, chain length of EHPC strains grown for human challenge was associated with experimental colonization rates as assessed during human challenge (study number 11/NW/0592 and 15/NW/0931).

3.3.2.3.2 Pneumococcal chain length is serotype and isolate specific

Chain length of selected pneumococcal strains obtained from colonized individuals Table 3-5) was investigated. All strains were distinguishable by capsular serotype, MLST-type (ST-number) and by isolate (I-number).

Chain length was significantly different between serotypes (ANOVA, $P< 0.0001$, Figure 3-12 A). Longest chains were indicated for SPN24F (6.03 ± 1.08), followed in descending order by SPN23A (3.19 ± 0.31), SPN15B (2.35 ± 0.21), SPN15A (2.16 ± 0.35) and SPN22F (2.02 ± 0.18). Chain length data was further available for three isolates of SPN15B (ST1262). Statistical comparison revealed that chain length was significantly different between the isolates (ANOVA, $P < 0.0001$, Figure 3-12 B). The result implicates that chain length is determined by more than capsular type.

Table 3-5: Cohort of pneumococcal strains

Isolate number	serotype	ST-number	PspA
8175	15A	58	fam2
8082	15B	1262	fam2
8142	15B	1262	fam2
8286	15B	1262	fam2
8276	22F	433	fam1
8211	22F	433	fam1
8314	23A	438	fam2
8224	23A	438	fam2
8229	24F	177*	fam2

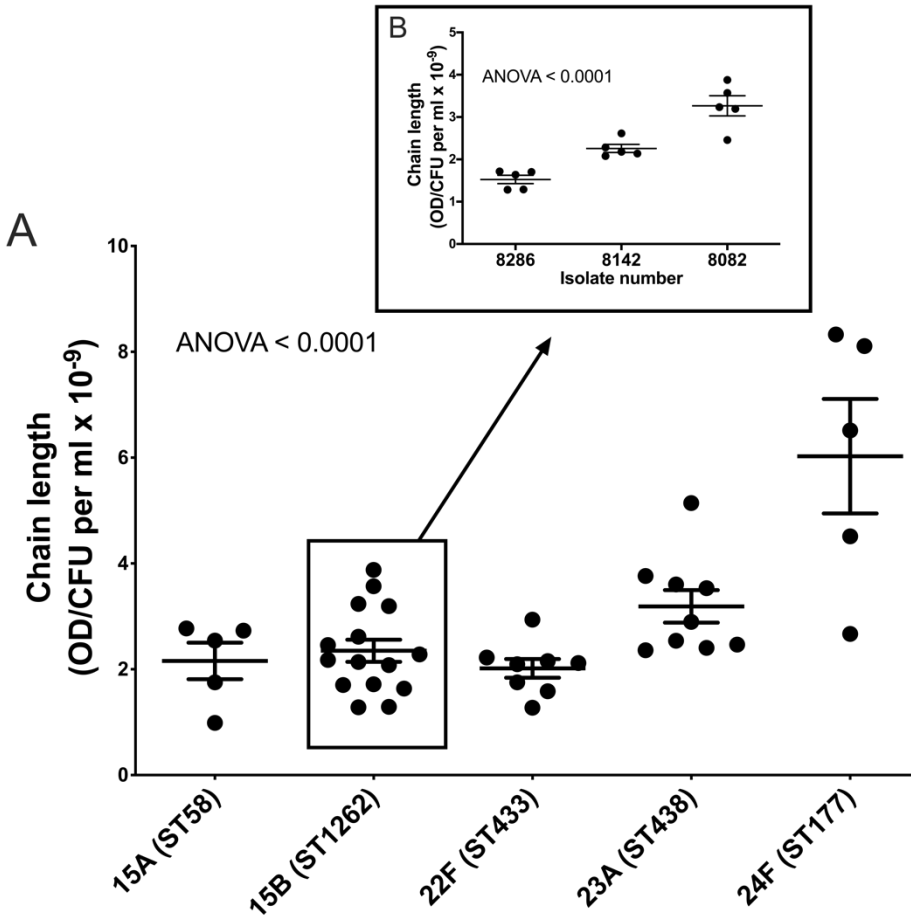


Figure 3-12: Chain length is serotype and isolate specific.

(A) Chain length of SPN15A (ST58), SPN15B (ST1262), SPN22F (ST433), SPN23A (ST438) and SPN24F (ST177) was analysed in Vegitone. (B) Chain length of three different isolates of pneumococcal strain SPN15B (ST1262). Chain length is expressed as ratio of optical density (OD) to colony forming units (CFU/ml). Bars represent the mean ± SEM of three or more independent experiments. Significance using one-way ANOVA. $P < 0.05$

3.3.2.3.3 Superior experimental colonization rate of pneumococcal strain SPN6B/BHN418 upon human challenge associates with pneumococcal chain length

Investigating the superior colonization rate of pneumococcal strain SPN6B/BHN418 I evaluated SPN6B/BHN418, SPN15B/SH8286, SPN23F/P1121 and SPN23F/P833 for pneumococcal chain length defined as the ratio of OD/CFU under the same conditions as used for pneumococcal challenge (Figure 3-13).

Evaluation of average chain length in Vegitone showed significant differences between the tested pneumococcal strains (ANOVA, $P<0.0001$). The highest ratio of OD/CFU (mean ± SEM) and therefore larger chains were observed with SPN6B/BHN418 ($2.28 \pm 0.14 \times 10^{-9}$). Significantly smaller chains were observed for SPN15B/SH8286 ($1.57 \pm 0.08 \times 10^{-9}$, $P=0.0003$). Average chain length of SPN23F/P833 ($0.96 \pm 0.12 \times 10^{-9}$) and SPN23F/P1121 ($0.49 \pm 0.04 \times 10^{-9}$) were significantly smaller when compared to SPN6B/BHN418 ($P<0.0001$, respectively).
The data demonstrate superior chain length of SPN6B/BHN418 grown in Vegitone over other strains tested.

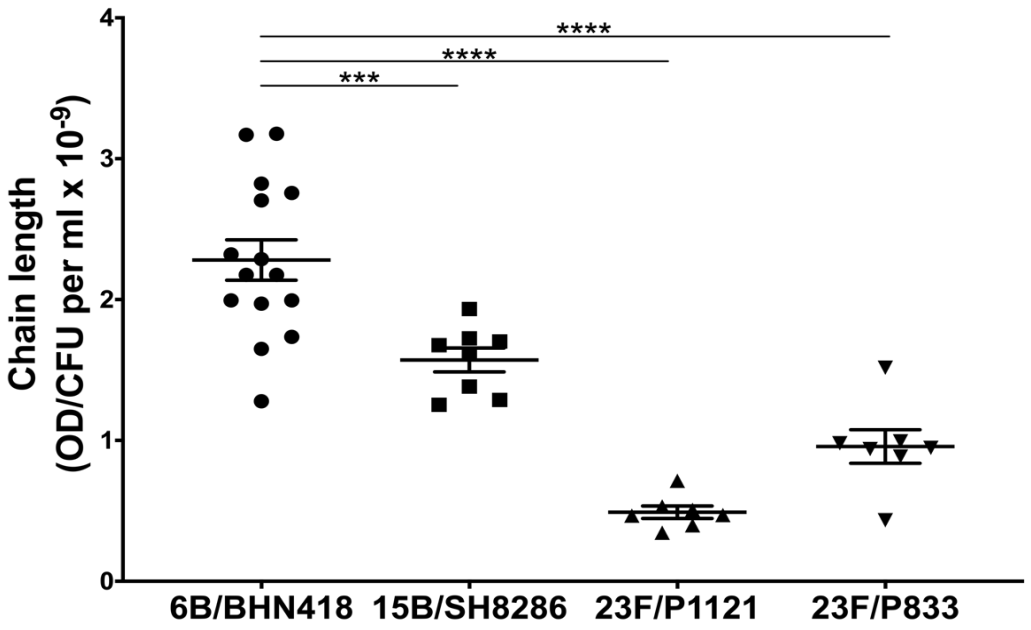


Figure 3-13: Pneumococcal strain SPN6B/BHN418 demonstrates superior chain length.

Chain length of pneumococcal strains SPN6B/BHN418 (circle), SPN15B/SH8286 (square), SPN23F/P1121 (triangle apex up) and SPN23F/P833 (triangle apex down) was analysed in Vegitone. Chain length was defined as the ratio of optical density (OD) to colony forming units (CFU/ml). Bars represent the mean ± SEM of three or more independent experiments. Significance was determined using one-way ANOVA with uncorrected Fisher's LSD post-hoc test. *P < 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

3.3.2.3.4 Proof of concept: Assessing pneumococcal chain length to estimate experimental pneumococcal colonization rates of serotype 3 strains prior to human challenge

3.3.2.3.4.1 Experimental approach

Tasked with the establishment of pneumococcal serotype 3 in the EHPC model, I assessed pneumococcal chain length defined as the ratio of OD/CFU to estimate experimental colonization rates prior to human challenge. A total of 19 SPN3 strains were selected from two pneumococcal strain collections (Pfizer and EHPC collection) and analysed for average chain length in Vegitone. Three of those showed poor growth behaviour in this type of media and were excluded from the analysis. To increase accuracy of pneumococcal CFU calculation of SPN3 strains, two methods of pneumococcal plating were used with colony forming units averaged over both methods.

Strains were selected for human challenge based on chain length as well as other criteria including clade, phylogeny and genomic analysis. Selected strains were used to expose human volunteers participating in an SPN3 dose ranging study (study number 19/NW/0238). Experimental colonization rates were determined by bacterial growth from nasal samples (nasal wash and where applicable nasal swab).

3.3.2.3.4.2 Average chain length of pneumococcal serotype 3 strains

Average chain length (mean ± SEM, OD/CFU x 10^{-9}) of pneumococcal serotype 3 strains was analysed (Figure 3-14). Strain SPN3/402-155 (ST180, clade 1a) was the only strain showing an average chain length (3.44 ± 0.53) above that of SPN6B/BHN418 (2.28 ± 0.14). Further strain SPN3/PFESP200 (ST180, clade 1b) and SPN3/PFESP231 (ST458, no clade) showed chain length similar to that of SPN6B/BHN418 (PFESP 200: 2.46 ± 0.24 and PFESP231: 2.27 ± 0.16). All other strains tested showed smaller average chain length when compared to SPN6B/BHN418.

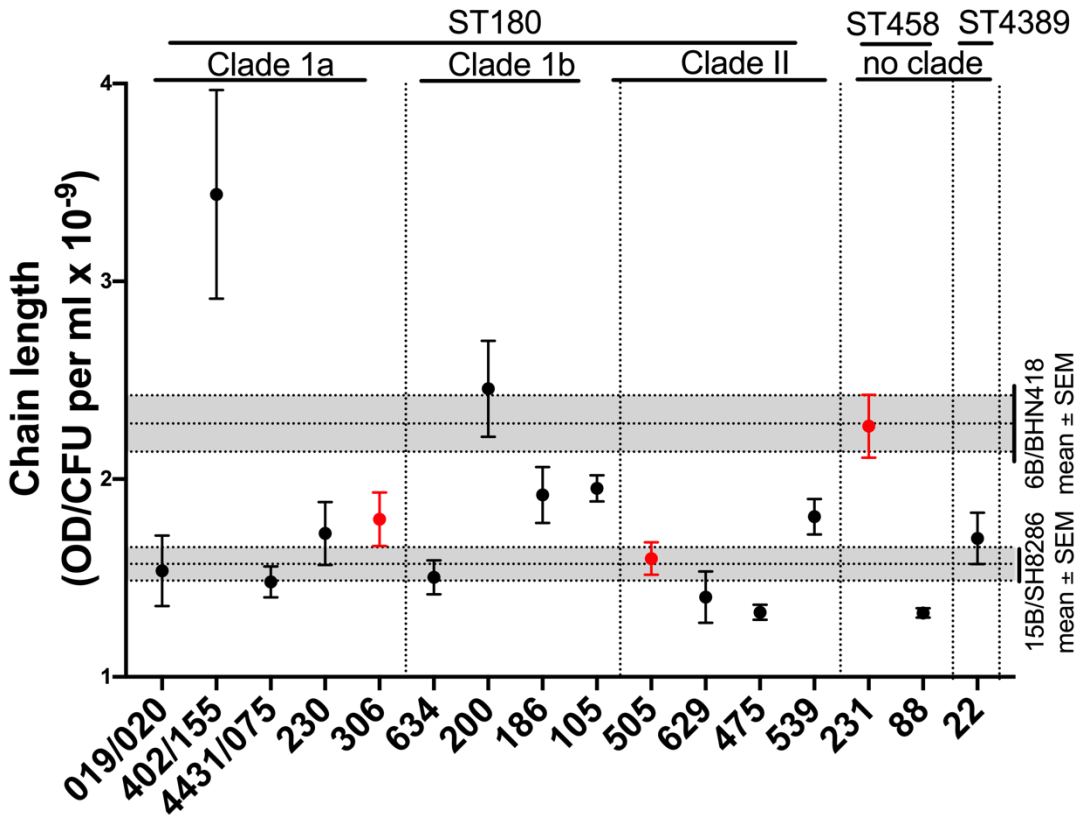


Figure 3-14: Chain length of pneumococcal serotype 3 strains.

Pneumococcal strains of serotype 3 were evaluated for average chain length in Vegitone prior to use in human challenge studies. Chain length was defined as the ratio of optical density (OD) to colony forming units (CFU/ml). Red data points mark those strains that were subsequently chosen for human challenge. Bars represent the mean ± SEM of ≥ 3 independent experiments.

3.3.2.3.4.3 Colonization rates of pneumococcal serotype 3 strains in the EHPC model

Three pneumococcal serotype 3 strains were chosen for human challenge using a dose ranging design: SPN3/PFESP231 (n = 20), SPN3/PFESP306 (n = 36) and SPN3/PFESP505 (n = 40) with doses ranging from 10,000 CFU/ nostril to 160,000 CFU/nostril. Average colonization rates (percentage of all challenged volunteers who were positive for growth of the challenge strain on Columbia blood agar from nasal samples at any time point post-challenge) and maximum colonization rates (highest percentage of challenged volunteers who were positive for growth of the challenge strain on Columbia blood agar from nasal samples at any time point post-challenge with any given dose) of SPN3 strains used for human challenge were determined.

The average colonization rates over all doses for each strain were: 45% (9/20, 95% CI: 23 – 66%) for SPN3/PFESP231, 41% (15/36, 95% CI: 24 – 57%) for SPN3/PFESP505 and 35% (14/40, 95% CI: 20-49%) for SPN3/PFESP306 (Figure 3-15 A). Interestingly, when analysing the dose response relationship of all three strains maximum colonization rates did not follow the same pattern as average colonization rates (Figure 3-15 B). SPN3/PFESP505 achieved the highest colonization rates with 70% (7/10, 95% CI: 41 – 98%, Dose: 160,000 CFU/nostril). Maximum colonization rates of SPN3/PFESP231 were lower with 60% (6/10, 95% CI: 29 - 90%, Dose: 20,000 CFU/nostril) followed by SPN3/PFESP306 with 50% (5/10, 95% CI: 19 - 80%, Dose: 160,000 CFU/nostril).

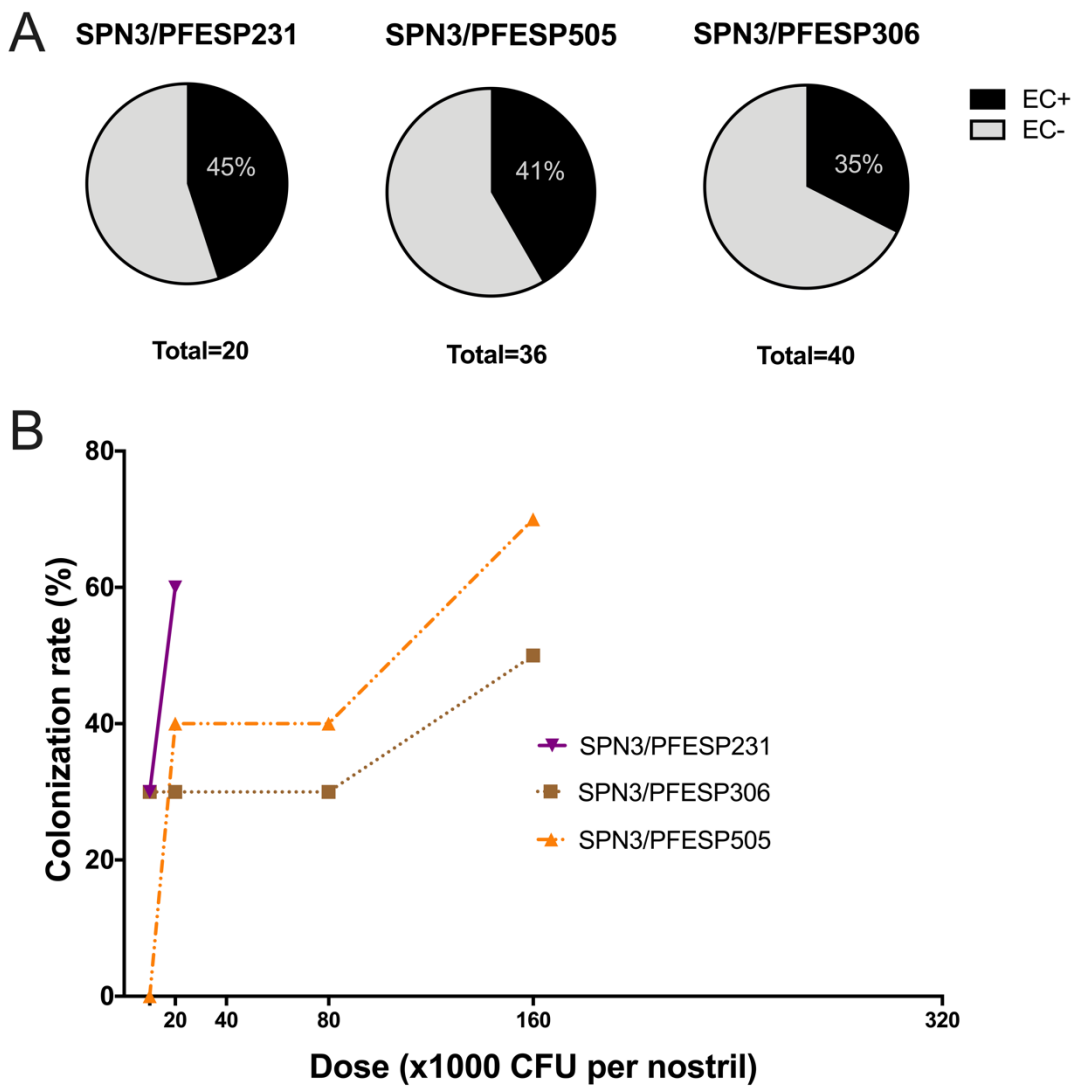


Figure 3-15: Colonization rates of pneumococcal serotype 3 strains in the EHPC model.

Young healthy non-smoking volunteers were intranasally challenged with one of three pneumococcal strains of serotype 3. (A) Average colonization rates independent of dose. (B) Dose-response relationship of pneumococcal strains SPN3/PFESP231 (triangle apex down), SPN3/PFESP505 (triangle apex up) and SPN3/PFESP306 (square). Each point represents the percentage of people colonized with the challenge strain. Colonization rates are defined as the percentage of volunteers who were positive for pneumococcal colonization. Volunteers were considered colonization positive if the challenge strain was grown from nasal samples on Columbia blood agar at any time point following challenge.

3.3.2.3.4.4 The predictive value of average pneumococcal chain length

I investigated whether average chain length (defined as the ratio of OD/CFU) of the pneumococcal serotype 3 strains used in the EHPC model were associated with their achieved experimental colonization rates (Figure 3-16 A).

Chain length, average colonization rates (averaged over all given doses) and maximum colonization rates (highest rate achieved with any given dose) of SPN3/PFESP231 were equal to that of SPN6B/BHN418. SPN3/PFESP231 further showed superior chain length and the highest average colonization rates amongst the tested SPN3 strains.

Notably, there was a strong correlation of chain length with average colonization rates achieved by EHPC strains ($P = 0.0071$, $R = 0.9190$, Figure 3-16 B). Correlation of chain length and maximum colonization rates was not significant (Figure 3-16 C), largely attributed to the success of SPN3/PFESP505 despite its small chain length value.

The data suggest, that while assessment of chain length relative to SPN6B/BHN418 may be used as one factor to estimate experimental colonization rates, other factors may need to be taken into consideration to achieve a more accurate prediction outcome.

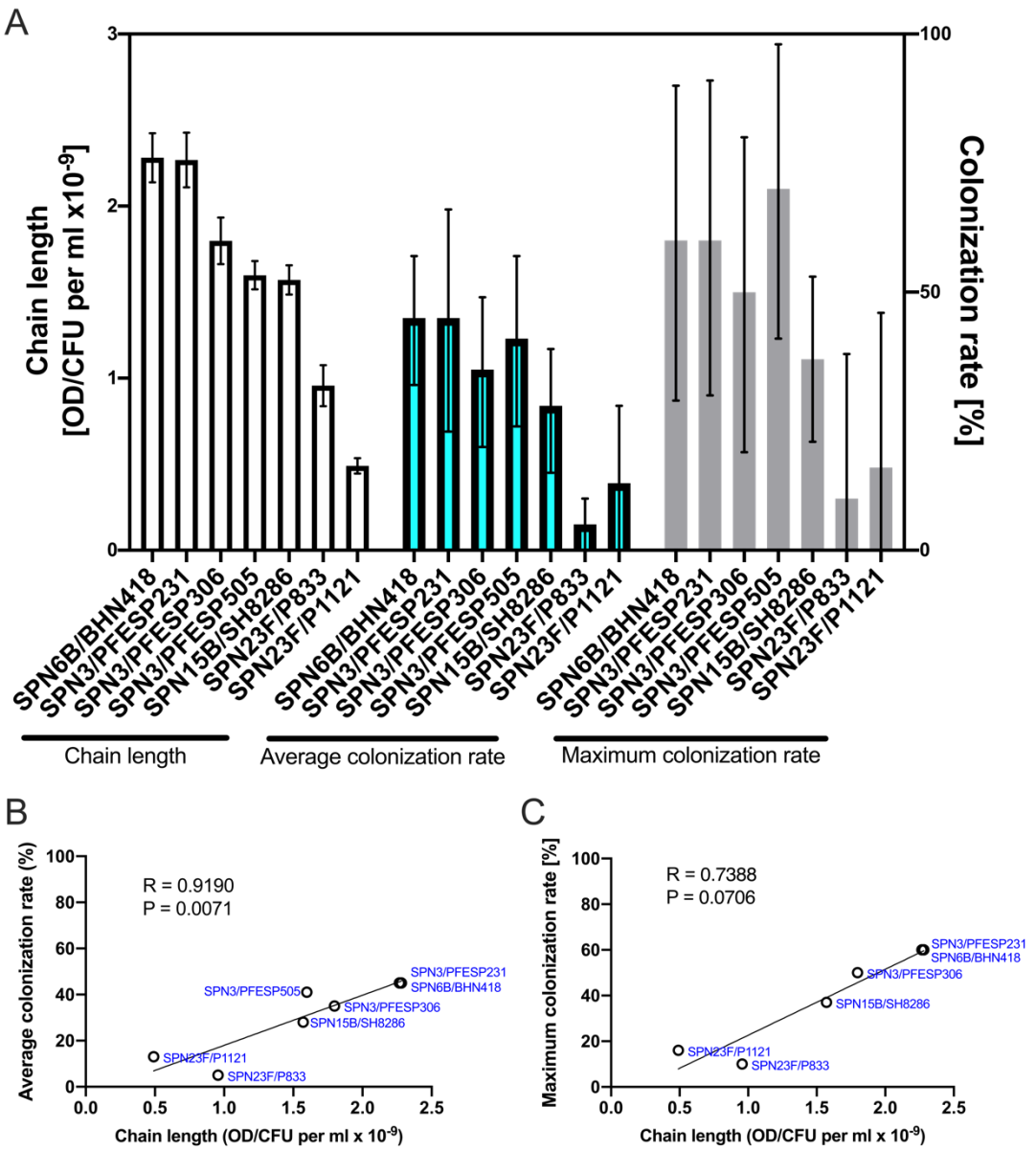


Figure 3-16: The predictive value of pneumococcal chain length

(A) Average experimental colonization rates (green), maximum experimental colonization rates (grey) and average chain length (white) of pneumococcal strains used for human challenge studies involving young healthy non-smoking volunteers. Error bars for experimental colonization rates represent the 95% confidence interval. Chain length is defined as the ratio of optical density (OD) to colony forming units (CFU/ml) and represented as mean ± SEM. (B/C) Correlation of average chain length of indicated pneumococcal strain with achieved (B) average and (C) maximum experimental colonization rates. Colonization rates are defined as the percentage of volunteers who were positive for pneumococcal colonization. Volunteers were considered colonization positive if the challenge strain was grown from nasal samples on Columbia blood agar at any time point following challenge. Average colonization rates = colonization rate averaged over all given doses. Maximum colonization rates = highest rate of colonization achieved with any given dose.

