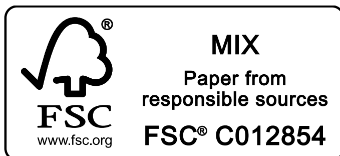
Host and microbe characteristics of pneumococcal colonization in elderly

Cassandra Lynn Krone



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PhD Thesis, University of Utrecht, the Netherlands

ISBN/EAN: 978-94-6108-515-3

Cover art: "*The Hunters and the Hunted*;" Linocut print, oil on kozo paper; ©2013 Cassandra L. Krone

Layout and design: Cassandra L. Krone

Printed by: Gildeprint Drukkerijn - www.gildeprint.nl

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Printing of this thesis was kindly supported by: Wilhelmina Children's Hospital, University Medical Center Utrecht; Infection and Immunity Center Utrecht; Astellas; Pfizer; GlaxoSmithKline; Yakult.

Host and Microbe Characteristics of Pneumococcal Colonization in Elderly

Gastheer- en pathogeen eigenschappen betrokken bij pneumokok-kenkolonisatie bij ouderen

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op

dinsdag 29 oktober 2013 des middags te 12.45 uur

door

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"Though my soul may set in darkness,
it will rise in perfect light.
I have loved the stars too fondly
to be fearful of the night."

Sarah Williams

dedicated to my parents
and Jamie

Committee

Prof.dr. J.A.G. van Strijp

Prof.dr. M.J.M. Bonten

Prof.dr. A.I.M. Hoepelman

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1

Chapter One

General Introduction

Streptococcus pneumoniae is a Gram-positive, lancet-shaped, lactic acid bacteria belonging to the phylum firmicutes (1). It is an aerotolerant anaerobe, a quality making it perfectly adapted to its host and niche in the upper respiratory tract (URT) and saliva of humans. Both George Sternberg and Louis Pasteur independently discovered this bacterium in 1881 when injecting human saliva into a rabbit, and subsequently isolating a lancet-shaped diplococcus from the blood of the sick rabbits (2-5). A similar chaining coccus bacterium had been described in 1875 and 1880, but it was Sternberg and Pasteur who demonstrated the pathogenicity of the bacteria (6). Lobar pneumonia was a common, and in the pre-antibiotic era an especially frightening disease, so many early microbiologists sought to determine the causative agent. By 1887 Koch's postulates had been satisfied by Albert Fraenkel and Carl Friedlander, establishing *S. pneumoniae* as a major cause of pneumonia (7). It was Fraenkel who gave it the name "pneumokokkus" and it has been referred to as the pneumococcus ever since.

S. pneumoniae structure and serotypes

Like all Gram-positive bacteria, *S. pneumoniae* has a cytoplasmic lipid membrane followed by a thick peptidoglycan cell wall. The cell wall is 6 layers thick and the main molecular components are peptidoglycan, teichoic acid, and lipoteichoic acid (1). Lipoteichoic acid is anchored in the cell membrane and can span the cell wall into the capsule (1). Teichoic acid is anchored in the cell wall (1). Both teichoic acid and lipoteichoic acid have attached bacterial adhesins (including choline-binding proteins) that mediate binding to human epithelial cells (see Figure 1) (8, 9). Some Gram-positive bacteria, including pneumococcus, also have an additional external layer, the capsule. The polysaccharide capsule of pneumococcus (except serotype 3) is covalently attached to the peptidoglycan of the cell wall (8). Currently, over 90 structurally different capsule types have been identified; these capsule types differentiate pneumococci from each other and as such are independently recognized by the host. The capsule types are called serotypes, because a serum-based test, called the quelling reaction, was originally used to type pneumococci. The capsule is also the most important virulence factor. It prevents phagocytosis by being highly charged and appears to interfere with the Fc region of IgG and complement component C3b interacting with their receptors on phagocytic cells (8). The capsule also prevents the pneumococcus from becoming trapped in neutrophil extracellular traps (NETs) (10).

Pneumococcal colonization

In his initial discovery, Sternberg noted that the saliva he was obtaining was from healthy, asymptomatic individuals (11). He also reported that different saliva isolates produced different results, not all resulted in sickness and death of rabbits. Other scientists later confirmed his observations, thus establishing that the bacterium was a generally asymptomatic inhabitant of humans but not all humans carried the bacteria (11, 12). Due to this duality of being both pathogen and asymptomatic inhabitant, many