3.4 Discussion

Using the EHPC model I have shown that colonization rates varied greatly depending on the strain used for pneumococcal challenge. Amongst four pneumococcal strains (SPN15B/SH8286, SPN6B/BHN418, SPN23F/P1121 and SPN23F/P833) tested between 2011-2017, SPN6B/BHN418 was the most efficient colonizer.

Detection of colonization is traditionally based on classical microbiology. Non-culture-based methods however have been recommended to complement microbiological detection [52]. In this study, molecular detection of pneumococcal genes (*lytA*/*cpsA* or *lytA*/*piaB*) in nasal wash did not significantly increase overall detection of colonization and had no effect on the pattern of colonization observed. Previous reports of colonization densities amongst different pneumococcal serotypes have been controversial [359, 360]. Comparison of pneumococcal duration during natural colonization events also presents a challenge as the exact onset of colonization is difficult to determine. Here, I compared colonization densities and duration (determined by classical microbiology) of pneumococcal strains SPN6B/BHN418 and SPN15B/SH8286 upon experimental human pneumococcal challenge over a 14-day period. The data showed no significant differences of colonization density and duration between the tested pneumococcal strains.

This is the first study to compare colonization efficiency of multiple serotypes in experimental human pneumococcal colonization. Pneumococcal growth is susceptible to environmental variability. Differences in growth behaviour could influence pneumococcal virulence, colonization and transmissibility [361, 362]. Notably, even under fixed culture conditions experimental variations in pneumococcal growth may occur. Such variability may be caused by differences in day-to-day culture handling,

batch and age of media or bacterial fitness. Interestingly, pneumococcal susceptibility to these factors seems to be particular notable in Vegitone, perhaps due its ingredients being plant rather than animal derived [363]. Nevertheless, methods of EHPC stock preparation as well as other critical EHPC processes such as challenge and detection of colonization have been standardised over a decade. Studies carried out over the last 10 years using the EHPC model and involving pneumococcal challenge of young healthy non-smoking volunteers with SPN6B/BHN418 have shown stable overall rates of colonization (ES (95% Cl): 0.47 (0.43,0.52)) [43, 105, 207, 248, 342]. The differences in experimental pneumococcal colonization rates observed are therefore unlikely to be due to experimental factors but rather either pathogen or host determinants of colonization.

The identification of microbial factors influencing colonization patterns is of great relevance clinically but also as guidance for researchers as they prepare to set up new challenge models. Despite this, there is presently little understanding of microbial factors driving colonization patterns in the human host. The presence of neutrophils at the human nasopharyngeal mucosa in the absence of pneumococcal colonization has previously been demonstrated [207, 209]. Here I show that neutrophil activity during the first 48 hours of colonization distinguished between two clearance profiles (nasal versus saliva clearers). The strong baseline neutrophil activation demonstrated by nasal clearers – perhaps related to the individual's microbiome profile or environmental factors – suggests an involvement of neutrophils in the early control of pneumococcal colonization. While it is also possible that increased neutrophil activity at baseline is the result of an acute inflammation such as is caused by viral infection, such acute inflammation is usually associated with an increase in TNF which has been shown to inhibit neutrophilic killing [210, 342]. Interestingly, absence of quick

clearance of the pneumococcus from the nose in addition to a delayed increase in neutrophil degranulation was the hallmark of successful establishment of colonization, further implicating the relevance of early neutrophil activity against pneumococcal colonization.

Pneumococcal resistance to non-opsonic NMK has been shown to positively correlate with colonization prevalence, with serotypes that show higher resistance to NMK commonly found colonizing the human nasopharynx at high rates [211]. The data presented here, however, do not support the association of high resistance to non-opsonic NMK and superior experimental colonization rates of SPN6B/BHN418. In fact, I observed higher resistance to NMK with strains which did not colonize well in the human challenge model. The data potentially highlights the different role of the pneumococcal capsule in phagocytosis and pneumococcal binding.

Pneumococcal adherence to the nasal epithelium has been described as the last step in establishing stable colonization [21, 309, 364]. Here I provide visual evidence of epithelial adherence during experimental human pneumococcal colonization. Animal models as well as *in vitro* models have demonstrated that the ability of the pneumococcus to adhere to nasal cells depends on serotype [211, 261, 330]. Surprisingly, the here presented retrospective analysis does not support an association of *in vitro* epithelial adherence with the ability of the pneumococcus to colonize using the EHPC model. The lack of association may be explained by the complexity of the colonization process, with adherence to epithelial cells being the last of many host-pneumococcal interactions during colonization. *Ex vivo* investigation of pneumococcal adherence to epithelial cells using microscopy has been reported here. While this approach is not suitable for retrospective association analysis, it can be used to investigate pneumococcal adherence patterns while accounting for the complexity of

the nasopharyngeal environment, thus providing an alternative approach for future studies.

The formation of pneumococcal chains has previously been demonstrated to provide a competitive advantage during colonization in mice [274, 365]. Here, I evaluated whether chain length associates with experimental pneumococcal colonization rates in the human model. The data demonstrate that superior experimental colonization rates of pneumococcal strain SPN6B/BHN418 were associated with a high magnitude of average chain length. The result agrees with work by Weiser and colleagues showing that increased chain length of pneumococcus promotes adherence and colonization in human cell lines and murine models [274]. Increased chain length is associated with greater surface area per particle, which could promote pneumococcal adherence to host surfaces, although not observed here with nasal cells in culture. A limitation of the experimental approach described here is the use of OD/CFU ratio as a definition for chain length. First described by Basset et al. [354] as a simple alternative to the assessment of pneumococcal chain length via microscopy, the method may be sensitive to other factors that can influence optical densities of liquid bacterial cultures (e.g., secreted proteins, pneumococcal appendages or pneumococcal fitness/survival during the incubation period). However, in congruence with data presented by Basset et al. [354] microscopic assessment of bacterial smears (Appendix 1, Figure 7-1 – Figure 7-4) suggests that pneumococcal chains appear longer and increased in frequency in pneumococcal samples with high OD/CFU ratio (>2 x 10^{-9} OD/CFU per ml). On the other hand, samples with very low OD/CFU ratio (<1 x 10^{-9} OD/CFU per ml) contain predominantly single and diplococci. Association of the magnitude of pneumococcal chains within liquid culture using semi-quantitative microscopy and

OD/CFU ratio (Appendix 1, Table 7-1) further supports the use of OD/CFU ratio as a quantitative marker of pneumococcal chain length.

Thus, considering the data presented, I hypothesised that assessment of chain length (defined as the ratio of OD/CFU) relative to SPN6B/BHN418 may be used to estimate the success of pneumococcal candidate strains in the human challenge model prior to human challenge. In a small pilot study, chain length was further shown to be isolate dependent, indicating the necessity of pre-screening pneumococcal isolates rather than serotype candidates. Feasibility of the proposed hypothesis was sought in the establishment of a serotype 3 EHPC model. Chain length as determined by OD/CFU ratio of pneumococcal serotype 3 strains was measured prior to human challenge. Out of 16 pneumococcal strain candidates three were selected for human challenge. Of those, strain SPN3/PFESP231 showed average chain length comparable to SPN6B/BHN418 and was thus the most likely strain candidate to achieve desired experimental colonization rates of 40% or more. Indeed, experimental challenge of young healthy non-smoking volunteers with SPN3/PFESP231 in a dose ranging study resulted in average colonization rates of 45% and maximum colonization rates of 60%, equal to those observed after challenge with SPN6B/BHN418.

Despite the apparent success, the predictive value of chain length for experimental pneumococcal colonization rates is limited. A hallmark of the EHPC model is the ability to define the optimal dose with which a person is challenged, thus preselection of pneumococcal strain candidates based on achievable maximum colonization rates is highly desirable. Here I show that pneumococcal strain SPN3/PFESP505 showed maximum colonization rates similar to those seen with SPN6B/BHN418, despite demonstrating smaller chain length. Pneumococcal strains of serotype 3 have been shown to evade vaccine induced immunity compared to other vaccine covered

serotypes [366]. In fact, strains belonging to the same clade as SPN3/PFESP505 (Clade II) have increased in numbers amongst both carriage and invasive strains after the introduction of PCV [367]. The ability of some pneumococcal serotype 3 strains to potentially evade pre-existing serotype specific immunity could give the bacterium an advantage over other strains and account for higher colonization rates - even in the absence of colonization facilitating microbial factors.

Indeed, given the complexity of microbial-host interactions it is highly unlikely that only one microbial factor would shape colonization rates. Future studies comparing isogenic pneumococcal variants differing only in chain length should measure the influential power of chain length on experimental colonization rates. Alternatively, the development of a multifactorial bioinformatic model may help to determine the impact of chain length on pneumococcal colonization success.

An important limitation of the studies presented here is the variability of dose range and number of volunteers challenged between the studies as well as the small number of serotypes and strains tested. This limitation is mainly caused by the fact that human challenge models require substantial clinical and laboratory infrastructure to ensure volunteer safety as well as precise challenge and bacterial detection, which is costly and logistically demanding.

The microbial properties tested here are not exclusive and other properties (both host and microbial derived) that influence colonization rates may exist.

Beyond differences in pneumococcal capsule type, pneumococcal strains vary remarkably in their genetic make-up. Presence or absence of microbial genes influencing the pneumococcal colonization process, may account for some of the variability in experimental colonization rates between pneumococcal strains.

Development of bioinformatic models to identify lineage specific colonization genes is currently under way [368] and may aid in future estimation of experimental pneumococcal colonization rates.

Pilus-1 expression has been indicated as a potential advantage for pneumococcal colonization [369]. In our study, presence of the pilus-1 operon and subsequent pilus-1 expression was observed only in SPN6B/BHN418 but not in any of the other EHPC strains [368, 370, 371], perhaps providing the strain with an additional advantage during the establishment of experimental colonization. On the other hand, pilus-1 was shown to be highly immunogenic [369]. Pre-existing anti-pilus-1 antibodies in challenged adults may abolish the advantageous effect of pilus-1 expression on the establishment of pneumococcal colonization.

The role of host immunity in the establishment of pneumococcal colonization has been highlighted many times over. Colonization is an immunising event and previous challenge can be protective against homologous re-challenge [8, 43]. Adults - such as those challenged in the described studies - have likely encountered pneumococcus numerous times throughout their life and as such should have some immunity (subject to circulating serotypes), potentially confounding the colonization rates reported here. While the author recognises the need for analysis of immunity patterns in the described cohorts, the question of confounding immunity is further complicated by the fact that to date no universal correlate of protection against pneumococcal colonization has been identified in humans. High baseline levels of blood capsular specific IgG memory B-cells have been found to associate with protection against acquisition of SPN6B/BHN418 in the human challenge model [253], but this has so far not been replicated with other serotypes. In addition, while baseline polysaccharide specific total IgG levels against SPN15B are reportedly higher than those against SPN6B,

antibody levels prior to pneumococcal challenge did not associate with protection against experimental colonization with either serotype [8, 43].

The relevance of host inflammatory responses to pneumococcus in the early control of colonization has previously been demonstrated [353]. A recent study further demonstrated that epithelial sensing of pneumococcal strain SPN6B/BHN418 is less pronounced and leads to lower inflammatory responses when compared to SPN23F/P1121 *in vitro* [192]. Inadequate epithelial sensing in response to SPN6B/BHN418 may be a confounding factor for the superior colonization rates observed with this strain and may in part explain its ability to efficiently colonize at higher doses compared to other strains. I therefore recommend further investigation of epithelial sensing and resulting inflammatory profiles of the here described pneumococcal strains to investigate their potential effect on colonization patterns.

As a key step in the life cycle of the pneumococcus, transmission of this bacterium is able to shape colonization patterns in the human community profoundly and has been shown to depend on microbial, host as well as environmental factors [309]. The EHPC model, however, uses direct challenge to introduce the pneumococcus to the host, thereby potentially limiting the transferability of the reported results to clinical settings.

Investigating the biology behind the superior colonization rates of pneumococcal strain SPN6B/BHN418 in the EHPC model, I demonstrate a positive association of average chain length and experimental human pneumococcal colonization rates. Further, the use of chain length assessment relative to SPN6B/BHN418 for the preselection of EHPC candidate strains was feasible and may help to reduce research waste.

Chapter 4 The human pneumococcal carriage model - Establishing markers of host susceptibility

4.1 Introduction

The pneumococcus is a respiratory pathogen, able to cause invasive disease as well as acute respiratory tract infection (pneumonia) and inner ear infections (otitis media). In 2015 around 138 million cases of clinical pneumonia and 0.9 million pneumonia death were recorded in young children globally [372]. Pneumonia is also a frequent cause of hospitalisations of the elderly, people with chronic lung disease and asthmatics [66]. With nasopharyngeal colonization being the prerequisite of disease [364], its control is key. Implementation of pneumococcal vaccination programs have had an impressive effect on pneumococcal colonization and disease occurrence of vaccine type strains in all age groups [86, 373]. This demonstrates that disruption of colonization can disrupt transmission and lead to herd protection. Pneumococcal conjugate vaccines induce polysaccharide-specific IgG immunoglobulins, a response that has been associated with vaccine effects against pneumococcal colonization and disease [86, 258].

Meanwhile, unintended vaccination consequences such as serotype replacement and the subsequent rise of non-vaccine type related mortality are a stark reminder of the importance to improve current or identify new control strategies against the pneumococcus. Though considered the first step to disease, pneumococcal colonization is also an immunizing event [43]. As such, experimental pneumococcal colonization is uniquely placed to identify host control mechanisms and susceptibility factors in the human host. Here, I investigate three potential markers of host susceptibility to pneumococcal colonization, previously suggested to play a role during human pneumococcal colonization: sIgA against pneumococcal pilus-1 protein RrgB, agglutination of the pneumococcus in nasal fluid and pneumococcal adherence to the nasal epithelium [258, 309, 374].

4.2 Methods

Volunteer recruitment, pneumococcal challenge and detection of colonization

Volunteer recruitment, pneumococcal challenge and detection of colonization was conducted as outlined in Chapter 2. Briefly, unless otherwise indicated healthy non-smoking young adults were recruited, consented, screened and challenged with 80000 CFU/nostril of SPN6B/BHN418. Volunteers were then followed up and colonization was assessed using classical microbiological detection.

Identification of nasal clearance profiles

Methods used to collect and determine the presence of pneumococcus in saliva as well as the subsequent identification of nasal clearance profiles are described in detail in Chapter 3.

Agglutination assay

Assessment of pneumococcal agglutination in nasal fluid was performed based on a protocol previously described by Mitsi et al. [258]. Briefly, SPN6B/BHN418 stock aliquots (grown as described in Chapter 2, Section "Bacterial Stock preparation") were thawed and washed with PBS. 2 µl of bacteria was incubated with 48 µl of concentrated nasal wash supernatant (1 ml of nasal wash concentrated to 50µl using vacuum concentrator (RVC2-18) and dialysed overnight in PBS using Slide-A-Lyser Dialysis Units (Thermo Fisher). Antiserum to group 6 as well as reference human serum (lot 89SF, FDA, USA) were used as positive controls and anti-Hep-A purified human IgG

was used as a negative control (using sepharose and pooled sera from HepA vaccinated volunteers). Baseline agglutination was established using unstimulated bacteria in PBS. Samples were vortexed lightly and incubated for 1.5 hour at 37°C, 5% CO_2. Bacteria were fixed with paraformaldehyde and analysed on a Flow Cytometer (LSRII; BD Biosciences). Bacterial population was gated in the Forward scatter (FSC) and Sideward scatter (SSC) dot plot referring to cell size and granularity. Gating strategy was developed based on unstimulated control bacteria. Agglutination was quantified by calculating the proportion of the bacterial population with altered FSC and SSC and values were expressed as % of agglutination, as previously described [375]. All samples were analysed in duplicate and 30,000 events were acquired using FacsDiva Software 6.1. Analysis was performed using FlowJo software version 10.0 (Tree Star Inc.).

Anti-pneumococcal capsular polysaccharide IgG ELISA

Anti-pneumococcal capsular polysaccharide antibodies were determined by using WHO internationally standardised method and reagents. Briefly, 96-well ELISA plates were coated using 5 μg/mL of purified 6B polysaccharide (Oxford Biosystems) for 5 hours at 37°C. Wells were blocked with 10% foetal bovine serum in PBS (PBS-F) for 1 hour at 37°C. Plates were washed 3 times with PBS containing 0.05% Tween-20 between each step. Samples were diluted in PBS supplemented with 10% foetal bovine serum containing 10 μg/mL CWPS Multi (Oxford Biosystems) and incubated for 30 minutes at 37°C. Human reference serum (lot 89SF, FDA, USA) was used as standard. Diluted/adsorbed samples were then transferred to pre-coated plates and incubated overnight at 4°C. Antibody detection was performed using an alkaline phosphatase-

labelled polyclonal goat anti-human IgG secondary antibody (A3187, Sigma), followed by addition of p-nitrophenyl phosphate (Sigma). All samples were run in triplicate. Results are expressed as µg/mL calculated using the assigned IgG concentrations in reference serum 89SF.

Anti-pilus-1 IgA ELISA

Anti-pilus-1 IgA antibodies were determined as previously described with minor modifications [374]. Briefly, 96-well ELISA plates were coated using 0.1 µg/well recombinant RrgB protein (Clade I produced by lab of Prof. Jeff Weiser, USA; Clade II produced by Biomatik, USA) in PBS overnight at 4°C. Plates were washed 3 times with PBS containing 0.05% Tween-20. Washing was repeated after each step up to the application of substrate solution. Plates were blocked with blocking buffer (PBS, 0.1% Tween-20, 1% BSA) for 1 hour at room temperature. Samples and standard (sIgA from human colostrum, Sigma #I1010) were diluted in PBS and washed wells were incubated with diluted samples and standards for 1 hour at room temperature. Bound anti-RrgB IgA was measured using goat anti-human IgA coupled with Biotin (1:5000, BioRad) and HRP coupled Streptavidin (1:5000, Life technologies), each incubated for 1 hour at room temperature. TMB was used as HRP substrate and incubated for 5 minutes on a microplate shaker (600 rpm/minute) protected from light. Colour reaction was stopped by adding 2N H_2S0_4 in a 1:1 ratio. Absorbance was measured at 450 nm using FLUOstar Omega and analysis software Omega MARS 3.10 (BMG Labtech). IgA levels were expressed as arbitrary units, calculated using the standard curve and corrected for inter-plate variation using an internal control.

MUC5AC ELISA

ELISA for MUC5AC was performed as outlined in Chapter 2.

Cell culture and pneumococcal adherence assay

Cell culture of Detroit 562, NEC and NPE cultures was performed and cells were used for pneumococcal adherence assay as outlined in Chapter 2.

Statistical analysis

Graphical and statistical analysis were performed as outlined in Chapter 2. For volunteer data spanning multiple time points, statistical analysis was performed using repeated measure ANOVA (parametric). Where matched data points were missing, a linear mixed-effects model, fit using restricted maximum likelihood (REML), was performed instead.

4.3. Results

4.3.1 Nasal secretory IgA against pneumococcal protein pilus-1

4.3.1.1 Experimental approach

The relationship between secretory IgA (sIgA) against the pilus-1 protein RrgB in nasal wash and pneumococcal colonization was investigated. Three sequence variants (Clades) of RrgB are known to be expressed amongst the pneumococcal species. The here used piliated challenge strain SPN6B/BHN418 was determined to express Clade II RrgB, while SPN15B/SH8286 was found to not contain the pilus-1 islet [371, 376].

In total 65 young healthy non-smoking volunteers participating in challenge studies with SPN6B/BHN418 and SPN15B/SH8286 (study numbers 11/NW/592, 18/NW/481, 15/NW/931) were selected for this study. A breakdown of the cohort as well as cohort demographics can be found in Table 4-1. Details on challenge, sampling and analysis time points are depicted in Figure 4-1. Nasal wash samples were analysed prior to and at Day 7 after challenge for sIgA against RrgB-Clade I (n=47) and RrgB-Clade II (n=65). Day 7 was chosen as it presented the latest available time point prior to repeated pneumococcal challenge throughout all analysed cohorts (Figure 4-1). Analysis was performed blinded for colonization status.

In addition, the long-term kinetics of sIgA against Clade II-RrgB levels were investigated. In total 25 young healthy non-smoking volunteers challenged multiple times with SPN6B/BHN418 over a period of 6 months (Cohort 1 and 2) were included into the analysis. Briefly, during Stage 1 volunteers were exposed to pneumococcus SPN6B/BHN418 at Day 0 and optionally at Day 14. Volunteers were given antibiotics

to help clear colonization before being invited for a repeat challenge with SPN6B/BHN418 3-6 months after first challenge. Colonization was determined throughout Stage 1 and Stage 2. Positive colonization was defined as growth of pneumococcus on Columbia blood agar from nasal wash at any time point throughout the study stage. Based on this classification four groups were identified: Group 1: those colonization negative throughout Stage 1 and Stage 2 (EC-/EC-, n = 9), Group 2: those colonization negative in Stage 1 but susceptible in Stage 2 (EC-/EC+, n = 1), Group 3: those susceptible throughout Stage 1 and Stage 2 (EC+/EC+, n = 3), Group 4: those susceptible in Stage 1 but colonization negative in Stage 2 (EC+/EC-, n = 12).

Table 4-1: Breakdown of analysed cohort

	n	Median Age in years (range)	Gender distribution (Female/Male)
Cohort 1[a]	6	25 (21-29)	4/2
Cohort 2[b]	19	22 (18-48)	11/8
Cohort 3[c]	22	22 (20-32)	14/8
Cohort 4[a]	18	21 (18-49)	13/5
Total	65	22(18-49)	42/23

[a] Challenge (Stage 1) with SPN15B/SH8286 (Cohort 4) or SPN6B/BHN418 (Cohort 1) followed by challenge with SPN6B/BHN418 3-6 months after initial challenge (Stage 2). [b] Challenge with SPN6B/BHN418 at Day 0, Day 14 (Stage 1) and 3-6 months after initial challenge (Stage 2). [c] Challenge with SPN6B/BHN418 at Day 0 (Stage 1)

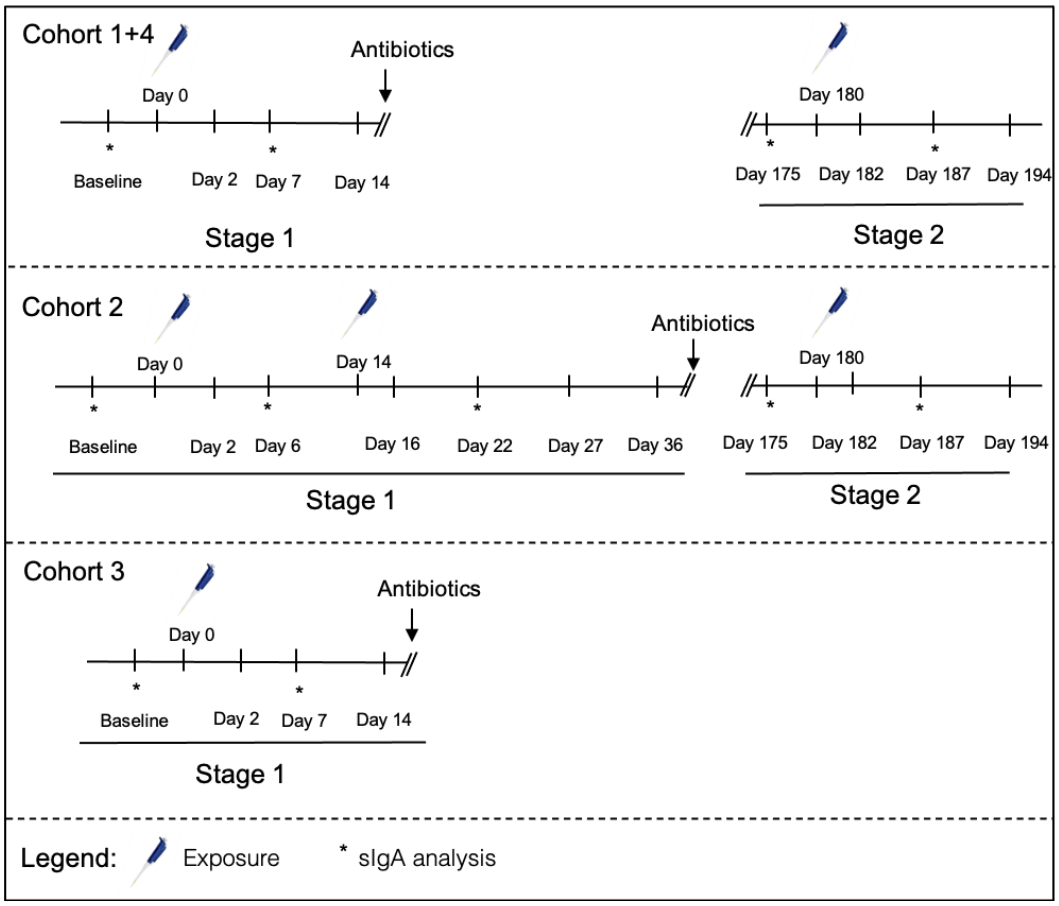


Figure 4-1: Study layout of cohorts analysed for anti-RrgB sIgA.

For Cohort 1 and Cohort 4 volunteers were challenged at Day 0 and 3-6 months after initial challenge (Day 180). Nasal sampling was performed at baseline, Day 2, Day 7 and Day 14 (Stage 1) and Day 175, Day 182, Day 187 and Day 194 (Stage 2). For cohort 2 volunteers were challenged at Day 0, Day 14 and 3-6 months (Day 180) after initial challenge. Nasal sampling was performed at baseline, Day 2, Day 6, Day 16, Day 22, Day 27 and Day 36 (Stage 1), Day 175, Day 182, Day 187 and Day 194 (Stage 2). For cohort 3 volunteers were challenged at Day 0 and nasal sampling was performed at baseline, Day 2, Day 7 and Day 14. Antibiotics were prescribed after conclusion of Stage 1 to help clear colonization.

4.3.1.2 Baseline levels of anti-pilus-1 sIgA in nasal wash do not associate with host susceptibility to human pneumococcal colonization

Baseline levels of sIgA against Clade II-RrgB and Clade I-RrgB prior to first experimental challenge with piliated strain SPN6B/BHN418 (expressing Clade II-RrgB) were measured in nasal wash and associated with colonization outcome (Figure 4-2).

Baseline levels of sIgA against Clade II-RrgB in those who became colonized (colonization positive, EC+) were not significantly different from those who did not become colonized (colonization negative, EC-). Similar, baseline levels of sIgA against Clade I-RrgB in those colonized were not significantly different from those not colonized. The data indicate no protective effect of baseline levels of anti-RrgB sIgA against colonization with piliated strain SPN6B/BHN418.

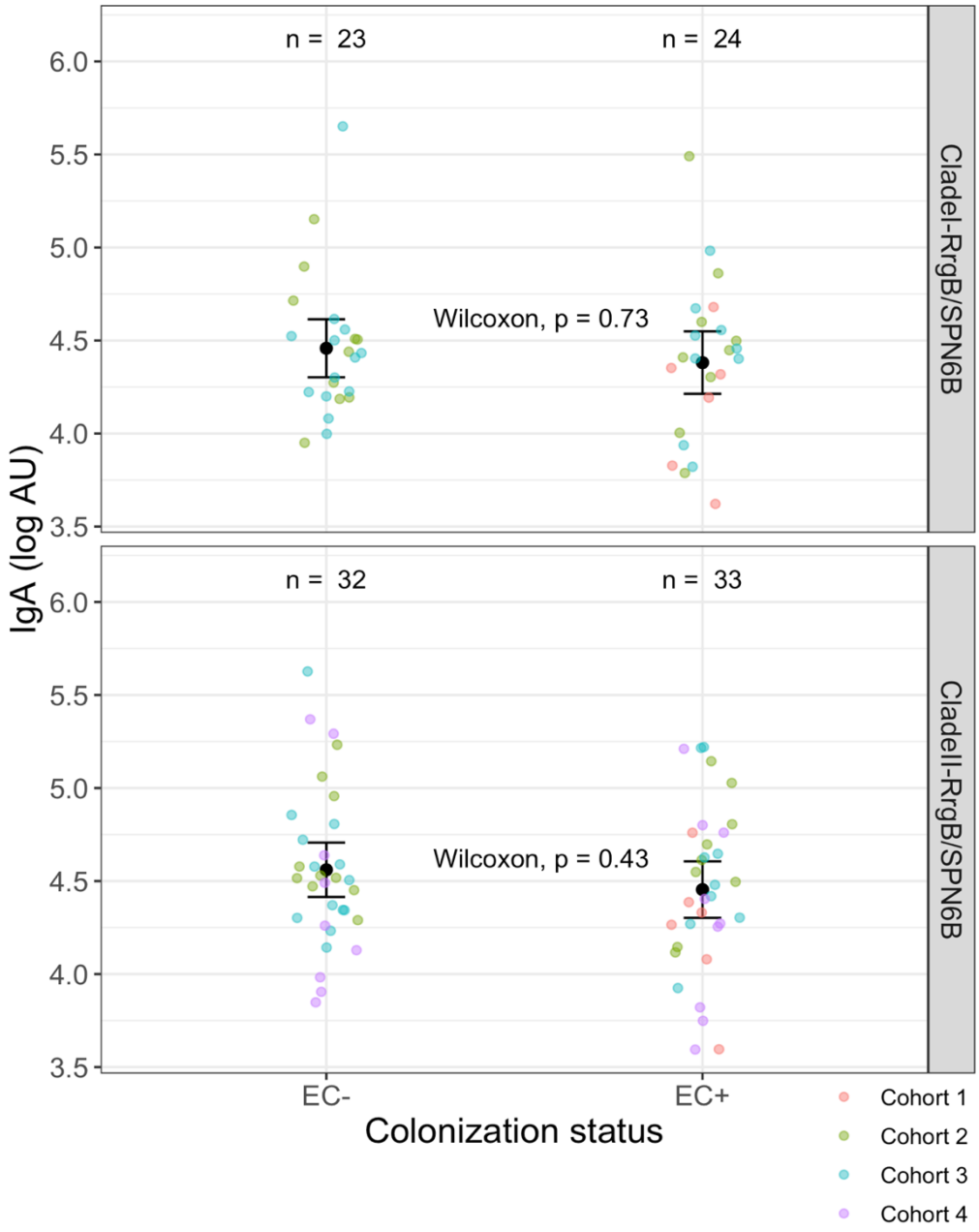


Figure 4-2: Baseline levels of anti-pilus-1 sIgA in nasal wash do not associate with host susceptibility to human pneumococcal colonization

Levels of sIgA against Clade II-RrgB (**lower panel**) and sIgA against Clade I-RrgB (**upper panel**) in nasal wash at baseline (prior to challenge with pneumococcus) stratified by colonization status after first challenge with piliated strain SPN6B/BHN418 (Clade II-RrgB). Participating volunteers were young healthy non-smoking adults. Colonization was defined as growth of the pneumococcal challenge strain from nasal wash at any time point following challenge (colonization positive = EC+, colonization negative = EC-). Significance levels were determined using unpaired two-sample Wilcoxon (= Mann Whitney) test.

4.3.1.3 Pneumococcal colonization elicits sIgA antibody response against pneumococcal protein pilus-1 in the human nose

I investigated whether pneumococcal challenge induces a humoral response of the IgA type against pneumococcal protein pilus-1 in the nose. To this effect, levels of sIgA against Clade I-RrgB and Clade II-RrgB prior to and seven days after first challenge with either SPN6B/BHN418 or SPN15B/SH8286 were analysed in nasal wash.

First, the impact of challenge with piliated strain SPN6B/BHN418 (Clade II-RrgB) on levels of sIgA against Clade II-RrgB was investigated (Figure 4-3, lower panel). In those colonized levels of sIgA against Clade II-RrgB were significantly increased (Wilcoxon, P = 0.019) 7 days after challenge (64155 ± 13242 AU) when compared to baseline (45895 ± 8309 AU). In those not colonized levels of sIgA against Clade II-RrgB did not differ significantly between baseline and day 7 after challenge.

Second, the impact of challenge with piliated strain SPN6B/BHN418 (Clade II-RrgB) on levels of sIgA against Clade I-RrgB was investigated (Figure 4-3, upper panel). Levels at Day 7 after challenge did not differ significantly from baseline in those colonized with piliated SPN6B/BHN418 as well as in those not colonized.

Third, the impact of challenge with non-piliated SPN15B/SH8286 on levels of sIgA against Clade II-RrgB was investigated (Figure 4-3, middle panel). Levels at Day 7 after challenge did not differ significantly from baseline in those colonized with non-piliated pneumococcus as well as in those not colonized.

The data demonstrates an induction of homologous anti-RrgB sIgA in those colonized with piliated pneumococcus SPN6B/BHN418 as early as seven days after challenge.

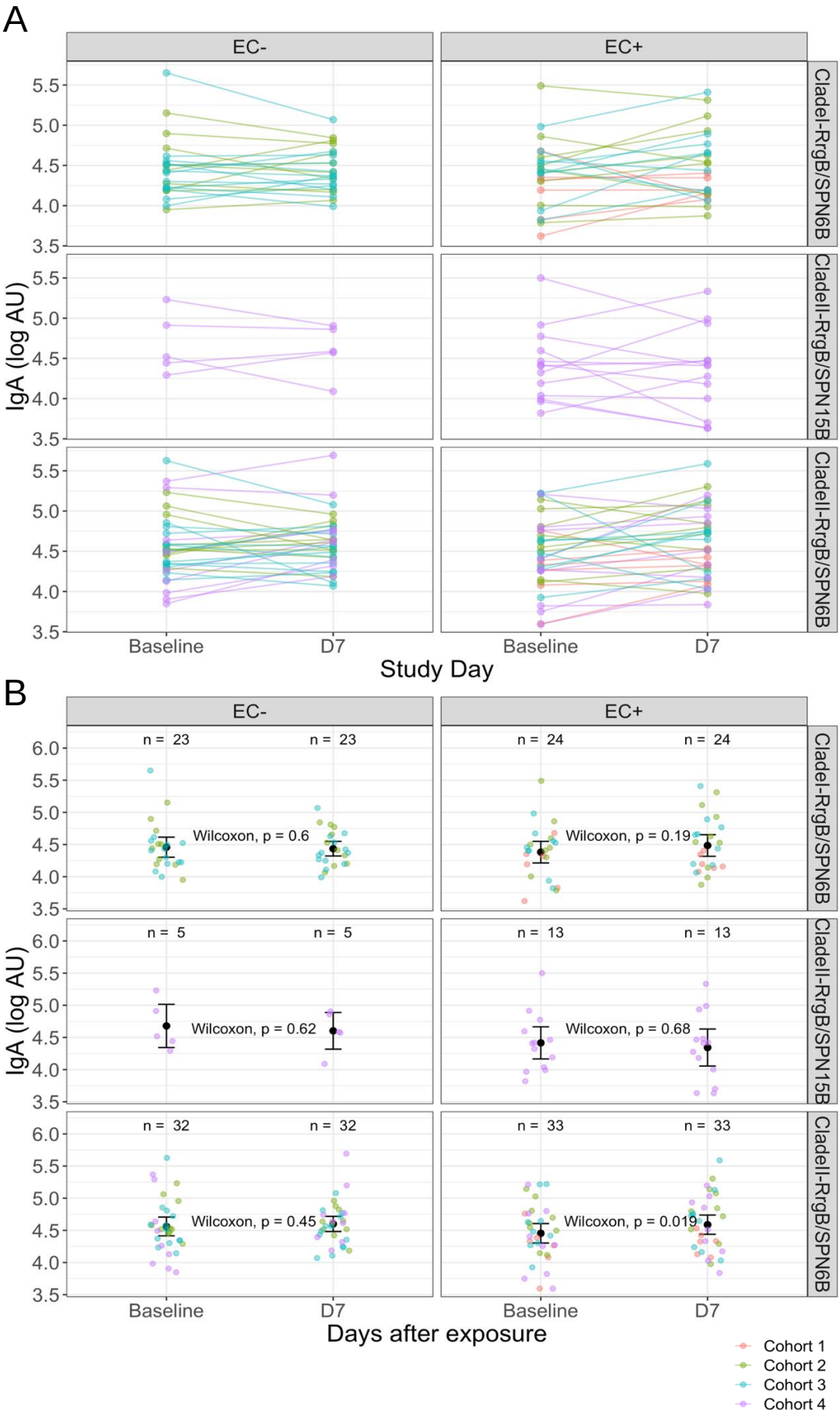

A
EC-
EC+
CladeI-RrgB/SPN6B
CladeII-RrgB/SPN15B
CladeII-RrgB/SPN6B
IgA (log AU)
5.5
5.0
4.5
4.0
3.5
Baseline
D7
Study Day
B
n = 23
n = 24
n = 5
n = 13
n = 32
n = 33
Wilcoxon, p = 0.6
Wilcoxon, p = 0.19
Wilcoxon, p = 0.62
Wilcoxon, p = 0.68
Wilcoxon, p = 0.45
Wilcoxon, p = 0.019
6.0
Days after exposure
Cohort 1
Cohort 2
Cohort 3
Cohort 4

Figure 4-3: Pneumococcal colonization elicits sIgA antibody response against pneumococcal protein pilus-1 in the human nose.

Mucosal levels of sIgA against Clade II-RrgB in young healthy non-smoking volunteers challenged with piliated strain SPN6B/BHN418 expressing Clade II-RrgB (lower panel) or non-piliated strain SPN15B/SH8286 (middle panel). Mucosal levels of sIgA against Clade I-RrgB in young healthy non-smoking volunteers challenged with SPN6B/BHN418 (upper panel). Levels were measured in nasal wash samples prior to and seven days after first challenge with the indicated challenge strain. Paired levels are shown in Figure A while mean ± SEM is depicted in Figure B. Colonization was defined as growth of the pneumococcal challenge strain from nasal wash at any time point following challenge (colonization positive = EC+, colonization negative = EC-). Significance was determined using paired Wilcoxon test.

4.3.1.4 Mucosal sIgA levels against pilus-1 remain elevated for up to 6 months after pneumococcal challenge

Kinetics of sIgA against Clade II-RrgB were measured in young healthy non-smoking volunteers participating in pneumococcal challenge studies involving repeated challenge with piliated strain SPN6B/BHN418 (Stage 1 and Stage 2). Volunteers susceptible throughout Stage 1 and Stage 2 as well as those colonization negative in Stage 1 but susceptible in Stage 2 were not statistically analysed due to the low number of volunteers identified into those groups.

Volunteers colonized in Stage 1 but colonization negative in Stage 2 showed a significant difference in levels of sIgA against Clade II-RrgB throughout the study period (REML, P = 0.0064, Figure 4-4). Significant increase in sIgA levels to homologous pilus-1 was detected from Day 22 after first challenge with piliated strain SPN6B/BHN418 when compared to baseline levels (Fisher's LSD; Day 22: P = 0.0091). Levels remained elevated for up to 6 months after the initial colonization episode (Fisher's LSD; Day 175: P = 0.0072, Day 187: P = 0.0083). In contrast, volunteer who were negative for colonization with piliated strain SPN6B/BHN418 throughout both stages showed no significant difference in levels of sIgA against Clade II-RrgB at any time point (RM Anova, P = 0.3497).

The data indicate that after initial colonization with piliated strain SPN6B/BHN418 homologous levels of anti-RrgB sIgA remain elevated for up to six months in those colonized.

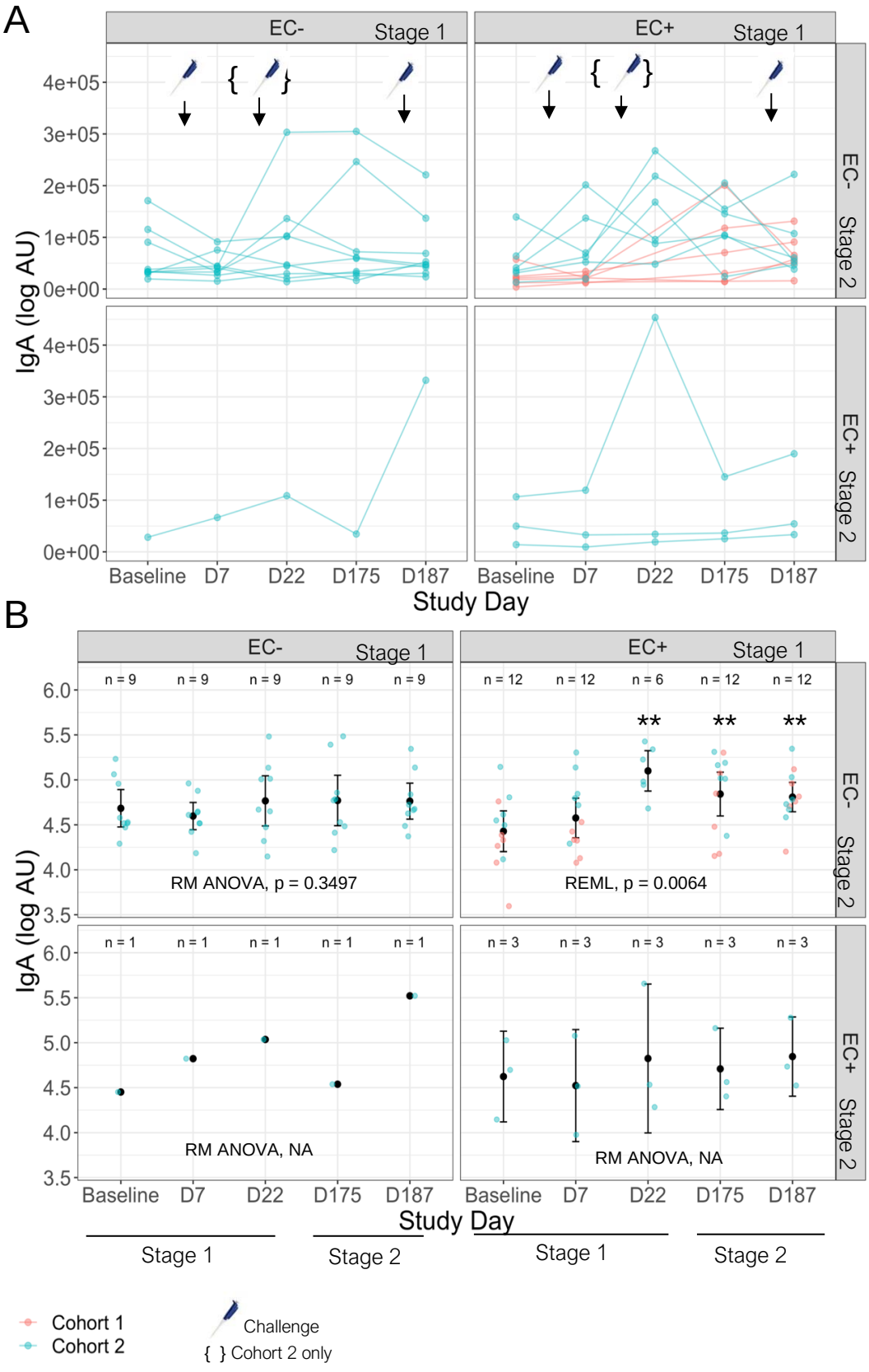

A
EC-
Stage 1
EC+
Stage 1
EC- Stage 2
EC+ Stage 2
IgA (log AU)
4e+05
3e+05
2e+05
1e+05
0e+00
Baseline
D7
D22
D175
D187
Study Day
B
n = 9
n = 12
n = 6
**
RM ANOVA, p = 0.3497
REML, p = 0.0064
n = 1
n = 3
RM ANOVA, NA
Stage 1
Stage 2
Cohort 1
Cohort 2
Challenge
{ } Cohort 2 only

Figure 4-4: Mucosal sIgA levels against pilus-1 remain elevated for up to 6 months after pneumococcal challenge

Levels of sIgA against Clade II-RrgB was measured in nasal wash at baseline (prior to challenge) and 7, 22, 175 and 187 days after first challenge (Day 0) with piliated strain SPN6B/BHN418 expressing Clade II-RrgB. Challenge with SPN6B/BHN418 was repeated at Day 14 (only for Cohort 2) and 3-6 months (Day 180) after first challenge. Participating volunteers were young healthy non-smoking adults. Colonization was defined as growth of the pneumococcal challenge strain from nasal wash at any time point following challenge (colonization positive = EC+, colonization negative = EC-). Paired levels are shown in Figure A while mean ± SEM is depicted in Figure B. Significance was determined using repeated measure ANOVA or - where data points are missing - linear mixed-effects model, fit using restricted maximum likelihood (REML) with uncorrected Fisher's LSD post-hoc test. *$P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$.

4.3.2 Pneumococcal agglutination by host molecules

4.3.2.1 Experimental approach

The role of nasal mucins and antibodies in the formation of pneumococcal aggregates (pneumococcal agglutination) was studied. To this effect baseline nasal washes obtained from young healthy non-smoking EHPC volunteers were analysed for levels of MUC5AC and PS6B-specific IgG by ELISA (study number 14/NW/1460). Nasal washes were also analysed for their capacity to form aggregates with pneumococcal strain SPN6B/BHN418 using flow cytometry. Agglutination was correlated with the levels of nasal MUC5AC and PS6B-specific IgG to investigate which of those molecules likely contributes to pneumococcal agglutination.

Further, the role of pneumococcal agglutination in the early control of and protection against experimental pneumococcal colonization was investigated. To this effect, baseline levels of agglutination of SPN6B/BHN418 in nasal wash were stratified based on experimental colonization status (determined from nasal wash by classical microbiology) as well as pneumococcal clearance profiles (detection of pneumocccus in saliva, as defined in Chapter 3.3.2.1) of volunteers challenged with pneumococcal strain SPN6B/BHN418.

4.3.2.2 Levels of nasal MUC5AC correlate with the formation of pneumococcal aggregates in nasal wash

Baseline capacity of nasal wash to form aggregates with SPN6B/BHN418 was analysed (Figure 4-5 A). Agglutination correlated significantly with levels of nasal MUC5AC (R = 0.5373, P= 0.0032, Figure 4-5 B), but not with levels of nasal PS6B-specific IgG (R = 0.3072, P = 0.0606, Figure 4-5 C). The results implicate an association of nasal MUC5AC with the process of pneumococcal agglutination.

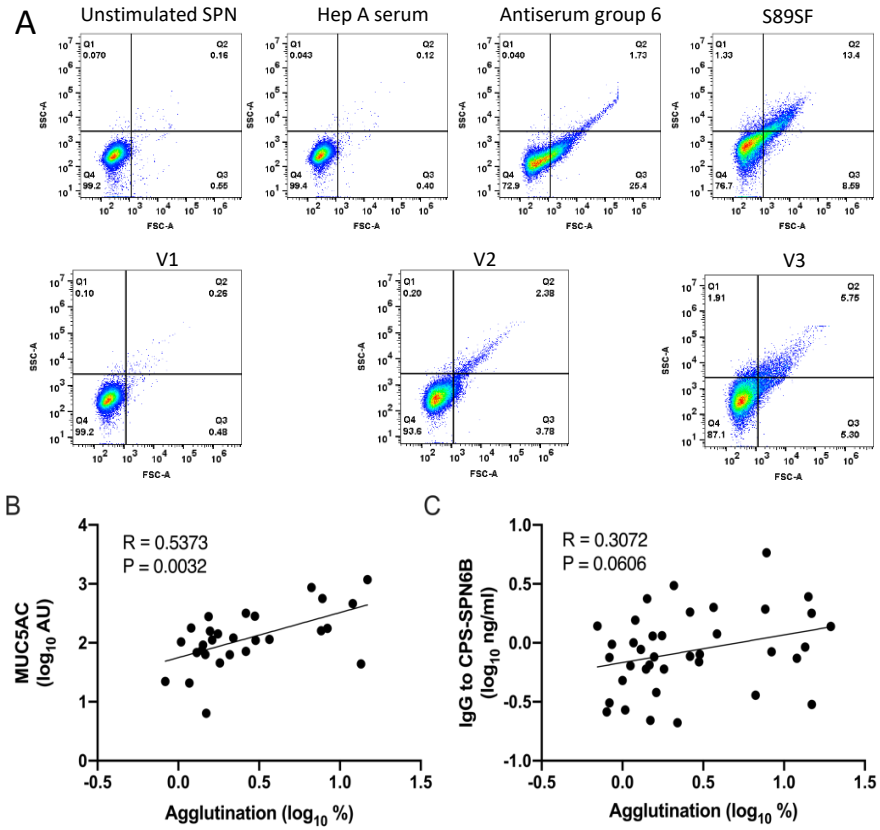


Figure 4-5: Levels of nasal MUC5AC correlate with the formation of pneumococcal aggregates in nasal wash.

(A/B) Flow cytometry gating and analysis strategy including controls and exemplary volunteer plots (V1-V3). Gating was based on unstimulated pneumococcus (SPN). Agglutination of SPN by anti-Hep A purified human IgG served as negative control. Agglutination of SPN by antiserum group 6 or human reference serum 89SF served as positive controls. Agglutination was quantified as the proportion of bacterial population with altered FSC and SSC (Q1+Q2+Q3) corrected by baseline agglutination (unstimulated SPN). (B/C) Correlation of the percentage of agglutination of pneumococcal strain SPN6B/BHN418 in baseline nasal wash samples obtained from young healthy non-smoking volunteers with (B) nasal MUC5AC and (C) nasal polysaccharide specific IgG levels at baseline. All samples were analysed in duplicates. Significance determined using Spearman correlation. Significance levels $P < 0.05$.

4.3.2.3 Pneumococcal agglutination capacity of nasal fluid does not associate with susceptibility to pneumococcal colonization in unvaccinated individuals

The relative importance of pneumococcal agglutination regarding the early control of and protection against pneumococcal colonization was investigated. There was no significant difference in the percentage of aggregate formation with SPN6B/BHN418 (agglutination) between “saliva clearers” (presence of pneumococcal DNA in saliva at 1 hour post challenge) and “nasal clearers” (absence of pneumococcal DNA in saliva at 1 hour post challenge) in baseline nasal wash samples (Figure 4-6 A). Notably, there was also no difference in baseline agglutination of SPN6B/BHN418 between those colonization negative and those colonization positive after challenge with the homologous strain (Figure 4-6 B).

In congruence with earlier published results [258] levels of 6B polysaccharide-specific IgG did not associate with protection against or control of colonization (Appendix 2, Figure 7-5).

The data implicate no association between agglutination of SPN6B/BHN418 and early control of and protection against colonization with the homologus strain in unvaccinated individuals.

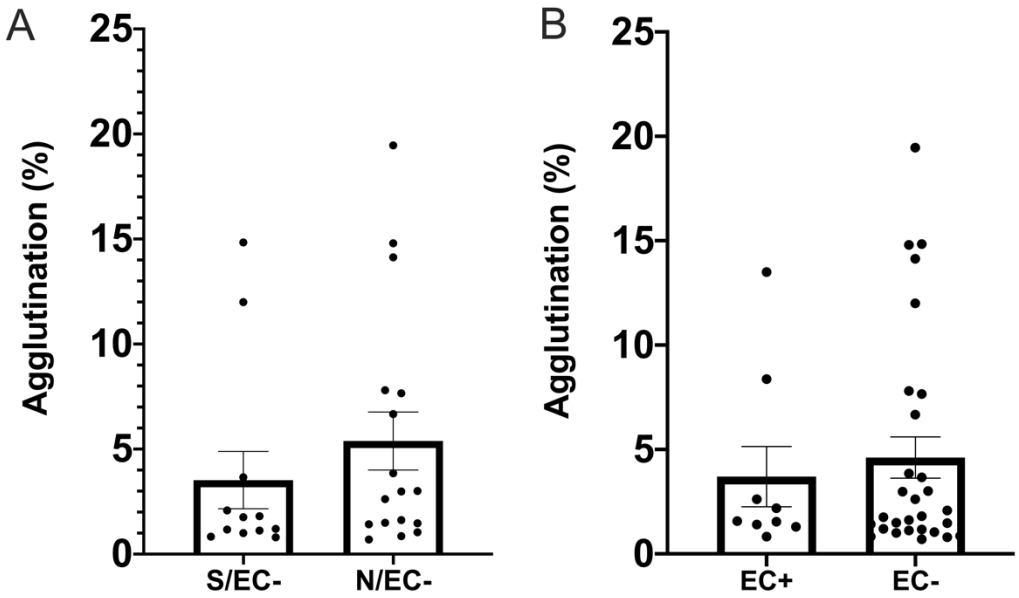


Figure 4-6: Pneumococcal agglutination capacity of nasal fluid does not associate with susceptibility to pneumococcal colonization in unvaccinated individuals

Agglutination of SPN6B/BHN418 in nasal wash at baseline was stratified by (A) pneumococcal clearance profile and (B) colonization status after challenge with SPN6B/BHN418. Participating volunteers were young healthy non-smoking adults. Pneumococcal clearance profiles were determined by the presence of pneumococcal DNA in saliva using molecular methods (S/EC- = presence of pneumococcal DNA in saliva at 1 hour post challenge; N/EC- = absence of pneumococcal DNA in saliva at 1 hour post challenge). Colonization status (colonization negative = EC- ; colonization positive = EC+) was determined as the growth of the pneumococcal challenge strain from nasal wash on blood agar plate at any time point following challenge. Bars represent mean ± SEM. Significance was determined using Mann-Whitney test. Significance levels $P < 0.05$.

4.3.3 Pneumococcal adherence to nasal epithelial cells

4.3.3.1 Experimental approach

In chapter 3 I showed that superior experimental colonization rates of pneumococcal strain SPN6B/BHN418 were not associated with a superior ability of the strain to adhere to the nasal epithelium. Here I investigated the relationship between pneumococcal adherence to nasal epithelial cells and host susceptibility to human pneumococcal colonization.

Nasal cells were collected prior to or 28 days after pneumococcal challenge and cultured for up to one month in an undifferentiated state. In total 34 nasal cell samples were collected from young healthy adults and 7 from volunteers over 50 years of age (study numbers: 16/NW/0031, 14/NW/1460, 17/NW/0029 and LSTM tissue Bank). One culture was excluded from analysis due to abnormal growth behaviour. Where volunteers were challenged with SPN6B/BHN418 after nasal cell collection, adherence assays were performed blinded towards the volunteer's status of colonization.

4.3.3.2 Low pneumococcal adherence to nasal epithelial cells associates with reduced colonization susceptibility in the elderly

The relationship between adherence of SPN6B/BHN418 to primary nasal epithelial cells (NECs) and human susceptibility to experimental pneumococcal colonization was investigated. Importantly, cells obtained from healthy individuals prior to and after challenge with SPN6B/BHN418 showed differences in SPN6B/BHN418 adherence levels (Figure 4-7 A). Similar increased levels of SPN6B/BHN418 adherence were observed when cells were cultured for longer time (Figure 4-7 B). Thus, all here described comparisons were matched by time point of cell collection and where applicable by culture passage.

Average pneumococcal adherence (mean ± SEM) to NECs, collected prior to pneumococcal challenge from young healthy individuals, was not significantly different between those who became colonized ($1.3 \times 10^5 \pm 1.7 \times 10^4$ CFU) and those who did not ($1.4 \times 10^5 \pm 2.4 \times 10^4$ CFU; Figure 4-7 C). However, when comparing adherence levels between individuals over 50 years of age (elderly) and young healthy controls significant difference in adherence levels of SPN6B/BHN418 were observed (Wilcoxon, $P = 0.0014$; Figure 4-7 D). In congruence with experimental pneumococcal colonization rates [1], NECs obtained from individuals of older age showed on average lower levels of pneumococcal adherence when compared to those obtained from young individuals. The result indicates that levels of pneumococcal adherence to nasal cells may associate with lower colonization susceptibility of the elderly.

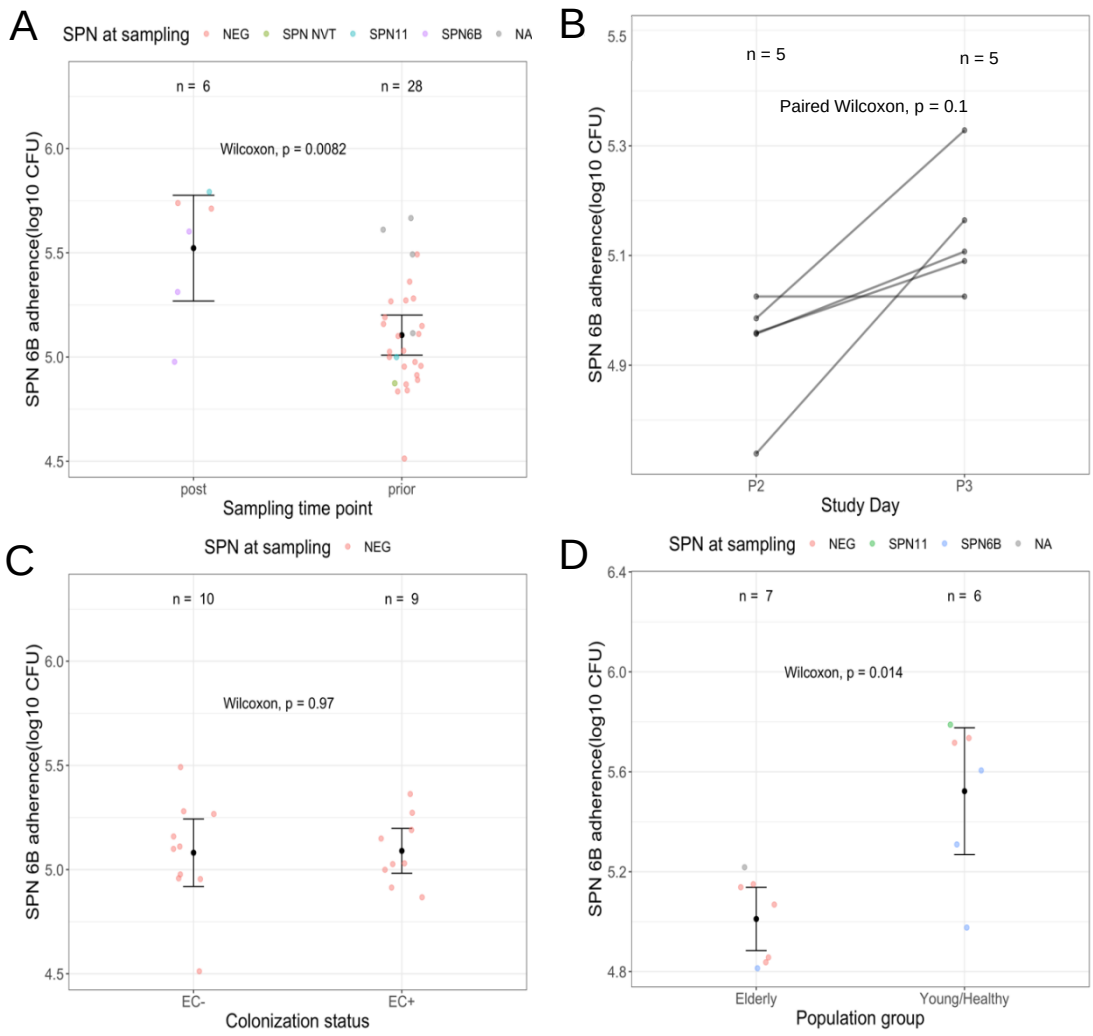


Figure 4-7: Low pneumococcal adherence to nasal epithelial cells associates with reduced colonization susceptibility in the elderly

Levels of pneumococcal adherence stratified by (A) nasal cell sampling time point (unpaired, prior = before challenge, post = Day 28 after challenge), (B) passage number at which the assay was performed (paired, P2 = 27 days into culture, P3 = 37 days into culture) or (C) by experimental colonization status. (A/B/C) Cells used were undifferentiated primary nasal cells (NECs) obtained from young healthy adults. (B/C) Cells were collected prior to pneumococcal challenge. (D) Levels of pneumococcal adherence to NECs collected at Day 28 after challenge in different population groups. (All) Cells were seeded at a concentration of $1x10^5$ cells/well in a 24 well plate. Cells were cultured in the presence of penicillin/neomycin/streptomycin prior to experiment. $1x10^6$ CFU of pneumococcus was added for 3 hours after 7 days of cell culture or when confluency was reached. Colours depict colonization status of volunteers at time point of nasal cell sampling. Significance levels $P < 0.05$. Significance level was determined using (A/C/D) unpaired two-sample Wilcoxon test or (B) paired sample Wilcoxon test.

4.4. Discussion

Success of pneumococcal vaccination programs worldwide have highlighted the impact of serotype-dependent protective antibodies against pneumococcal disease and colonization [86, 373, 377]. However, limitations of current polysaccharide-based vaccines also highlight the need for broader serotype independent control mechanisms.

Naturally acquired sIgA against pneumococcal pilus-1 protein RrgB may mediate the majority of piliated pneumococcal binding to mucus, thereby inhibiting the establishment of colonization [374, 378]. As seen with other pneumococcal proteins, RrgB is not universally conserved, instead, three known sequence variants (clades) of RrgB exist amongst the pneumococcal species [378]. RrgB Clade II, expressed by the here used challenge strain SPN6B/BHN418, is the rarest amongst the clade variants [379]. Piliated strains are relatively scarce amongst the pneumococcal species. In fact, pilus-1 is expressed in 21–27% of clinical pneumococcal strains [380]. In addition, pilus-1 is most commonly (though not exclusively) found in strains covered by the current available PCV vaccines and in those found in colonized children of very young age [374]. Investigating the role of pre-existing anti-RrgB sIgA against experimental human pneumococcal colonization I found no association between levels of anti-RrgB sIgA and host susceptibility. However, experimental colonization with SPN6B/BHN418 induced homologous anti-RrgB sIgA as early as seven days after challenge with levels remaining elevated for up to six months after challenge. A limitation of this study is the measurement of antibody responses to pneumococcal colonization as early as seven days after pneumococcal exposure in adults. Human antibody responses can be distinguished into primary and secondary responses, the former occurring in antigen naïve subjects with antibody titres usually increasing

several weeks after antigen contact. In contrast secondary immune responses occur in reaction to subsequent antigen contact and are defined by the activation of memory B-cells, resulting in faster and stronger antibody responses. Thus, early responses to pneumococcal challenge measured here are likely to be the result of immune memory responses. This is further supported by the fact that pneumococcal colonization of piliated strains was shown to be common during early childhood [374]. Thus, the adults challenged here are likely not naïve to the pilus-1 antigen. Indeed, analysis of levels of anti-RrgB sIgA prior to controlled pneumococcal exposure showed measurable levels of sIgA against Clade I and Clade II RrgB in all volunteers, indicating prior natural exposure to piliated pneumococcus.

Pneumococcal capsular polysaccharide is highly immunogenic and increased levels of capsular polysaccharide-specific IgG have been associated with protection against pneumococcal colonization in vaccinated and colonized individuals [43, 258]. The study of anti-RrgB sIgA in the context of repeated pneumococcal exposure presented here was unable to provide conclusive evidence of the potential protective effects of elevated mucosal immunoglobulin against pneumococcal protein pilus-1. A previously performed heterologous challenge study demonstrated no increased protection against colonization when volunteers were re-challenged with a different serotype 3-6 months after initial challenge. Notably however, in the mentioned study the initial challenge strain was not piliated, thus the observed lack of cross protection between serotypes does not allow for conclusions regarding the cross protective potential of anti-pilus targeting immunoglobulins [253]. Heterologous challenge studies including multiple challenges with piliated pneumococcal strains of different serotypes are needed to provide a clearer picture of the feasibility of RrgB targeting vaccination approaches. Sequence diversity of RrgB may further limit the efficiency of potential RrgB targeting

vaccine approaches. Data in this thesis suggest no significant cross-reactivity between Clade II-RrgB antigen and Clade I-specific antibodies in humans, corroborating prior observations made in mice [378]. While the lack of cross-reactivity certainly presents a limitation of potential RrgB targeting vaccines, it may not be impossible to overcome. A study conducted in mice, demonstrated that fusion proteins containing all three clade variants confers protection against pneumococcal disease [378].

It is thought that pneumococcal persistence in the nasopharynx requires binding to host cells and tissue. Thus, efficiency of immune exclusion - the process of crosslinking, entrapping and clearing pathogens - may be a decisive factor during colonization establishment. Previous publications have identified host molecules such as anti-pilus-1 sIgA and polysaccharide specific IgG as important pneumococcal binding and agglutination factors [258, 374].

Notably, in the study presented here, levels of polysaccharide specific IgG did not correlate with levels of pneumococcal agglutination in nasal wash. In addition, *ex vivo* agglutination of SPN6B/BHN418 by nasal wash did not associate with early pneumococcal clearance profiles or host susceptibility in volunteers challenged with SPN6B/BHN418. Notably, rapid pneumococcal removal - likely the result of mucociliary clearance - was observed in the absence of increased pneumococcal agglutination in those protected against colonization. A previous study of pneumococcal agglutination conducted by this group, demonstrated a protective effect of agglutinating polysaccharide specific IgG against pneumococcal colonization in vaccinated individuals which was associated with increased levels of polysaccharide-specific IgG [258]. In congruence with the results observed here, no such effect was shown in volunteers who had not received a pneumococcal vaccine. Thus, protective

effects of pneumococcal agglutination may be most relevant in settings where mucociliary clearance is impaired (e.g. smoking individuals) or mucosal antibodies are increased (e.g. in vaccinated individuals) [258].

Direct interactions with oligosaccharides present on mucins have been demonstrated for many pathogens [138]. Data presented in this thesis show that levels of nasal MUC5AC (the most abundant mucin in the nasopharynx) positively correlated with agglutination of the encapsulated strain SPN6B/BHN418 in nasal wash.

Possible interaction sites between mucins and the pneumococcus have been identified in the past [300, 301], however scientific evidence of direct interactions between the pneumococcus and mucins is conflicting. Evidence of electrostatic repulsion between mucin biofilms and encapsulated pneumococcus has been demonstrated [4, 261]. However, data of *in vitro* pneumococcal binding assays with upper respiratory tract mucin suggest that, depending on expression level of the pneumococcal capsule, direct interactions between mucins and encapsulated pneumococcus may still occur [313]. Another possible mechanistic explanation for the here observed correlation may involve pneumococcal interactions with the mucus interactome – a multiprotein complexes centred around airway mucins [381]. Proteins described to directly interact with mucins include immunoglobulins, defensins, complement and lysozyme [381, 382].

Future research into the details of pneumococcal-mucus interactions is needed to answer open questions abouts its relevance and identify players and mechanisms alike. Nevertheless, the data presented indicate that nasal MUC5AC may be an important molecule contributing to the formation of pneumococcal aggregates and subsequent clearance of the pneumococcus.

Pneumococcal adherence to the epithelium is believed to be the last step in the establishment of stable colonization. Molecular mechanisms as well as molecules involved in epithelial attachment have been extensively described [364, 383]. However, the relevance of epithelial adherence for human susceptibility to pneumococcal colonization remains unclear. Here, I show that in congruence with reduced experimental pneumococcal colonization rates in the elderly [1], pneumococcal adherence to nasal cells obtained from donors of older age (+50) was significantly lower than compared to a younger control group. In contrast, in a group of young healthy individuals, levels of pneumococcal adherence to nasal cells did not associate with susceptibility of the host to become colonized. While I can only speculate as to the cause of the lower pneumococcal adherence rate in elderly individuals observed here, reduced expression of adherence mediating molecules or changes in the nasopharyngeal niche resulting in a more challenging environment may serve as possible explanations.

To my knowledge this is the first study to investigate the association of epithelial adherence and host susceptibility to human pneumococcal colonization. An important limitation of the presented approach is the use of primary nasal cell cultures which require prolonged culture times. It has previously been shown that primary nasal epithelial cells alter gene expression under prolonged culture conditions [322, 323]. Such alterations could potentially equalise inter-individual differences between samples that would otherwise differentially influence colonization outcome *in vivo*. Further analysis comparing proteomic data *in vivo* and *in vitro* may shed more light into the question which nasal cell features are retained during culture, thus, enabling a more targeted use of these cells for pneumococcal research. *Ex vivo* microscopy of nasal scrapes obtained from challenged individuals may present a potential alternative

to investigate the relative importance of epithelial adherence for human susceptibility to pneumococcal colonization. Alternatively, models of differentiated nasal cell cultures have been shown to mirror *in vivo* epithelium more closely while retaining population characteristics [317-319]. Such a cell culture model of reconstituted nasal epithelium with an air liquid interface was implemented in the LSTM laboratories but showed low rates of success (Appendix 3).

The control of colonization is key to reduce mortality caused by the pneumococcus. Here I investigated the effect of three mucosal host factors on host susceptibility to human pneumococcal colonization. The presented results highlight nasal MUC5AC as a molecule of interest in the control of pneumococcal colonization, due to its association with the mechanism of pneumococcal agglutination.

Chapter 5 : Effect of nasal mucins on experimental human pneumococcal colonization

5.1 Introduction

Mucus is the first layer of defence against external pathogens such as bacteria and viruses. Those protective effects are the sum of mechanical, physical as well as component specific characteristics. Mucins are an integral part of mucus, largely determining its protective characteristics. Of the more than 17 known mucin genes, 11 have been identified in the human airways [384]. Mucins are known to play an integral role in the defence against respiratory pathogens. They function as a physical barrier and play an important role during mucociliary clearance [167, 374]. Further protective effects of mucins against respiratory pathogens may include direct effects such as antimicrobial properties [165-167], indirect effects on inflammatory immune responses [167, 168] or protective effects caused by an enrichment of bacteriophages in the nasopharyngeal niche [169].

On the other hand, rich in carbohydrates, mucin proteins may also promote bacterial survival, growth and biofilm formation in an otherwise nutrient poor niche [262-268]. Whether mucin – pneumococcal interaction ultimately is favourable for host or pathogen is key question.

Here, I aim to determine whether nasal MUC5AC protects against or facilitates pneumococcal colonization in volunteers challenged experimentally with SPN6B/BHN418.

5.2 Methods

Volunteer recruitment, pneumococcal challenge and detection of colonization

Volunteer recruitment, pneumococcal challenge and detection of colonization was conducted as outlined in Chapter 2. Briefly, young healthy non-smoking volunteers were recruited, consented, screened and challenged with 80000 CFU/nostril of SPN6B/BHN418. Volunteers were then followed up and colonization assessed using microbiological detection unless otherwise specified.

Complementary EHPC data sets

A large collaborative EHPC data set, including molecular colonization status, viral infection status, abundance of nasal operational taxonomic units (OTUs) and cytokines was used. Methods used to obtain this data have been previously described [210, 385].

MUC5AC ELISA

ELISA for MUC5AC was performed as outlined in Chapter 2.

Pneumococcal - mucin growth assay

Detroit 562 cells were cultured according to ATCC guidelines and seeded into a 24 well plate at 5 x 10^6 cells/well. Pneumococcus was grown as outlined in Chapter 2. Bovine serum albumin (A7906, Sigma) and commercially available mucin mixtures (mucin from porcine stomach (GM, M2378, Sigma) and mucin from bovine submaxillary gland (SM, 499643, EMD Millipore)) were prepared at 10 mg/ml in

saline or *in vivo* mimicking chemically defined media (CDM) as previously described [386] with or without glucose (CDM-G or CDM, respectively) and stored at -20°C degree (from here on referred to as condition mixtures). On day of assay, condition mixtures were thawed, fresh media prepared and where appropriate condition mixtures diluted to desired concentration of 1 mg/ml. Pneumococcal adherence assay was performed as described in Chapter 2 with the following alterations. After washing cells with $HBSS^{+/+}$, 200 µl of condition mixture (containing SM, GM or BSA) was added to wells with and without Detroit 562 cells. according to plate layout and experimental design. For mock control the respective condition media (saline, CDM or CDM-G) was used. Bacteria were thawed, diluted to $5x10^6$ CFU/ml and 200 µl added to each well. Adherence assay was resumed as previously described. Growth was measured as the fold change of total CFU retrieved after 3 hours of incubation. Results were corrected for the dose of bacteria added prior to incubation. For each condition a minimum of two biological replicates were analysed.

Statistical analysis

Graphical and statistical analysis were performed as outlined in Chapter 2. For volunteer data spanning multiple time points, significance analysis was performed using non-parametric Friedman with uncorrected Dunn's post-hoc test.

5.3 Results

5.3.1 Nasal MUC5AC and experimental colonization susceptibility

5.3.1.1 Experimental approach

Baseline levels of MUC5AC in nasal wash measured by ELISA were analysed for 96 volunteers, (study numbers 15/NW/0146 and 14/NW/1460, conducted between 2015-2017) separated into three cohorts. Cohorts were comparable regarding volunteers age, sex and gender distribution (Table 5-1). Volunteers belonging to cohort 2 and 3 were part of the control arm of a vaccination study and thus received intramuscular vaccination with tetravalent influenza vaccine three days prior to or post pneumococcal challenge in combination with a nasal saline spray (Figure 5-1). Colonization status was determined by classical microbiology and molecular methods. All volunteers were young healthy non-smoking adults who received 80,000 CFU/nostril of pneumococcal strain SPN6B/BHN418 at Day 0. Importantly, nasal MUC5AC levels prior to experimental challenge were not differentially influenced by time point of study conduct (Figure 5-2).

Table 5-1: Characteristics of analysed cohorts

	n	mean age (years)	Gender distribution (Female/Male)	Colonization outcome (POS/NEG)
Cohort 1	20	29	13/7	7/13
Cohort 2	33	21	20/13	18/15
Cohort 3	43	21	29/14	21/22

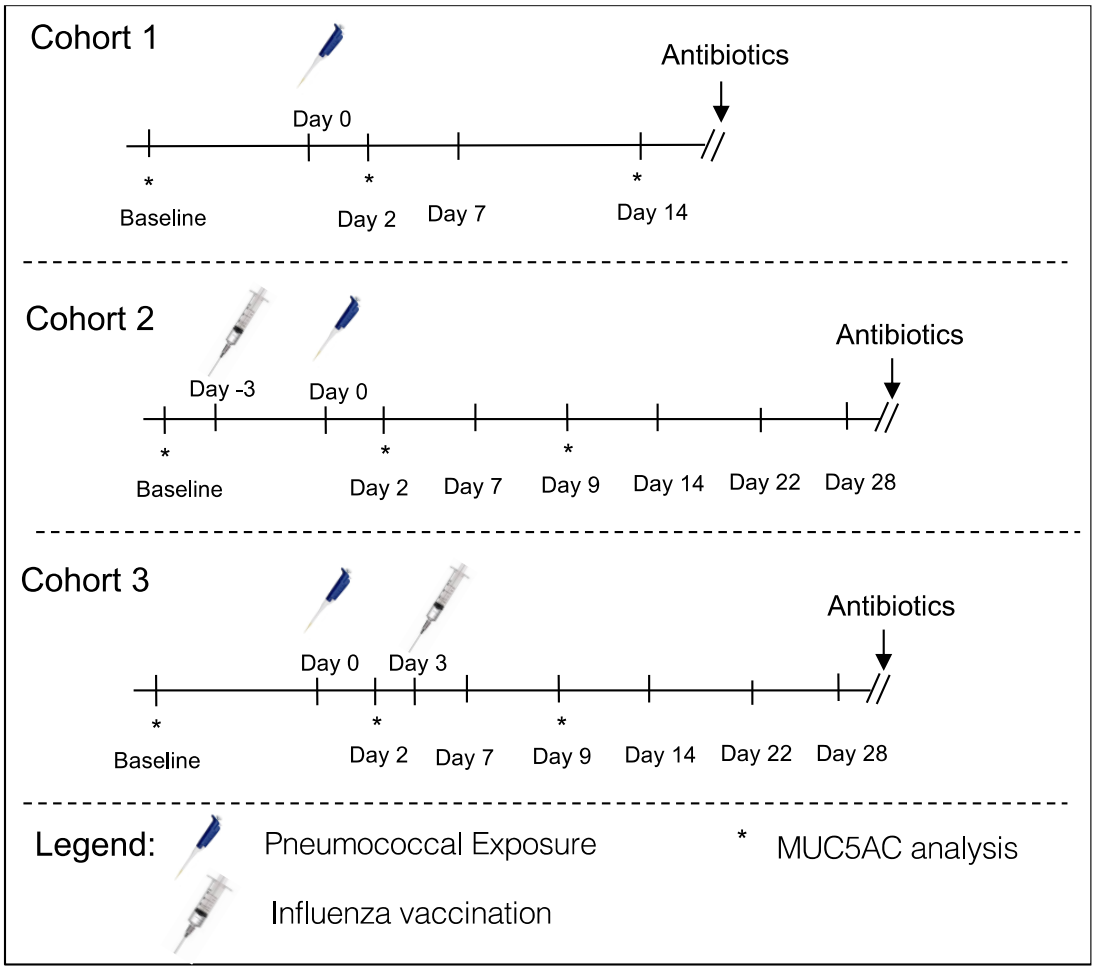


Figure 5-1: Study layout of cohorts analysed for MUC5AC.

Young healthy non-smoking volunteers received 80,000 CFU/nostril of pneumococcal strain SPN6B/BHN418 at Day 0. Volunteers were vaccinated with intramuscular tetravalent influenza vaccine in combination with a nasal saline spray three days prior to (Cohort 2) or three days post (Cohort 3) pneumococcal challenge. Nasal samples were obtained at baseline for all cohorts and at Day 2, 7, 9, 14, 22 and 28 after pneumococcal challenge for cohort 2 and 3 and at Day 2,7 and 14 after pneumococcal challenge for Cohort 1.

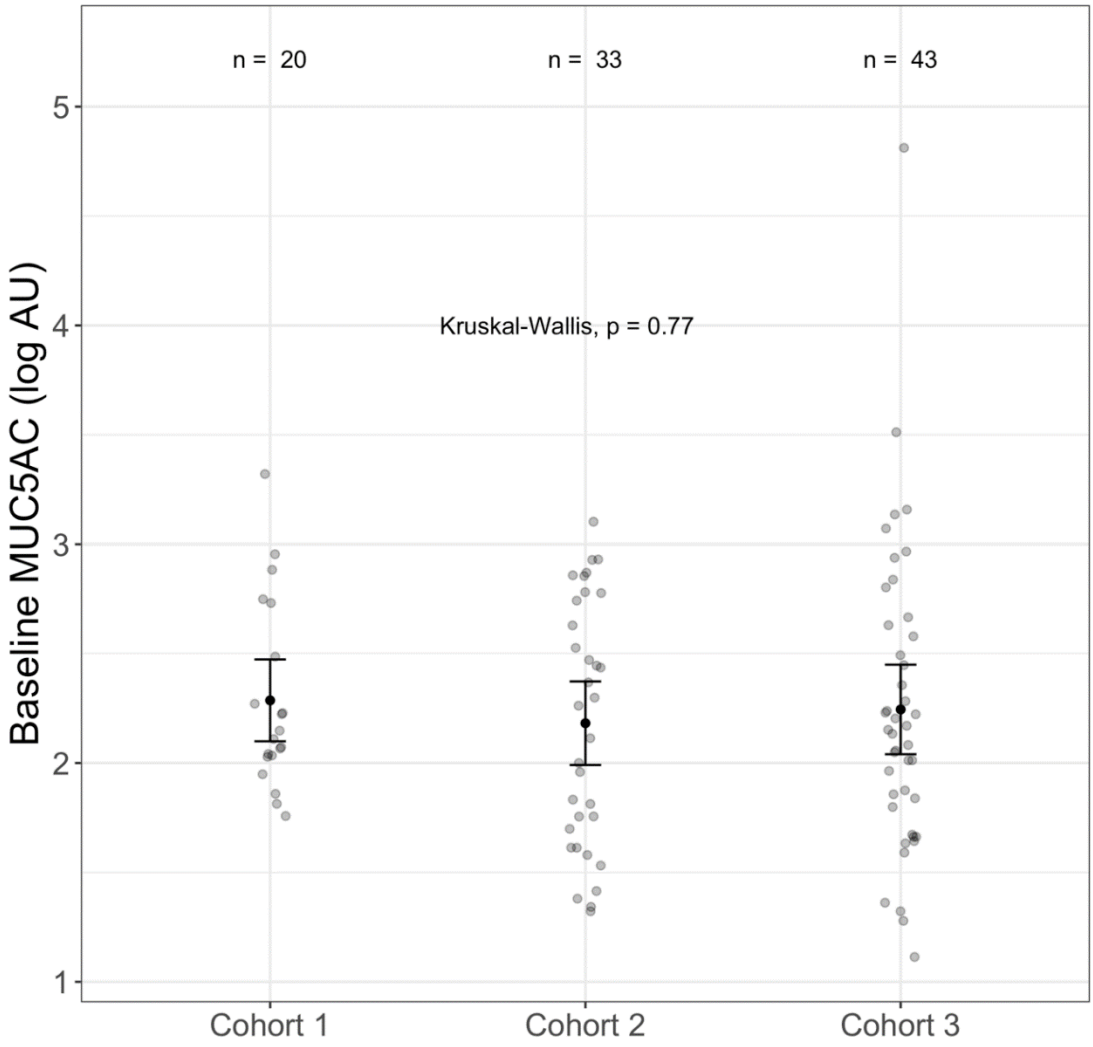


Figure 5-2: Effect of study time on levels of MUC5AC in nasal wash.

Baseline MUC5AC levels were measured in nasal wash samples of Cohort 1 (study conducted Summer 2015), Cohort 2 (study conducted in Winter 2016) and Cohort 3 (study conducted in Winter 2017). All samples were obtained from young healthy non-smoking adults. Mean ± SEM is shown. Significance determined using Kruskal - Wallis test. Significance levels $P < 0.05$.

5.3.1.2 High MUC5AC levels at baseline are associated with increased pneumococcal load after challenge

I investigated the effect of nasal MUC5AC on experimental pneumococcal colonization outcome in three cohorts. First, baseline levels of MUC5AC in nasal wash were associated with experimental pneumococcal colonization status after challenge as determined by classical microbiology.

No difference in nasal MUC5AC levels were observed between colonization positive and colonization negative individuals when all three cohorts were combined (Figure 5-3 A). Notably, asymmetric distribution of data points belonging to cohort 2 was observed between the two groups. Thus, analysis was repeated by separating the three cohorts (Figure 5-3 B). For cohort 1 and 3, no significant difference in nasal MUC5AC levels between colonization positive and negative individuals was observed. For cohort 2, a trend for higher MUC5AC levels was observed in individuals who were colonization positive compared to colonization negative individuals ($P = 0.055$).

To investigate the effect of nasal MUC5AC on experimental pneumococcal colonization density, data, separated by cohorts, was stratified using a combination of microbiology and molecular detection. Cohort 1 was excluded from the analysis as no molecular colonization data was available. Data was grouped into: those susceptible with densities over the threshold for bacterial detection by classical microbiology (EC+), those susceptible with densities under the threshold for bacterial detection by

classical microbiology but detected by molecular methods (EC+-) and those negative for experimental pneumococcal colonization (EC-).

For Cohort 3, no significant difference in MUC5AC levels between the three groups was observed (Figure 5-3 C). For Cohort 2, MUC5AC levels in nasal wash were significantly different between the three groups (Kruskal Wallis, P = 0.023, Figure 5-3 C). Volunteers belonging to the EC+- group showed significantly lower levels of MUC5AC prior to pneumococcal challenge when compared to volunteers of the EC+ group (Dunn's, P = 0.0069). Similar to the results presented earlier, there was no difference in MUC5AC levels between volunteers of the EC+ group and those negative for colonization (EC-). The relationship between nasal MUC5AC and experimental pneumococcal colonization density was further corroborated by a significant correlation between baseline MUC5AC levels in nasal wash and pneumococcal densities (determined by molecular detection) on Day 2 after challenge (n = 17, P = 0.0465, R = 0.4926, Figure 5-3 D).

Taken together the data suggest MUC5AC as a marker of experimental pneumococcal density *in vivo* in a subset of volunteers.

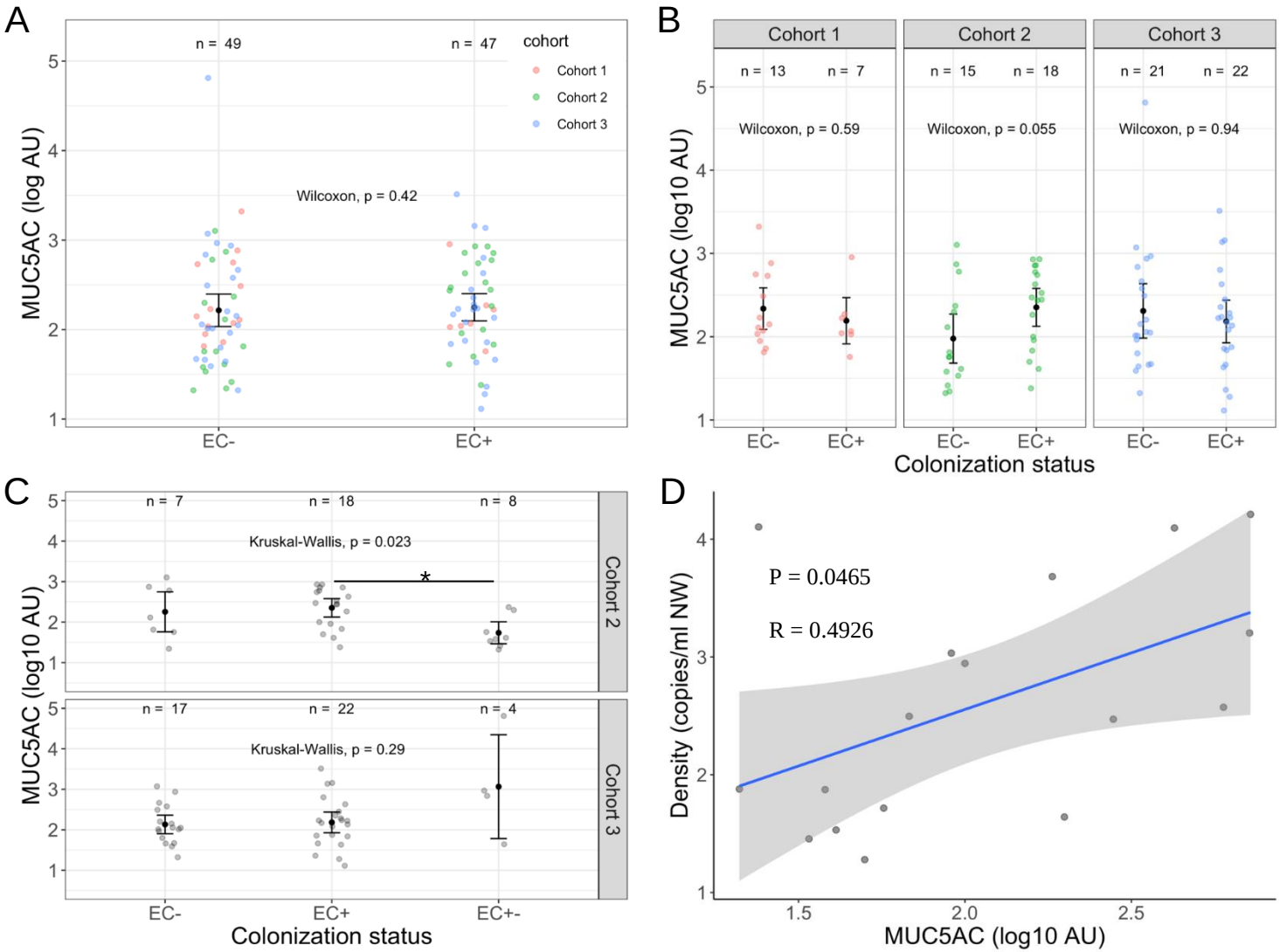

A
n = 49
n = 47
cohort
Cohort 1
Cohort 2
Cohort 3
Wilcoxon, p = 0.42
MUC5AC (log AU)
EC-
EC+
B
Cohort 1
Cohort 2
Cohort 3
n = 13
n = 7
n = 15
n = 18
n = 21
n = 22
Wilcoxon, p = 0.59
Wilcoxon, p = 0.055
Wilcoxon, p = 0.94
MUC5AC (log10 AU)
EC-
EC+
Colonization status
C
n = 7
n = 18
n = 8
Kruskal-Wallis, p = 0.023
*
Cohort 2
n = 17
n = 22
n = 4
Kruskal-Wallis, p = 0.29
Cohort 3
MUC5AC (log10 AU)
EC-
EC+
EC+-
Colonization status
D
P = 0.0465
R = 0.4926
Density (copies/ml NW)
MUC5AC (log10 AU)

Figure 5-3: High MUC5AC levels at baseline are associated with increased pneumococcal load after challenge.

(A) MUC5AC levels in nasal wash at baseline stratified by colonization status (colonization negative = EC- and colonization positive = EC+) (B) with additional stratification based on cohort. Colonization status was determined by growth of the challenge strain from nasal wash on Columbia blood agar at any time point following challenge with SPN6B/BHN418 (C) MUC5AC levels in nasal wash at baseline stratified by colonization status as defined by combination of molecular detection of the pneumococcal *lytA* gene and growth of challenge strain on Columbia blood agar from nasal wash at any time point following challenge with SPN6B/BHN418 (EC+ = *lytA* positive and growth positive, EC- = *lytA* negative and growth negative, EC+- = *lytA* positive and growth negative). (A/B/C) Significance was assessed using two-sample Wilcoxon or Kruskal-Wallis test with uncorrected Dunn's post-hoc. *$P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$. Bars represent mean ± SEM. (D) Correlation of MUC5AC levels in nasal wash at baseline and pneumococcal density on Day 2 following pneumococcal challenge as determined by molecular detection of *lytA* in Cohort 2. Those with negative colonization status on Day 2 were excluded. Significance was assessed using spearman correlation. Significance levels $P < 0.05$. All nasal wash samples were obtained from young healthy non-smoking volunteers.

5.3.1.3 Nasal factors influencing MUC5AC secretion *in vivo*

5.3.1.3.1 No significant impact of pneumococcal colonization on nasal MUC5AC levels

Aiming to investigate whether pneumococcal colonization induces nasal MUC5AC secretion in humans MUC5AC levels were measured in nasal wash before and up to 2 weeks post challenge (Figure 5-4). There was no significant difference of nasal MUC5AC levels over time independent of colonization status.

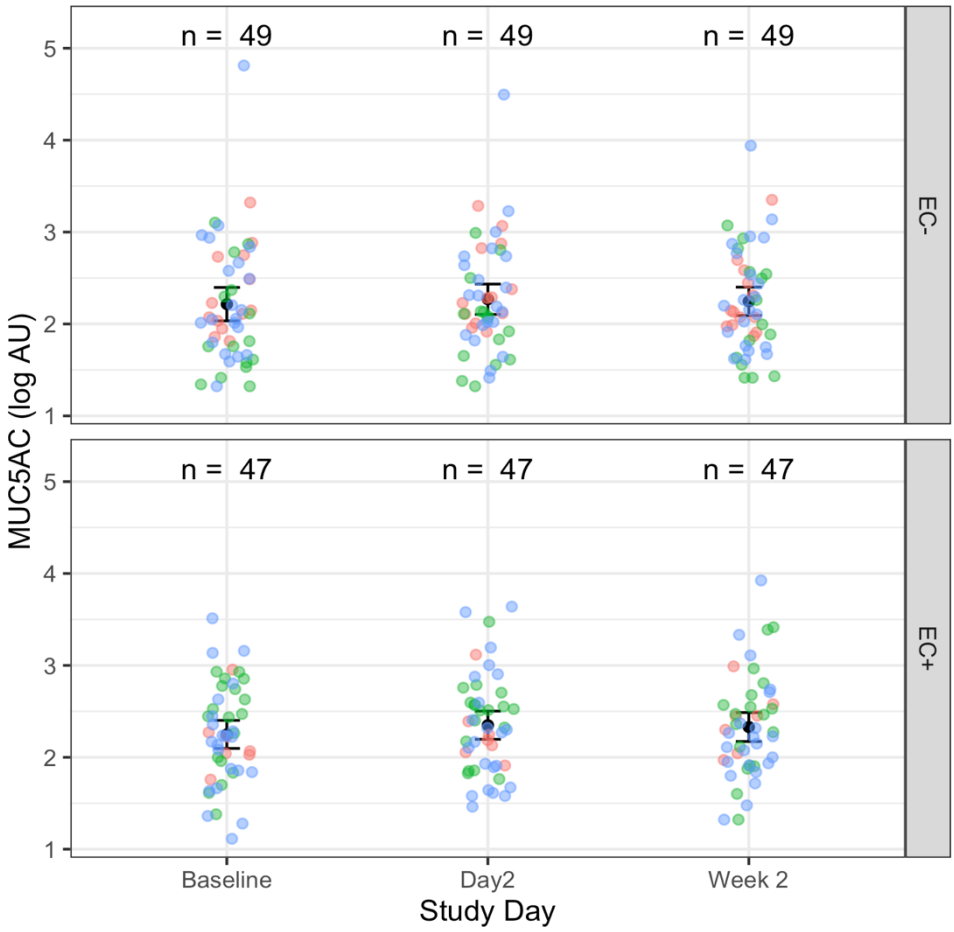


Figure 5-4: No significant impact of pneumococcal colonization on nasal MUC5AC levels
MUC5AC kinetics in nasal wash over two weeks stratified by colonization status as determined by growth of the pneumococcal challenge strain from nasal wash on blood agar at any time post challenge (colonization positive = EC+, colonization negative = EC-). Nasal wash samples were obtained from young healthy non-smoking volunteers. Mean ± SEM is shown. Significance levels determined using Friedman test with uncorrected Dunn's post-hoc. Significance level $P < 0.05$.

5.3.1.3.2 Increased nasal MUC5AC levels in those infected with respiratory virus

I investigated the effect of respiratory virus infection on nasal MUC5AC levels (Figure 5-5). A collaborative data set determining the presence of respiratory viruses at baseline in EHPC nasal samples was used. Virus data was matched with baseline MUC5AC data for 123 volunteers, of which 105 were negative, while 18 (17%) were positive for any of the tested respiratory viruses. Levels of MUC5AC in nasal wash were significantly higher (Wilcoxon, P = 0.012) in those positive for any respiratory virus at baseline compared to those who were negative for viral colonization. This concluded that presence of respiratory viruses is associated with higher levels of nasal MUC5AC.

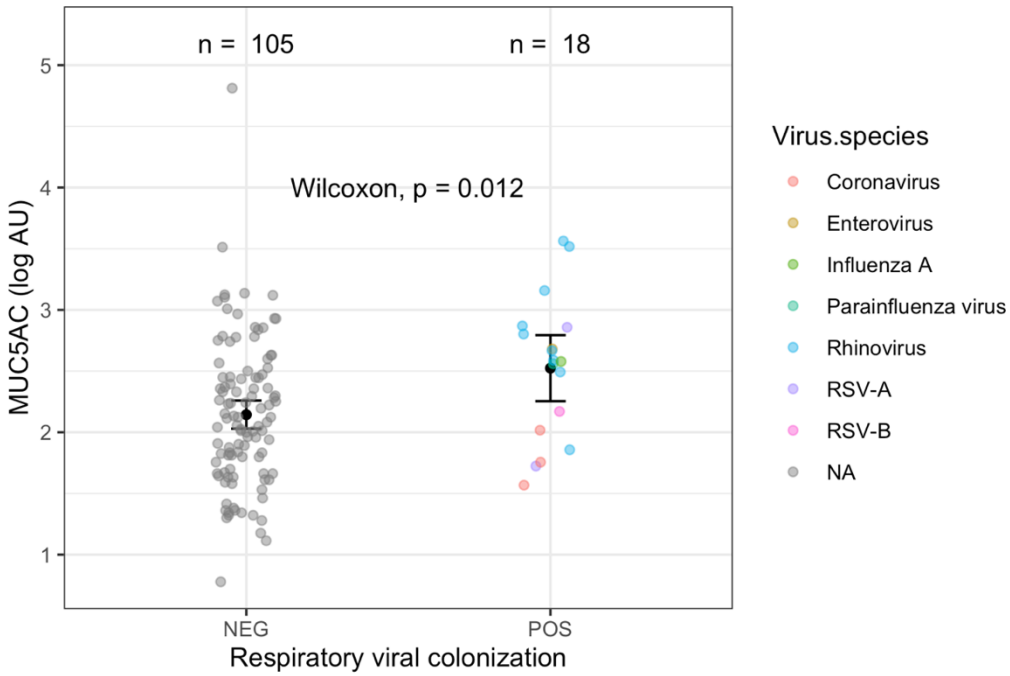


Figure 5-5: Increased nasal MUC5AC levels in those infected with respiratory virus

MUC5AC levels in nasal wash at baseline stratified by those positive for any respiratory virus (POS) and those negative for viral colonization (NEG). Nasal wash samples were obtained from young healthy non-smoking volunteers. Mean ± SEM is shown. Significance was assessed using two-sample unpaired Wilcoxon test. Significance level $P < 0.05$.

5.3.1.3.3 Inflammation influences levels of MUC5AC in the human nose

A data set determining the abundance of 30 cytokines in baseline nasal secretions of EHPC volunteers (Figure 5-6) was used to determine which cytokines are influencing nasal MUC5AC levels (Table 5-2). Cytokine data was matched with baseline MUC5AC data for 54 volunteers. After accounting for multiple testing cytokines involved in epithelial organ regeneration (hepatocyte growth factor, $P = 0.0001$, $R = 0.5022$), inflammatory cell recruitment (tumour necrosis factor alpha, $P = 0.0006$, $R = 0.4544$) and immunoregulation (Interleukin 10, $P = 0.0003$, $R = 0.4741$) showed significant correlation with levels of MUC5AC in nasal wash. Another 17 cytokines showed significant correlations with nasal MUC5AC ($P<0.05$) but did not reach the significance threshold of multiple testing adjustment. The data indicate a positive relationship between inflammation and levels of MUC5AC in the human nose.

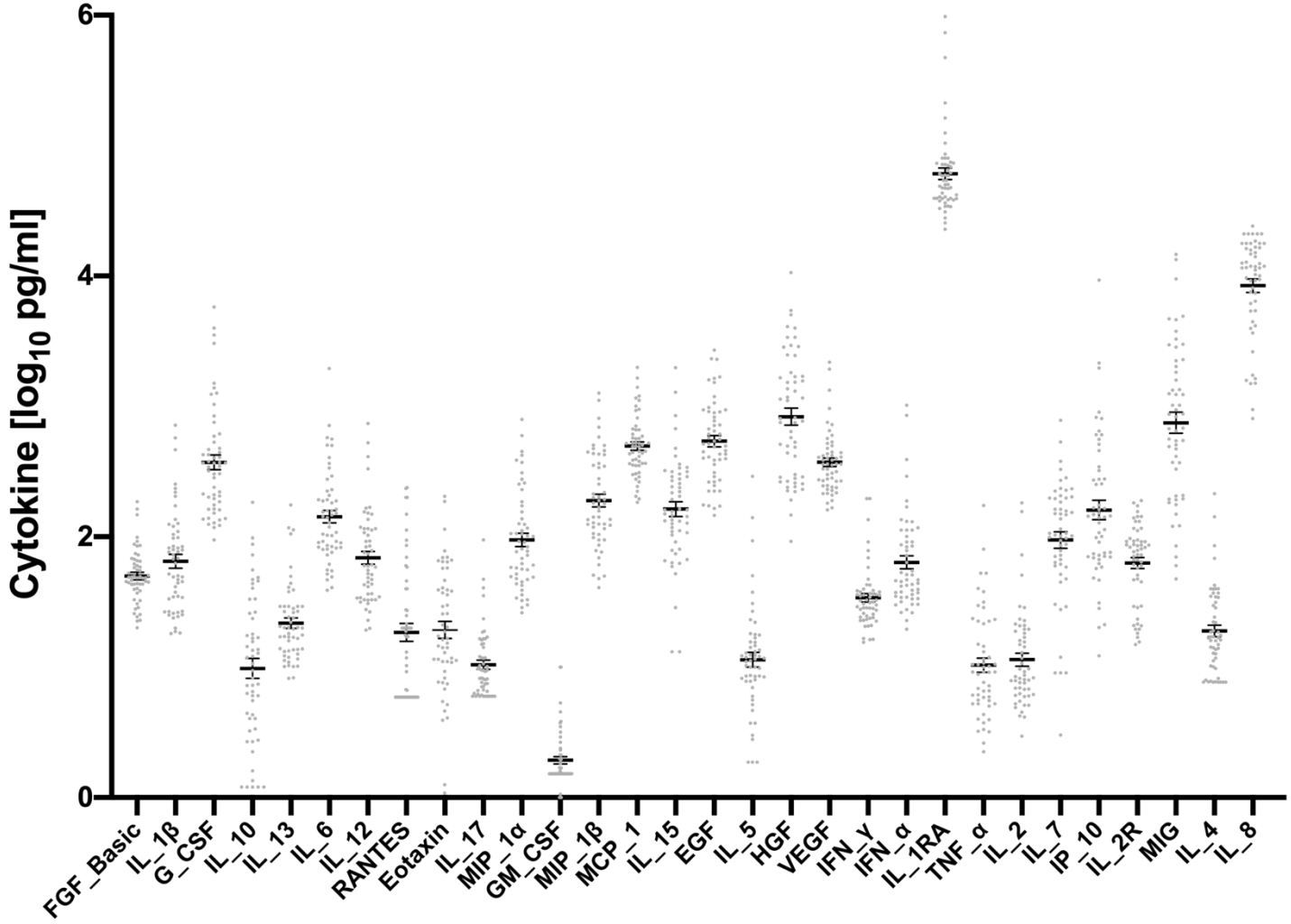


Figure 5-6: Abundance of 30 nasal cytokines prior to human challenge. Cytokines were measured in nasal fluid prior to pneumococcal challenge by 30-plex Luminex and expressed as pg/ml. Mean ± SEM is shown. Volunteers were healthy young adults enrolled into EHPC studies (n=54).

Table 5-2: Inflammation influences levels of MUC5AC in the human nose.

Growth factors	p-value	r-value	P < multiple testing adjusted P	Main Role [a]
HGF	0.00034977	0.48128875	TRUE	Hepatocyte Growth Factor
VEGF	0.00660923	0.37558261	FALSE	vascular endothelial growth factor
FGF_Basic	0.03904741	0.28992262	FALSE	basic fibroblast growth factor

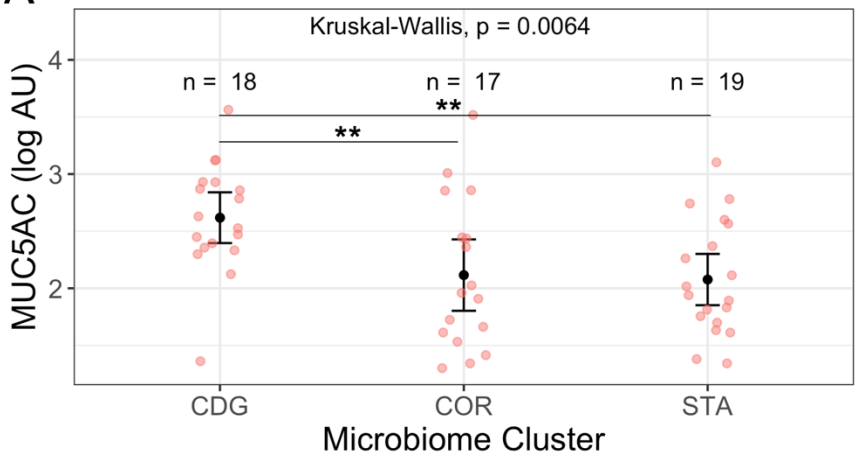
Cytokines/ Chemokines	p-value	r-value	P < multiple testing adjusted P	Main Role [a]
IL_10	0.00100749	0.44700398	TRUE	(Anti-) Inflammatory, Immune suppression
TNF_a	0.00165355	0.429635	TRUE	(Pro-) Inflammatory
MIP_1b	0.00366153	0.39971039	FALSE	chemoattractant
MIP_1a	0.00448108	0.39165554	FALSE	Inflammatory
G_CSF	0.00513084	0.38614354	FALSE	Differentiation and activation of granulocytes
IL_6	0.01008453	0.35716548	FALSE	(Pro-) Inflammatory and costimulatory action
Eotaxin	0.01070065	0.35449568	FALSE	eosinophil-specific chemoattractant
IL_2R	0.01685473	0.33328054	FALSE	Proliferation
IL_1b	0.02051997	0.32364188	FALSE	(Pro-) Inflammatory
MIG	0.0318143	0.30105435	FALSE	activation of cell-mediated responses
RANTES	0.03271965	0.29955275	FALSE	(Pro-) Inflammatory
IL_7	0.03543535	0.29524628	FALSE	Homeostasis, differentiation, and survival
IL_17	0.04760217	0.27876717	FALSE	(Pro-) Inflammatory

[a] based on information sourced from Sino Biological.Inc [387]

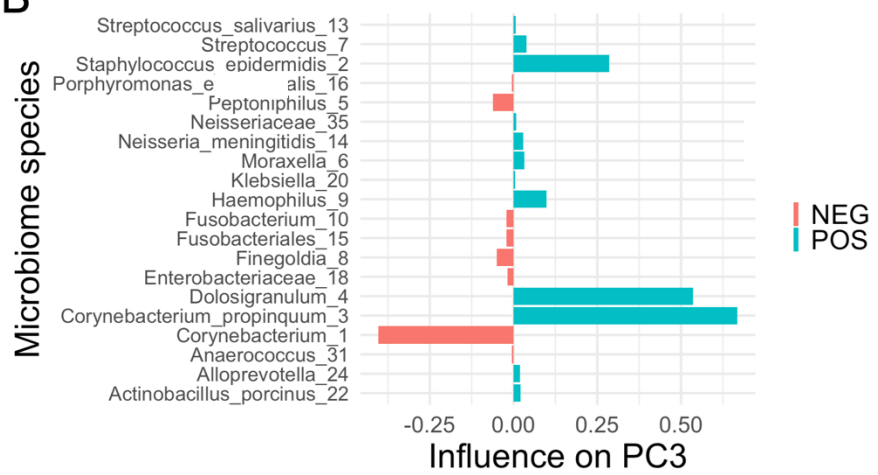
5.3.1.3.4 The local microbiome influences levels of MUC5AC in the human nose

A data set detailing the relative abundance of nasopharyngeal microbiome species in baseline nasal wash samples was utilized to investigate the influence of the local microbiome on MUC5AC levels in the nose prior to pneumococcal challenge. Microbiome data (485 OTUs) and cluster identification was matched with baseline MUC5AC data for 64 volunteers. First, OTU clusters[1] as defined previously [385] were associated with MUC5AC levels in the nose at baseline. OTU clusters with less than 5 samples were excluded from analysis (FUS: n=3, HPH: n=3, PEP/MIX: n=3, STR: n=1). Mucin abundance was significantly different (Kruskal-Wallis, P=0.0064) between the three remaining clusters (CDG: n= 18, COR: n=17, STA: n=19, Figure 5-7 A). Highest levels of MUC5AC were identified for those volunteers within the CDG cluster compared to COR cluster (Dunn's, P = 0.0085) and STA cluster (Dunn's, P = 0.0042). Second, correlations between baseline levels of nasal MUC5AC and nasopharyngeal OTUs were investigated using a principal component analysis approach. Briefly, levels of MUC5AC correlated (R > 0.3) with one principal component (PC). The top 20 influences on this component (loadings) were identified (Figure 5-7 B), of which the 10 strongest influences were selected for individual correlation analysis with MUC5AC. After adjusting for multiple testing, significant correlation was noted for Corynebacterium propinquum_3 (P = 0.0023, R = 0.4133, Figure 5-7 C and D). Another 2 OTUs showed significant correlations with baseline MUC5AC levels (P<0.05, Figure 5-7 C) but did not reach the significance threshold of multiple testing adjustment. Taken together, the data indicate an influence of certain microbiome species on levels of MUC5AC in the human nose.

A



B



C

OTU	p-value Spearman	r-value Spearman	P < multiple testing adjusted P
Corynebacterium_propinquum_3	0.002322559	0.413327927	TRUE
Staphylococcus_epidermidis_2	0.008516775	-0.339533865	FALSE
Finegoldia_8	0.017976551	-0.33676906	FALSE
Dolosigranulum_4	0.055097033	0.26505927	FALSE
Neisseria_meningitidis_14	0.056683963	0.307723455	FALSE
Peptoniphilus_5	0.143646925	-0.203612613	FALSE
Moraxella_6	0.67781156	0.082124284	FALSE
Streptococcus_7	0.80446559	0.033836899	FALSE
Corynebacterium_1	0.86168688	-0.023175276	FALSE
Haemophilus_9	0.890806425	0.019510321	FALSE

D

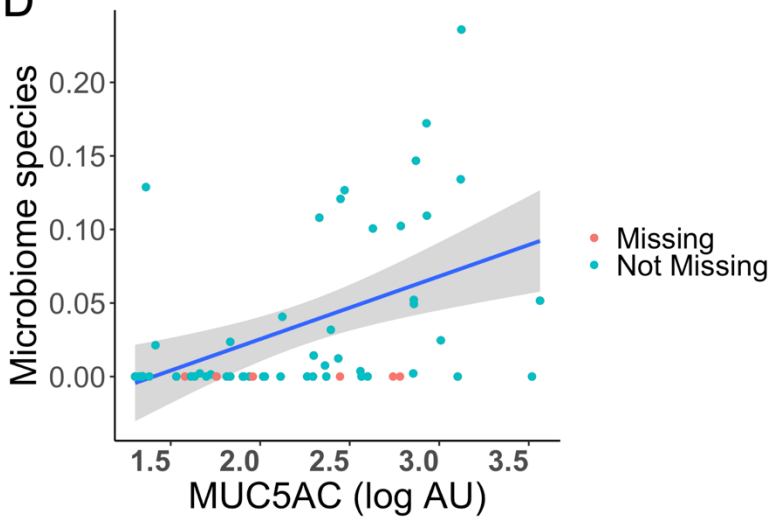


Figure 5-7: The local microbiome influences levels of MUC5AC in the human nose.

(A) Association of MUC5AC in nasal wash and microbiome clusters characterized by Corynebacterium_3 and Dolosigranulum_4 (CDG); Corynebacterium_1 (COR) and Staphylococcus_2 (STA). Mean ± SEM is shown. Significance determined using Kruskal-Wallis and uncorrected Dunn's post-hoc test. *P < 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001. (B) Impact of nasal microbiome species on principle component analysis. Colours represent the direction of impact. (C) Correlation analysis of the 10 most impacting nasal microbiome species and MUC5AC levels. Grey background indicates those with P< 0.05. (D) Spearman correlation of relative abundance of Corynebacterium propinquum_3 and MUC5AC levels. Nasal wash samples for MUC5AC and microbiome analysis were obtained from young healthy non-smoking volunteers. All analysis were performed using baseline data. Red dots indicate volunteers there no reading (relative abundance = 0) of this species amongst the nasal microflora was obtained.

5.3.2 *In vitro* effects of mucins on SPN6B/BHN418 growth and survival

5.3.2.1 Experimental approach

In the previous chapters I demonstrated a positive correlation of baseline nasal mucin protein MUC5AC and experimental pneumococcal density following pneumococcal challenge. Here, I aim to study the effects of mucins on pneumococcal growth and survival in an *in vitro* environment.

The rate of pneumococcal survival (fold change to challenge dose) was measured in different nutritional environments (saline or chemically defined media) with or without substitution of glucose as nutrient source. The types of substituted mucins were as follows: commercially available crude mucin mixtures of mucin from porcine stomach (GM) or mucin from bovine submaxillary glands (SM). Two concentrations were used (5 mg/ml and 0.5 mg/ml). Settings with mucin treatment were compared with the respective untreated control (Mock).

5.3.2.2 Effects of mucins under nutrient limiting conditions *in vitro*

First, I investigated pneumococcal survival in the presence and absence of mucins in saline. At 1 hour of incubation, pneumococcal survival was significantly different between the conditions (Kruskal-Wallis, $P < 0.0001$, Figure 5-8 A). Treatment with GM showed no improvement of survival when compared to non-treated conditions independent of dose. However, treatment with high concentrations (5 mg/ml) of SM increased survival significantly ($P = 0.0055$), while 10 times lower concentrations of

SM did not affect pneumococcal survival. Treatment with bovine serum albumin (BSA) showed increased pneumococcal survival (P = 0.0246) compared to non-treated conditions. At three hours of incubation, treatment with BSA or 5 mg/ml SM still showed significantly improved survival when compared to non-treated conditions (P = 0.0025 and P = 0.02, respectively; Figure 5-8 B).

Second, I investigated pneumococcal growth in the presence and absence of mucins using a specifically designed chemically defined media (CDM) mimicking the nasal environment. Under nutrient limiting conditions pneumococcal growth was significantly different between the conditions after 3 hours of incubation (Kruskal-Wallis, P = 0.0326; Figure 5-8 C). High concentrations (5 mg/ml) of GM and SM resulted in a significant increase of pneumococcal growth when compared to non-treated conditions (P = 0.0094 and P = 0.0257, respectively), while lower concentrations did not alter pneumococcal growth significantly. Under non-nutrient limiting conditions (CDM substituted with glucose, CDM-G), pneumococcal growth was not significantly different between the conditions (Kruskal-Wallis, P = 0.2752, Figure 5-8 D). BSA had no significant effect on pneumococcal growth independent of media used.

The data indicate that mucins can increase pneumococcal growth and survival *in vitro*.

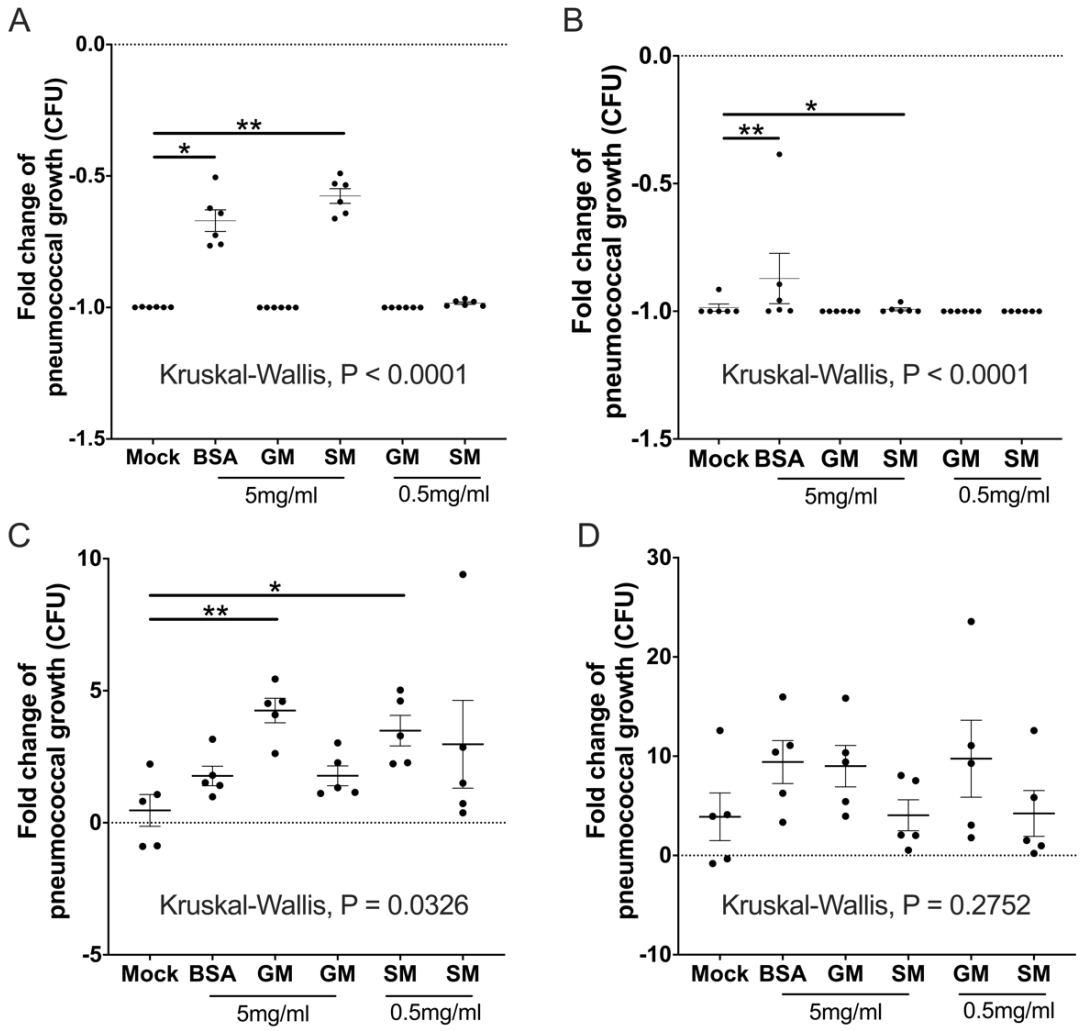


Figure 5-8: Effect of mucins on pneumococcal growth and survival *in vitro*.

(A/B) Survival of pneumococcus SPN6B/BHN418 was measured as colony forming units in saline after one hour (A) or three hours (B) of incubation in the presence of bovine serum albumin (BSA), mucin from porcine stomach (GM) or mucin from bovine submaxillary glands (SM). (C/D) Growth of pneumococcus SPN6B/BHN418 measured as the total amount of SPN6B/BHN418 recovered after 3 hours of incubation. Incubation was carried out in chemically defined media with (C) no-carbon source (CDM) or (D) glucose (CDM-G) in the presence of bovine serum albumin (BSA), mucin from porcine stomach (GM) or mucin from bovine submaxillary glands (SM). (A-D) Bacterial starting concentration = $5x10^6$/ml. Settings with treatment were compared with the respective untreated control (Mock). Data depicted as mean ± SEM of a minimum of five independent experiments. P-values were assessed using Kruskal-Wallis with uncorrected Dunnett's test. *P < 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.

5.3.2.3 Presence of nasal cells increases pneumococcal growth

The effect of nasal cells on pneumococcal growth was assessed using the experimental setup of a pneumococcal adherence assay. Notably, pneumococcal growth (total of adhered and non-adhered bacteria) was increased in the presence of nasal cells compared to growth systems where nasal cells were absent (Figure 5-9). Significant increase (Mann-Whitney test, $P < 0.05$) of bacterial growth in the presence of nasal cells was observed under both mucin treated and non-treated conditions. Significant increase in bacterial growth in the presence of nasal cells was further seen when using CDM as well as CDM-G growth media.

The data implicate the hypothesis that nasal cells may provide an additional nutrient source for pneumococcal growth independent of the nutritional status of the environment.

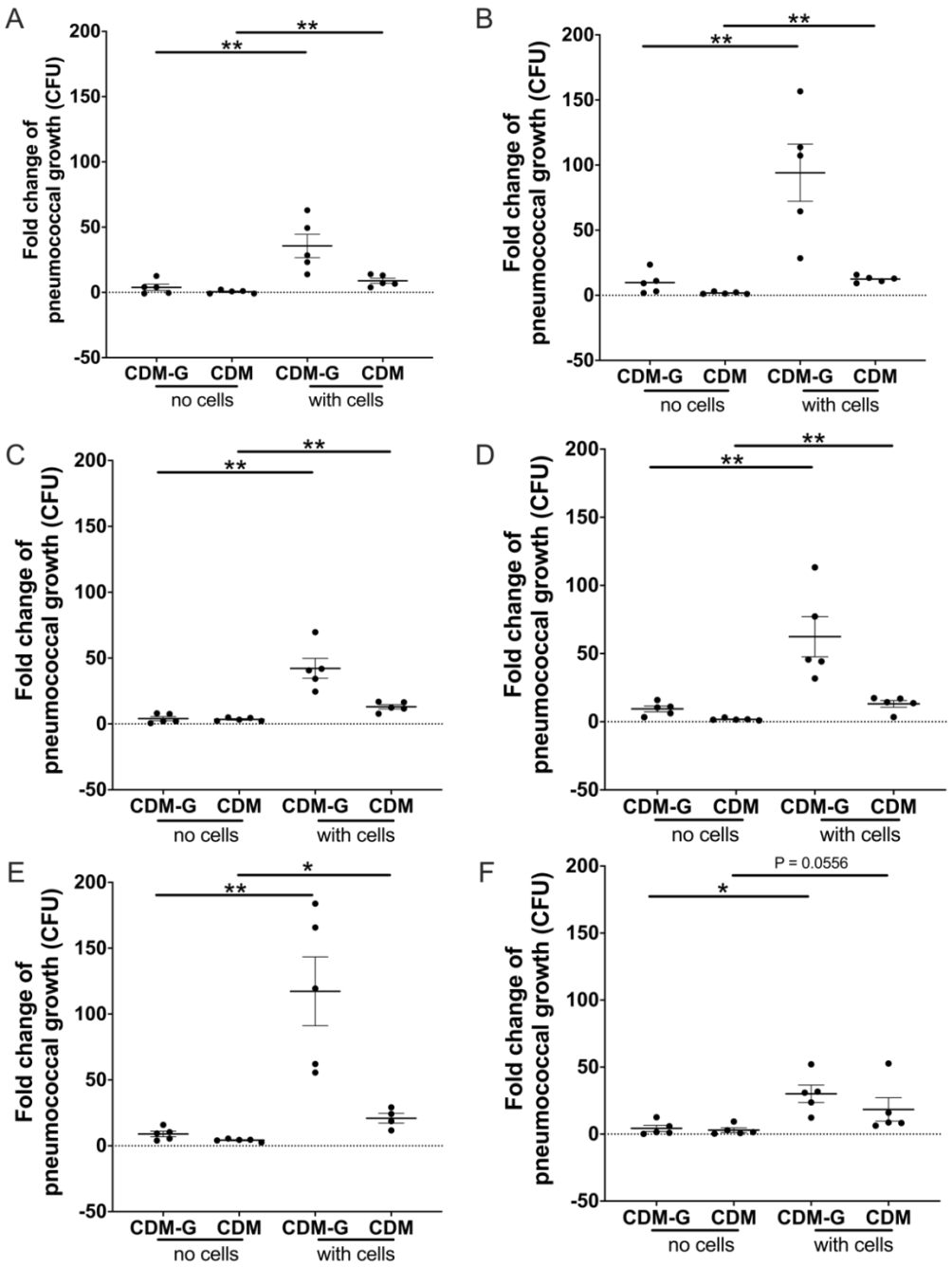


Figure 5-9: *In vitro* effect of nasal cells on pneumococcal growth.

Growth of pneumococcus SPN6B/BHN418 was compared in chemically defined media with no-carbon source (CDM) or chemically defined media with glucose (CDM-G) with or without Detroit 562 cells. Cells were seeded in a 24 well plate at $5x10^6$ cells/well and grown until confluency. $1x10^6$ CFU of pneumococcus was added for 3 hours to 24 well plates with or without Detroit 562 cells with (A) no-treatment, 5 mg/ml of (B) bovine serum albumin, (C) mucin from porcine stomach or (E) mucin from bovine submaxillary glands and 0.5 mg/ml of (D) mucin from porcine stomach or (F) mucin from bovine submaxillary glands. Growth was measured as fold change of challenge dose to the total of recovered colony forming units after three hours of co-incubation. Data is depicted as mean ± SEM of a minimum of five independent experiments. P-values using Mann-Whitney test. *$P < 0.05$, ** $P \leq 0.010$

5.4. Discussion

Control of pneumococcal colonization is one of the key milestones aimed at by health care professionals. Mucus and in extension mucins are a vital part of the human defence system against all kind of foreign pathogens including the pneumococcus. However, degradation of mucins by the pneumococcus may increase the availability of nutrients in this otherwise nutrient-poor niche [262-268], as well as influence pneumococcal adherence and internalisation [303-305]. Whether mucins are in fact protecting against or facilitating pneumococcal colonization is key question. Data presented in this chapter, demonstrate that, in a subset of volunteers, high baseline levels of MUC5AC in the nasopharynx associated with increased pneumococcal load after challenge. The association was corroborated by a positive directed correlation of baseline nasal MUC5AC and pneumococcal density 2 days after challenge, indicating MUC5AC as a marker of pneumococcal density in humans.

The lack of reproducibility of the presented association between nasal MUC5AC and experimental pneumococcal density during colonization is an important drawback of this study. Regulation of mucin genes and secretion is complicated as are its mediators and pathways. Climate conditions (such as humidity and temperature) as well as wall shear stress can affect mucin secretion [388]. In addition, inflammation, viral infection as well as the nasal microbiome have been shown to influence pneumococcal colonization outcome [385] and MUC5AC levels as demonstrated in this thesis and by others [267, 389, 390]. Given the complexity of pneumococcal interactions with nasal mucus as well as other colonization influencing factors *in vivo*, studies of causality are needed in order to determine the true relationship between nasal MUC5AC and

bacterial load during human pneumococcal colonization as well as to investigate potential mechanisms and factors involved.

Utilization of a variety of alternative carbon sources by the pneumococcus, including capsular polysaccharide, sialic acid, hyaluronic acid and mucins, has previously been demonstrated [266, 268, 316]. Use of sialic acid by the pneumococcus has also been associated with increased pneumococcal proliferation, colonization, and aspiration in mouse models of colonization and infection [267]. Thus, increased amounts of mucins in the human nose may facilitate pneumococcal growth and survival *in vivo*, leading to increased pneumococcal loads, as seen here during human pneumococcal colonization with SPN6B/BHN418. Assessing growth behaviour of SPN6B/BHN418 *in vitro*, mucins indeed increased pneumococcal survival and growth of strain SPN6B/BHN418 when compared to mock conditions. In congruence with previous published data [313] this effect was not observed in the presence of an alternative carbon source, suggesting that the pneumococcus only utilizes mucins under nutrient limiting conditions. Interestingly, bovine serum albumin (BSA) – while naturally lacking glycosylation – significantly increased pneumococcal survival and resulted in a small but non-significant increase in bacterial growth. Similar effects of serum albumin have been previously shown in other research settings [391-393]. BSA preparations are not always pure and may provide additional nutrients in the form of residual sugar and lipids thereby perhaps allowing for the effect seen here.

A limitation of this study is the use of commercially available crude non-human mucin preparations. While commonly used in research as alternative to human mucins, these preparations may have different composition and properties when compared to human nasal mucus. The use of recombinant mucins may provide a valid alternative, however, at present full-length recombinant mucins are extremely limited and expensive.

Alternative carbon sources in the shape of glycoproteins are not only available in nasal secretions, but also present on the apical surface of the nasal epithelium. Here I demonstrate that the presence of the nasal cell line Detroit 562 significantly enhanced pneumococcal growth compared to conditions where nasal cells were absent. The effect was further independent of the wider nutritional environment. This result indicates that nasal cells by themselves may provide or secrete alternative carbon sources used by the pneumococcus. While the nature of this alternative carbon source remains yet to be identified, it seems likely that cell surface and/or secreted mucins may play a role. Targeted depletion of mucins and other secreted factors may provide a path to studying this phenomenon further. Epithelial cell death in response to bacterial challenge may provide an alternative source of nutrients allowing for the facilitating effect of nasal cell presence on pneumococcal growth. However, the bacterial/cell ratio (MOI) used here heavily favoured cells over bacteria (MOI > 1:5). Previous publications showed no effect of pneumococcal challenge on epithelia cell survival for up to four hours when using experimental setups where the pneumococcus outnumbered cells up to an MOI of 37:1 [324, 394].

Mouse models and *in vitro* assays demonstrate the ability of the pneumococcus to trigger MUC5AC secretion [267, 395, 396], further underlining a role for MUC5AC during pneumococcal colonization. In contrast, the data presented here showed no stimulating effect of human pneumococcal colonization on nasal MUC5AC levels. Rapid degradation of glycosylated MUC5AC by the pneumococcus and other microorganisms colonizing the nasopharyngeal niche may mask the stimulating effect of pneumococcal colonization on mucin secretion *in vivo*, perhaps explaining the lack of impact seen here.

Increased pneumococcal load during colonization has been previously associated with increased pneumococcal transmission and disease [155, 267, 397-400]. Disruption of mucin utilization by the pneumococcus may reduce pneumococcal density, transmission and disease and present a key to easing the global pneumococcal burden. This notion is supported by a publication demonstrating that treatment with vinpocetine (an anti-stroke drug) has a direct inhibiting effect on MUC5AC expression in colonized mice, leading to a decrease in pneumococcal loads, pneumococcal induced inflammation and hearing loss in a well-established mouse model of otitis media [395].

Mucin are known to account for both mechanical and structural protective effects of human mucus. For the first time this study investigated the effect of nasal mucins on human pneumococcal colonization. The data presented here implicates MUC5AC as a marker of pneumococcal density in a subset of volunteers. While the association found here does not implicate direct causality, it is possible that the pneumococcus may use nasal MUC5AC as an alternative energy source *in vivo*, thus, facilitating bacterial growth and survival and leading to increased pneumococcal load as has been shown here and in other research settings [267].

Chapter 6 General discussion

6.1 Introduction

This thesis had the following aims:

1. To identify microbial markers enabling estimation of experimental pneumococcal colonization rates prior to human challenge thereby allowing more rapid expansion of the EHPC model and reducing research waste
2. To explore the relationship between mucosal host factors and susceptibility to human pneumococcal colonization thereby enhancing the understanding of host-pneumococcal interactions and identifying potential vaccine and intervention targets

A discussion of the individual findings as well as their limitations has been provided in each chapter. Therefore, here I will provide a brief summary of the main findings and their implications for current and future pneumococcal research.

6.2 Summary of main findings

The experimental human pneumococcal carriage model offers a unique opportunity to study human colonization, thereby enabling intervention and vaccine development. It also presents an alternative endpoint for vaccine testing. Colonization rates achieved by different serotypes implemented into the EHPC model determine costs as well as human resources necessary for conducting human challenge studies. To be considered successful, candidate strains must achieve colonization rates of 40% or more. Several attempts to establish new pneumococcal strains in the EHPC model were made in the past, but success remained elusive, highlighting the need to establish a tool that enables the preselection of suitable pneumococcal candidates for human challenge.

Chapter 3 of this thesis sought to identify such a tool to estimate experimental colonization rates of pneumococcal candidate strains prior to human challenge. Data of four dose ranging studies using different pneumococcal challenge strains were compared, revealing superior experimental colonization rates (45%) of SPN6B/BHN418. Further analysis of microbial characteristics demonstrated that SPN6B/BHN418 showed highest chain length (defined as OD/CFU ratio) amongst the tested pneumococcal strains. The implicated association between chain length and experimental colonization rates was further corroborated by a previous study demonstrating that increased chain length can facilitate pneumococcal adherence and subsequent colonization in a mouse model [274]. On the account of these results, I hypothesised that assessment of pneumococcal chain length relative to SPN6B/BHN418 may be used to estimate success of pneumococcal candidate strains in the EHPC model prior to human challenge. The hypothesis was tested when assessment of pneumococcal chain length was used to select SPN3 strain candidates

for human challenge. Results showed that strain SPN3/PFESP231, demonstrating similar chain length when compared to SPN6B/BHN418, achieved experimental colonization rates of 45% on average, equal to those observed after challenge with SPN6B/BHN418.

The success of pneumococcal vaccination campaigns through its effects on both disease and colonization is undisputable. Despite this, the global burden of pneumococcal disease remains high. The improvement of current target formulations (for example by increasing number of serotype coverage with one vaccination), as well as the search for new vaccines are on the forefront of pneumococcal research.

Chapter 4 of this thesis sought to investigate mucosal factors influencing host susceptibility to experimental human pneumococcal colonization, thereby providing insights into new avenues of controlling pneumococcal colonization and disease. Immune exclusion – the process of crosslinking, entrapping and clearing pathogens - has long been recognized as an important protective mechanism against the pneumococcus. Here, I show that levels of MUC5AC in the human nose positively correlate with the capacity of nasal wash to form aggregates with the pneumococcus *ex vivo*, implicating a role of nasal MUC5AC in this important defence mechanism.

The first layer of the nasal mucosa, mucus is the primary defence mechanism against many pathogens including the pneumococcus. As the main component of mucus, the role of mucins, however, is complex. While mucins provide a physical barrier and play an important role in innate defence mechanisms [165-169, 374], bacteria such as the pneumococcus may also use mucins to their own advantage.

Chapter 5 aimed to investigate the effect of human mucins on growth and survival of the pneumococcus as well as answer the question whether nasal MUC5AC ultimately protects against or facilitates pneumococcal colonization in humans. The data presented, show a facilitating effect of mucins on pneumococcal growth and survival *in vitro* as well as an association of high levels of nasal MUC5AC and increased experimental pneumococcal density *in vivo* in a subset of volunteers.

6.3 Implications and limitations

The rapid expansion of the EHPC model is highly desirable but is hampered by the lack of a reliable method to accurately estimate experimental colonization rates prior to human challenge. Pre-selection of pneumococcal strain candidates for human challenge, based on the assessment of chain length (defined as OD/CFU ratio) relative to SPN6B/BHN418, highlighted a serotype 3 candidate strain that demonstrated equal experimental colonization rates compared to the gold standard EHPC strain SPN6B/BHN418. Nevertheless, the predictive value of chain length as a sole factor for experimental colonization rates remains questionable.

Previous reports suggest the influence of microbial factors as well as host characteristics such as nasal microbiome composition, pathogen-pathogen interactions and viral infection status on human pneumococcal colonization [155, 169, 253, 258, 330, 385]. In addition, previous pneumococcal colonization episodes may affect experimental colonization through serotype specific and unspecific host defence mechanisms. In fact, a recent study performed in mice showed, that the pneumococcus may induce epigenomic changes in the host epithelium, thereby reversibly altering cellular responses to the pneumococcus [193]. Efficiency of host defence mechanisms as well as ability of the bacterium to avoid the same have been reported to be serotype dependent [192, 366]. Lack of efficient host immune defence against pneumococcal strains such as SPN6B/BHN418 or SPN3/PFESP505 may allow the bacteria to achieve higher colonization rates independent of colonization facilitating microbial factors. The existence of multiple influences on colonization rates presents a challenge to current models and methods used to predict and understand colonization efficiency and will need to be addressed in future research approaches.

Protective effects of mucins against airway pathogens have long been demonstrated *in vitro* and in mouse models [165-169]. Data in this thesis, now demonstrate a positive directed correlation between nasal MUC5AC and pneumococcal agglutination in nasal fluid of unvaccinated individuals. While the result may further corroborate the protective effect of nasal mucins, it remains unclear whether direct or indirect interactions of nasal MUC5AC with the pneumococcus contribute to the effect.

In the respiratory tract, where free nutrients are rare – the carbon rich mucins can also act as alternative energy source for pathogens such as the pneumococcus as shown in this thesis. So, do mucins ultimately protect against or facilitate pneumococcal colonization? While data presented here suggest a potential facilitating effect of nasal MUC5AC on pneumococcal colonization, the positive association between MUC5AC and pneumococcal density is to be interpreted with caution and in context of other factors influencing both mucin abundance and pneumococcal density. It is further important to note that while MUC5AC certainly is the most abundant mucin in the nasopharynx, other mucin proteins exist and may affect colonization outcome in one way or the other. Transmembrane mucins, have been shown to link to intracellular signalling pathways [401] and in case of MUC1 assimilate a protective role against pneumococcal disease [168]. In addition, the extracellular domain of several transmembrane mucins may - at release - activate EGF receptors thereby regulating mucin expression in a positive feedback loop [200]. Whether transmembrane mucins and in fact all mucins have an accumulating total effect or whether some mucins act protective while others act facilitating, remains unclear.

6.4 Future work

Using the EHPC model identification of both bacterial and human factors involved in pneumococcal colonization control offers to deliver imperative research and tools to reduce the global pneumococcal burden. Difficulties in accomplishing this goal may root in the complexity of pneumococcal interactions with the host as well as the nasal environment. In addition, current limitations on serotype availability for human challenge studies limits the impact of EHPC research and its use for vaccine testing. Considering this and the results of this thesis, the author proposes future work including but not limited to:

Chapter 3:

- the development or implementation of robust and high throughput methodology to measure pneumococcal chain length enabling the creation of larger data sets
- the challenge of human volunteers with otherwise isogenic strains only differing in chain length
- the use of multifactorial bioinformatic models to predict pneumococcal colonization rates and assess the weight of individual factors on colonization success

Chapter 4:

- Investigation of the effect of anti-RrgB sIgA on early pneumococcal control and clearance
- Investigation of protective effects of anti-RrgB sIgA involving multiple challenges and multiple piliated strains
- Investigation of epigenomic patterns associating with colonization susceptibility
- Performance of a genome wide association study to identify yet unknown host factors involved in pneumococcal colonization control

Chapter 5:

- the use of knock-out mice, human cell culture models or bioinformatic models to further dissect the role of individual mucosal factors in pneumococcal colonization such as MUC5AC

6.5 Conclusion

Understanding colonization of the human host by the pneumococcus is imperative in controlling the mortality rate associated with pneumococcal disease. Mice and *in vitro* models have for decades allowed a glimpse into the mechanisms of pneumococcal colonization and disease. The introduction of the EHPC model a decade ago allowed for the first-time controlled studying of the bacterium in its natural habitat. The here presented data lends credibility to the approach of using assessment of average pneumococcal chain length (defined as OD/CFU ratio) to pre-select pneumococcal candidate strains for human challenge studies. Association of mucosal host factors and host susceptibility to human pneumococcal colonization further implicate a dual role of human mucin 5AC during pneumococcal colonization, highlighting the need for further understanding of mucin-pneumococcal interactions during human colonization.

Chapter 7 Appendices

Appendix 1 – Evaluation of the ratio of optical density to colony forming units as a marker of pneumococcal chain length

Stock aliquots of pneumococcal strains used for EHPC challenge were immobilized on a microscopic slide, gram stained (exemplary images: Figure 7-1 to Figure 7-4) and the degree of chain formation was analysed using semi-quantitative microscopy while blinded for the pneumococcal strain. Semi-quantitative chain length assessment was then compared with quantitative assessment of OD/CFU ratio as described in Chapter 3 (Table 7-1). The results indicate that quantitative measurement of OD/CFU ratio may be used as a marker of the magnitude of chains (chain length) within a liquid culture of pneumococcus.

Table 7-1: Magnitude of pneumococcal chains in bacterial smears using two assessment methods

Strain	Semi-quantitative Chains/total SPN ratio[a]	Quantitative OD/CFU ratio[b]
SPN6B/BHN418	0.183 (601/3287)	2.28 ± 0.14
SPN15B/SH8286	0.127 (343/2702)	1.57 ± 0.08
SPN23F/P1121	0.028 (115/4042)	0.49 ± 0.04
SPN23F/P833	0.029 (102/3465)	0.96 ± 0.12

[a] Chains (>2 pneumococci) were identified by eye by an operator while blinded for the pneumococcal strain. Number of total SPN was measured using the particle analyse function in ImageJ. Shown is the the ratio of chains to total number of pneumococci of 3 images analysed. In brackets: the sum of pneumococcal chains and total pneumococci of three images analysed. [b] OD/CFU ratio [OD/CFU per ml $x10^{-9}$] of liquid pneumococcal cultures. Data shown are the calculated mean ± SEM of >3 independent experiments.

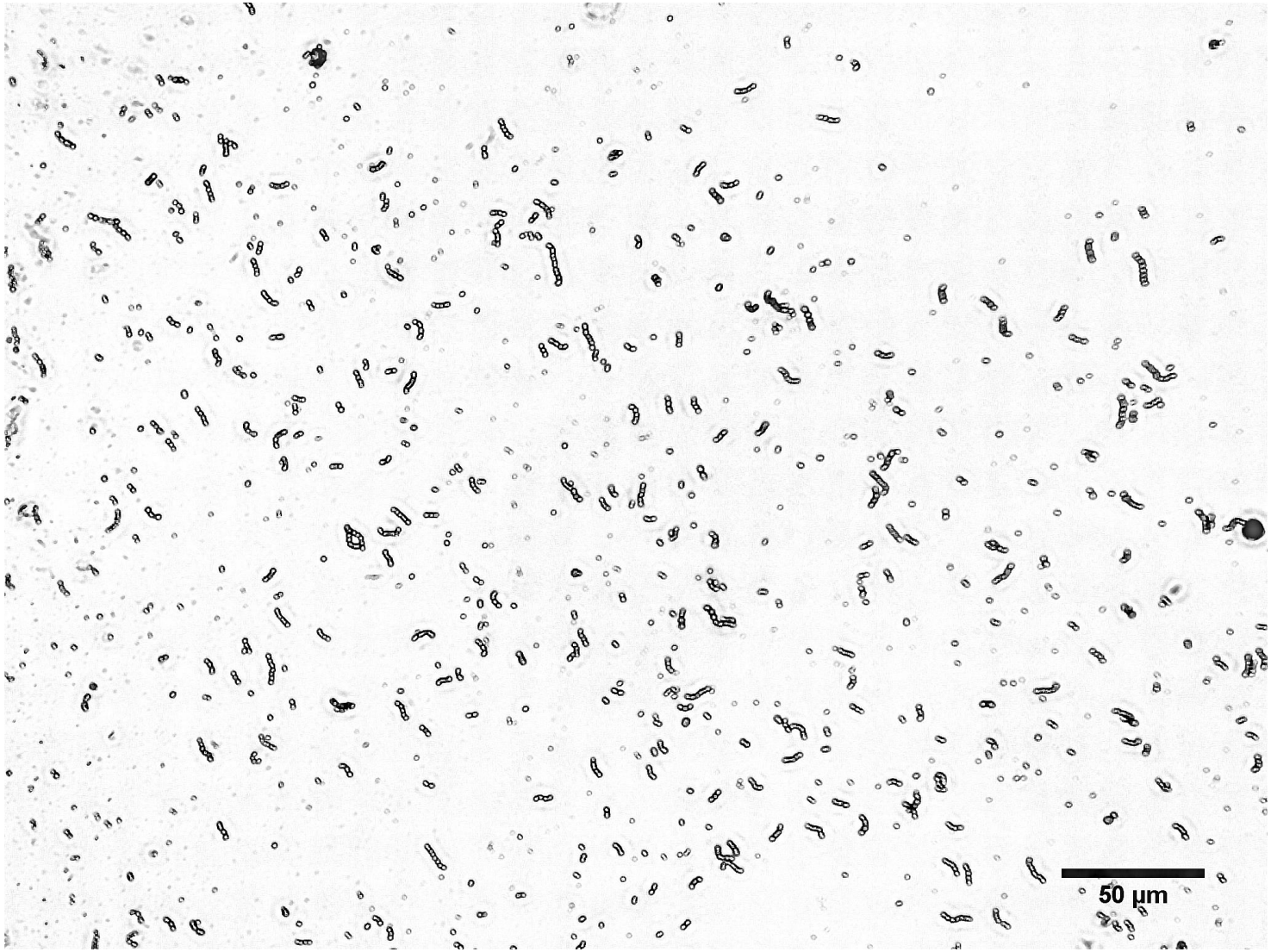


Figure 7-1: Visualisation of pneumococcal chains of strain SPN6B/BHN418.

Bacterial smear of a pneumococcal stock aliquot of SPN6B/BHN418 resuspended in saline, fixated on a microscopic slide and gram stained. Pictures were taken using EVOS XL Core and ImageJ. Picture was contrast enhanced and background subtracted using ImageJ functions.

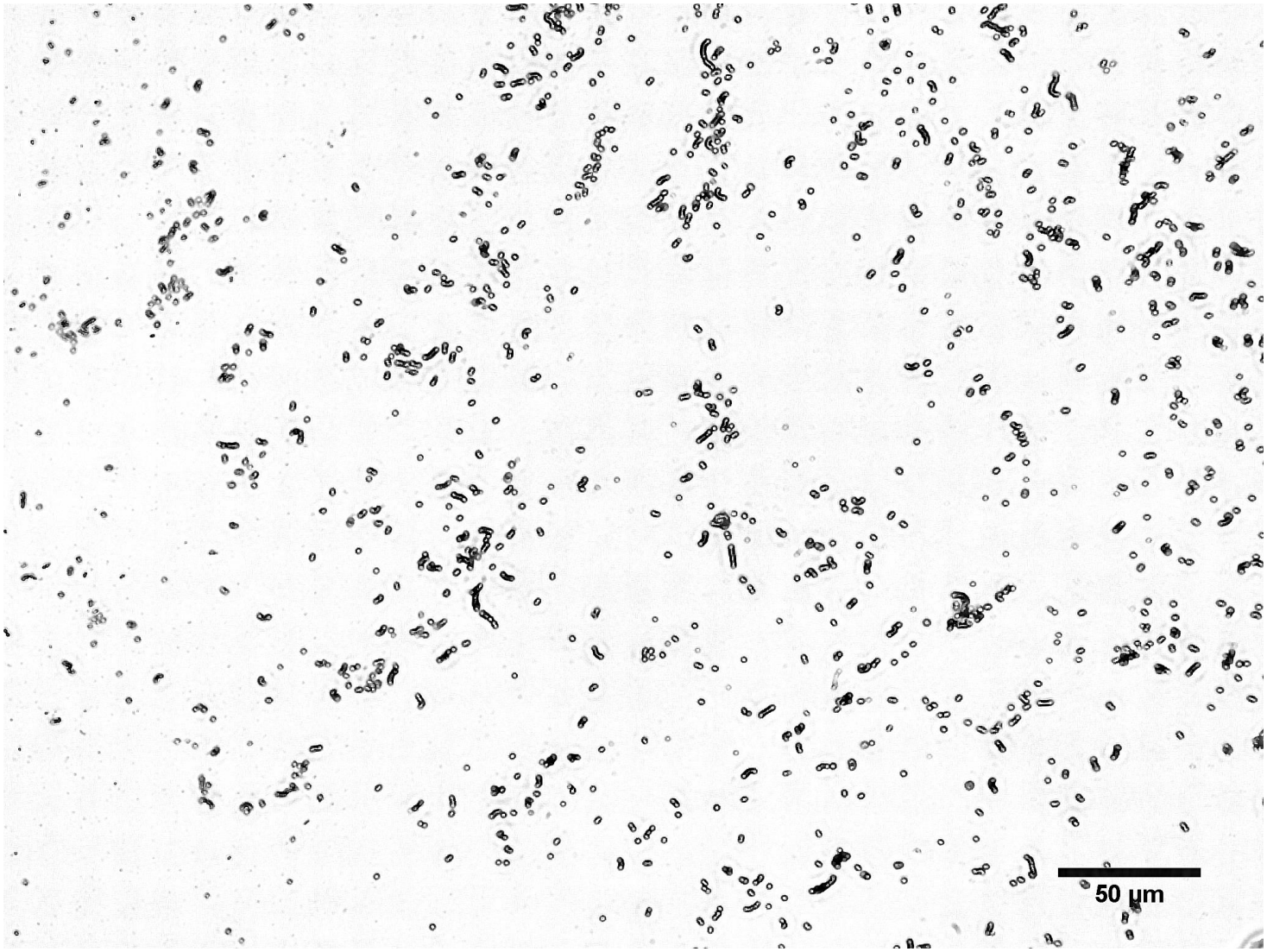


Figure 7-2: Visualisation of pneumococcal chains of strain SPN15B/SH8286.

Bacterial smear of a pneumococcal stock aliquot of SPN15B/SH8286 resuspended in saline, fixated on a microscopic slide and gram stained. Pictures were taken using EVOS XL Core and ImageJ. Picture was contrast enhanced and background subtracted using ImageJ functions.

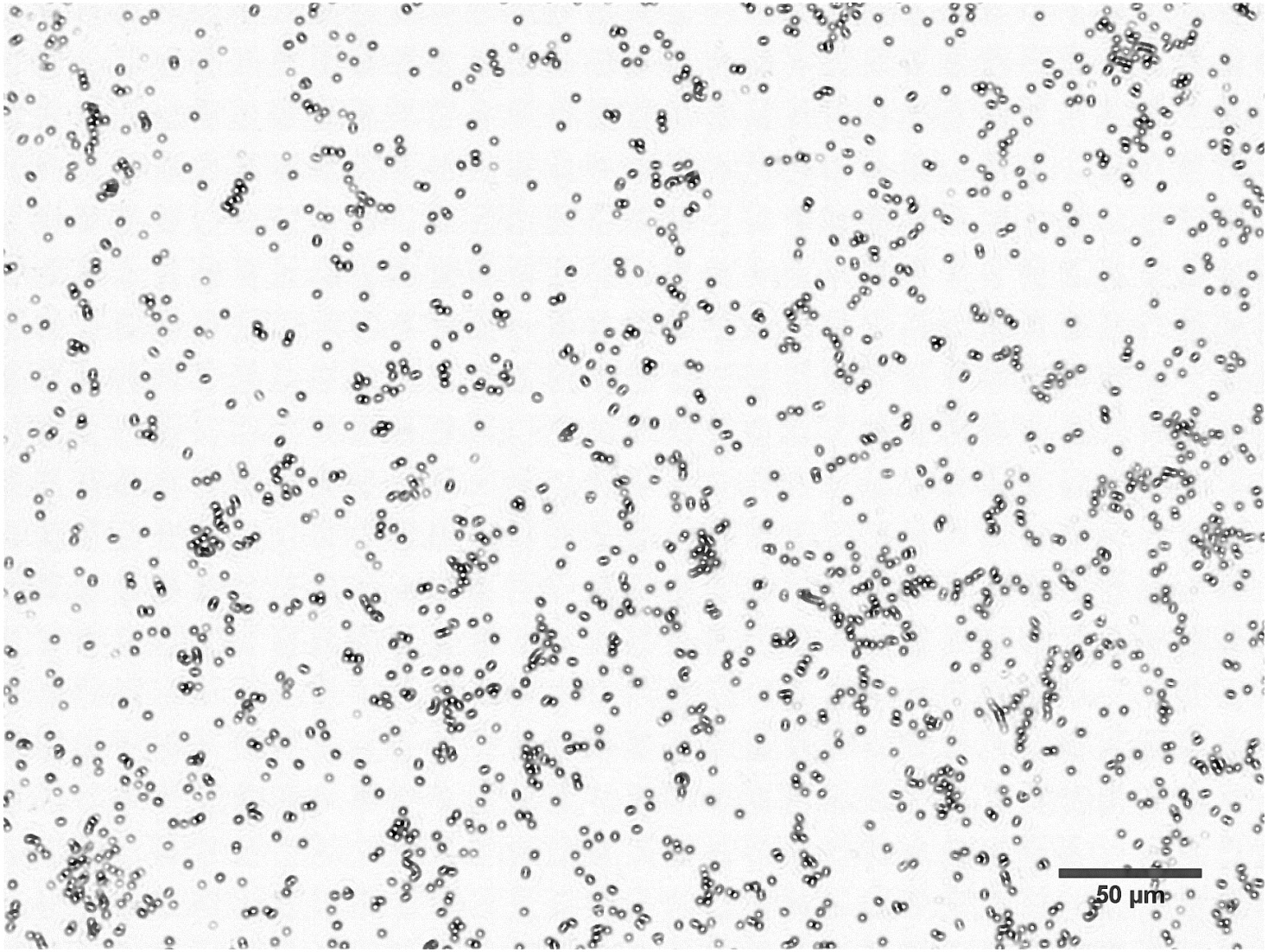


Figure 7-3: Visualisation of pneumococcal chains of strain SPN23F/P1121.

Bacterial smear of a pneumococcal stock aliquot of SPN23F/P1121 resuspended in saline, fixated on a microscopic slide and gram stained. Pictures were taken using EVOS XL Core and ImageJ. Picture was contrast enhanced and background subtracted using ImageJ functions.

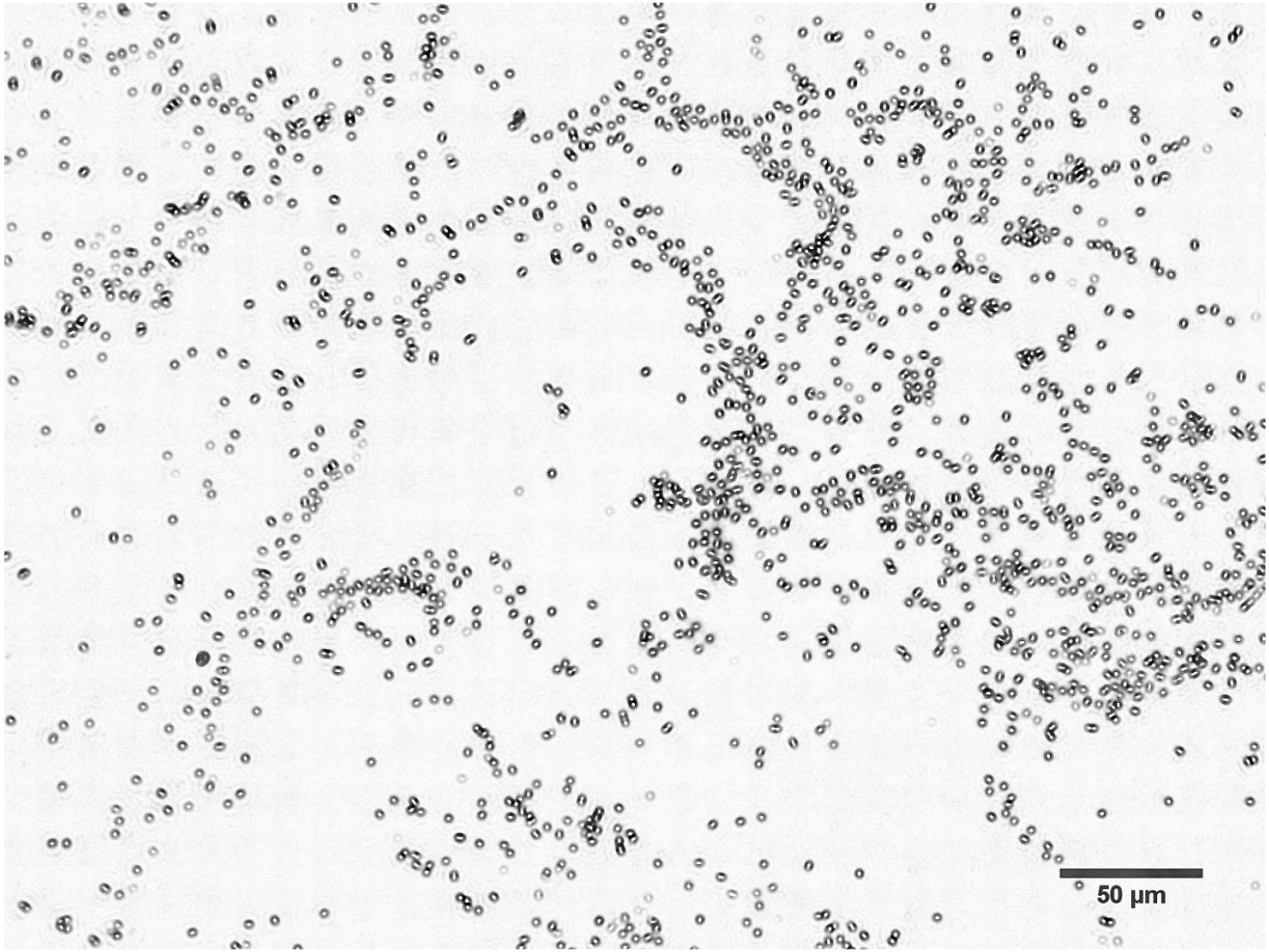


Figure 7-4: Visualisation of pneumococcal chains of strain SPN23F/P833.

Bacterial smear of a pneumococcal stock aliquot of SPN23F/P833 resuspended in saline, fixated on a microscopic slide and gram stained. Pictures were taken using EVOS XL Core and ImageJ. Picture was contrast enhanced and background subtracted using ImageJ functions.

Appendix 2 – Association of 6B specific polysaccharide IgG and host susceptibility

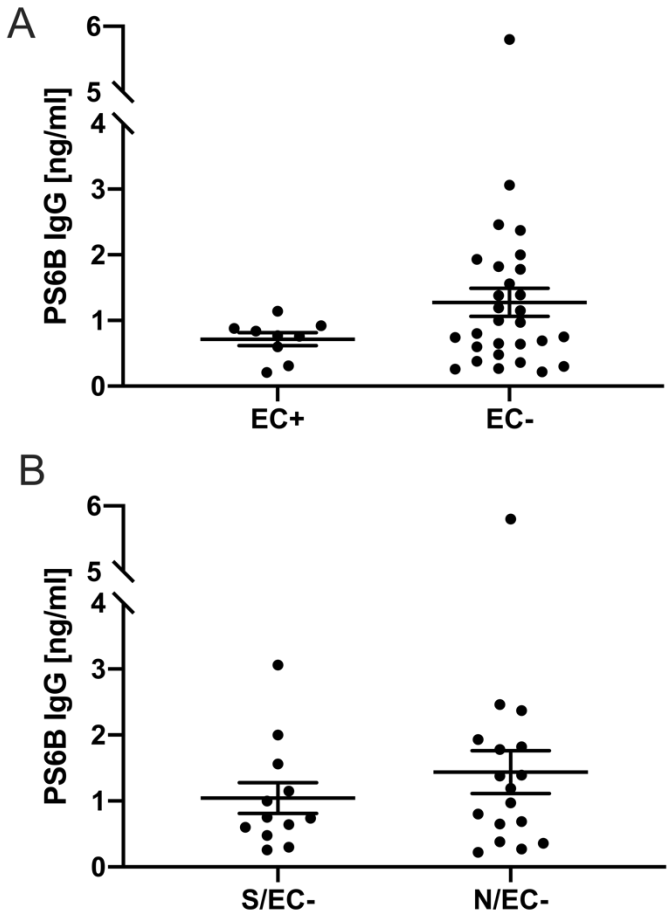


Figure 7-5: Association of mucosal IgG against 6B-specific polysaccharide with pneumococcal colonization.

Levels of SPN6B polysaccharide specific (PS6B) IgG antibodies in nasal wash at baseline stratified by (A) colonization status and (B) pneumococcal clearance profile after challenge with SPN6B/BHN418. Participating volunteers were young healthy non-smoking adults. Pneumococcal clearance profiles were determined by the presence of pneumococcal DNA in saliva using molecular methods (S/EC- = presence of pneumococcal DNA in saliva at 1 hour post challenge; N/EC- = absence of pneumococcal DNA in saliva at 1 hour post challenge). Colonization status (EC- = colonization negative; EC+ = colonization positive) was determined as the growth of the pneumococcal challenge strain from nasal wash on blood agar plate at any time point following challenge. Bars represent mean ± SEM. Significance was determined using Mann-Whitney test. Significance levels $P < 0.05$.

Appendix 3 - Reconstituted nasal epithelial cell model for pneumococcal research

Introduction

Nasal cell culture models can be used to study cause-effect relationships involving host-bacterial interactions. Primary nasal cell culture models have been reported to more closely mimic *in vivo* nasal cells when compared to the current standard of immortalised cell lines. Here I established two cell culture protocols in the LSTM laboratories: an air liquid interface nasal cell culture model of differentiated human nasal epithelial cells (NPE) and a model of undifferentiated primary nasal epithelial cells (NECs). Figure 7-6 summarizes the protocol used for primary cell culture.

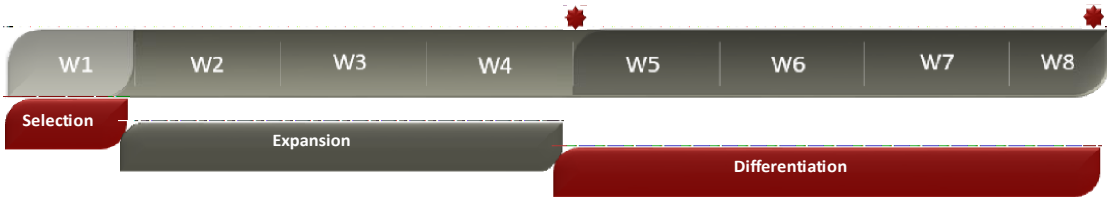


Figure 7-6: Schematic of growth protocol for primary epithelial cells.
Nasal epithelial cells were expanded and differentiated according to a method previously described [346]. Briefly, cells were obtained from human volunteers and cultured for 7 days in 12 well plates (passage 0). At week two undifferentiated cells were seeded into a T25 flask (passage 1) followed by a T75 flask (passage 2) and expanded for a maximum of 20 days. Undifferentiated cells (NEC) were then placed on 0.24 µm inserts and differentiation was initialised once confluency was reached. Differentiating cells (NPE) were kept in culture for a minimum of 30 days under air liquid interface conditions. Red stars indicate time points at which undifferentiated NECs or NPE were recommended to be used for assays.

Results

After establishment of the primary nasal cell culture protocol, cellular organisation was compared between Detroit 562, NEC and NPE cultures. Presence of secreted MUC5AC was found in all cell culture models tested (Figure 7-7 A). However, NPE

showed the highest amount of MUC5AC secretion when compared to Detroit or NEC cultures. NPE were also evaluated regarding epithelial organisation, permeability and mucin secretion throughout the nasal cell differentiation process (Figure 7-7 B). Cellular organisation developed gradually over the 30 days of differentiation. Ciliated cells, indicated by a positive α-tubulin staining, were not detectable until 30 days into the differentiation process, while permeability decreased over time (Figure 7-7 B and C). Seven days into differentiation tight junction protein ZO-1 was found uniformly distributed over the cell which was consistent with a high mean permeability of the epithelium (82.58 ± 2.57 mg/ml). Over time ZO-1 was found predominantly localised at the lateral side of the cells consistent with decreased permeability (Day 7: 39.24 ± 7.40 mg/ml, Day 30: 3.71 ± 1.31 mg/ml). Numbers of goblet cells actively secreting mucins increased gradually as evident by increased staining (Figure 7-7 B) and higher levels of MUC5AC protein in cell culture media over time (Figure 7-7 D).

The data demonstrate that the implemented NPE model is closely resembling *in vivo* epithelium in line with previous reports [317-320] and secrets a high amount of mucus. However, the success rate of NPE differentiation as defined by decreasing permeability over time was low (25%, 2 out 8 cultures).

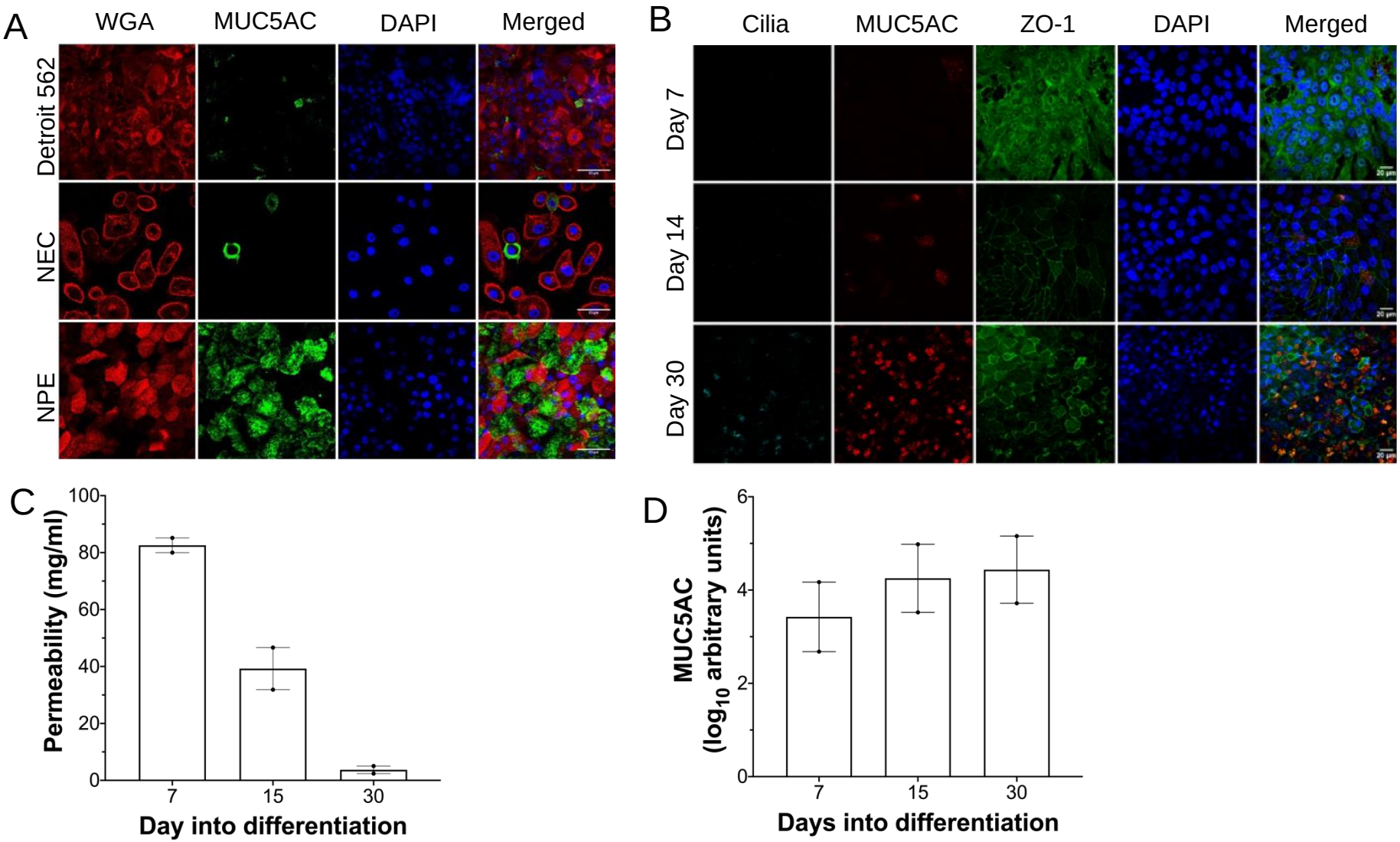

A
WGA
MUC5AC
DAPI
Merged
Detroit 562
NEC
NPE
B
Cilia
MUC5AC
ZO-1
DAPI
Merged
Day 7
Day 14
Day 30
C
Permeability (mg/ml)
100
80
60
40
20
0
7
15
30
Day into differentiation
D
MUC5AC
(log10 arbitrary units)
6
4
2
0
7
15
30
Days into differentiation

Figure 7-7: Comparison of cellular organisation of nasal cell culture models

(A) Detroit 562, NEC and NPE cultures were stained for presence of secreted mucin MUC5AC. Cell body was stained with wheat germ agglutinin (WGA, red) and cell nucleus with 4',6-diamidino-2-phenylindole (DAPI, blue). (B) NEC cells were obtained from a healthy adult donor and expanded into a primary cell culture. Differentiation was initialised at passage 2. Differentiating NPE cultures (Day 7, 15 and 30 into differentiation) were stained for presence of goblet cells (MUC5AC), degree of permeability (tight junction protein ZO-1) and presence of ciliated cells (α-tubulin). (C) Permeability was measured by adding fluorescent Dextran to the apical side of the epithelium for 3 hours, thereafter fluorescence intensity was measured. Permeability was calculated as the ratio of fluorescence measured in bottom well to fluorescence of the applied concentration of Dextran and expressed in percent. (D) Levels of MUC5AC protein in nasal cell culture media after 3 hours of incubation measured by ELISA.

Methods

Immunohistochemistry

Nasopharyngeal cell cultures were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Thereafter cells were permeabilised using 1% Triton for 10 minutes and blocked with blocking buffer (3% goat serum, 3% BSA in $HBSS^{+/+}$) for 1 hour at room temperature. Cells were incubated with primary antibodies in blocking buffer for 1 hour at room temperature. Thereafter cells were washed three times in distilled water and (where necessary) incubated with secondary antibodies in blocking buffer for 1 hour at room temperature. Antibodies used were: mouse MUC5AC (1:100, Abcam), goat anti-mouse IgG – Alexa Fluor 555 (1:1000, Thermo Scientific), rabbit ZO-1 (1:100, Thermo Scientific), goat anti-rabbit IgG – Alexa Fluor 488 (1:1000, Thermo Scientific), alpha-tubulin – Alexa Fluor 633 (1:20, Insight Biotechnology) and WGA - Rhodamine (1:500, Vector Laboratories). Thereafter cells were washed three times in distilled water and mounted using Vectashield Hard set mounting media with DAPI (Vector laboratories).

Permeability assay

Dextran-Fluorescein (FD4, Sigma) at final concentration of 1 mg/ml was added to the apical side of NPE cultures for 3 hours in which cultures were returned to the incubator and protected from light. After incubation, basal media was collected, and fluorescence measured using FLUOstar Omega and analysis software Omega MARS 3.10 (BMG Labtech). Permeability was measured as the ratio of fluorescence measured in the bottom well after 3 hours of incubation to fluorescence of the applied concentration of Dextran prior to incubation.

MUC5AC ELISA

Cell culture media (EMEM media (M5650, Sigma-Aldrich) supplemented with 1% heat-inactivated foetal bovine serum (FBS, Fisher Scientific) and 2 mM L-Glutamine (Sigma-Aldrich) was added to the apical side of the cells for 3 hours after which media was collected. ELISA for MUC5AC was performed as outlined in Chapter 2.

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