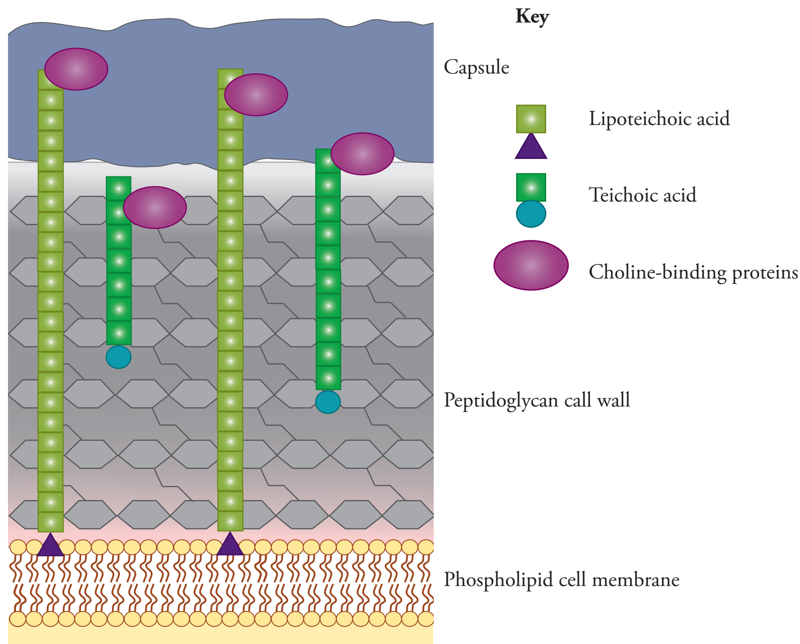


Figure 1: **Structure of *S. pneumoniae* cell wall**

epidemiological studies were performed to understand how the pneumococcus causes disease. The consensus in the pre-antibiotic era was that 45-60% of all adults were colonized with pneumococci, and that this colonization only occasionally led to disease (11, 12). Since then, pneumococcus is considered to be a pathobiont, that is a commensal or symbiont of the normal URT flora, that can, under certain circumstances, be a pathogen. Introduction of antibiotics led to decreased interest in studying carriage dynamics in people, since the disease manifestations were considered curable (13). The rise of antibiotic resistance and renewed interest in vaccine introduction for primary prevention in the 1970's caused a resurgence in epidemiological carriage studies.

Currently, pneumococcal colonization is considered to be highest in children, and declining with age due to shorter carriage duration associated with maturing immunity (14). URT colonization must occur, and a carrier state established, before developing pneumococcal disease (15, 16). Information on circulating serotypes and conjugate vaccine effects can simultaneously be monitored by carriage surveillance, since pneumococcal conjugate vaccine largely prevents vaccine serotype acquisition and colonization. Studying colonization dynamics is not only a useful epidemiologic tool, but also gives insight into evolution of virulence factors, niche composition, and host-microbe interactions. Colonization elicits weak serotype-specific immunity, and this coupled with the antigenic variation of pneumococci allows for repeated colonization by the same serogroups (12, 17). Modeling this interaction with taking into

account adaptive serotype-independent immunity and serotype-dependent immunity in part explains why coexistance of pneumococci occurs and why high antigenic variation is maintained in the species (17). The types of infection that *S. pneumoniae* causes are not very efficient ways for a bacterium to spread to new hosts, so colonization is also key to the survival of pneumococcus as a species (18). Experimental human colonization results in a brief mild rhinorrhea, indicating mild inflammation in the URT, which could contribute to the spread of the bacteria in the population (19). The question of why pneumococcus causes disease then has been hypothesized to be a byproduct of maintaining mechanisms that promote colonization (19).

Disease manifestations

S. pneumoniae is generally found in humans, and the reservoir for disease is human carriers (8, 14). The mode of transmission is droplet spread, direct oral contact, or contact with articles soiled with secretions of colonized individuals as mentioned above (8, 14, 18). The most common diseases resulting from a pneumococcal infection are pneumonia, in all ages, and otitis media, in young children (8, 14). Pneumococcal pneumonia is the most common cause of community-acquired pneumonia (CAP) (14). Invasive diseases such as bacteremia and meningitis can also occur, either as a complication of pneumonia or from direct spread from the nasopharynx into the bloodstream (8). Public health officials recommend to vaccinate those at risk as preventative measures, i.e. children under 5 years of age, elderly aged 65 years and older, and individuals with certain comorbidities, in particular immunodeficiencies (14). Pneumococcal CAP occurs in carriers who have a breakdown in immune defenses, and thus it is a sporadic disease (15, 20). The breakdown in immune defense can be intrinsic (i.e. immunodeficiency) or aided extrinsically (i.e. virus acquisition) (21). Outbreaks are uncommon and usually occur in crowded places such as prisons and nursing homes, usually are associated with a specific serotype, and generally in persons with underlying health conditions or in specific conditions like working in mines (20).

However, despite access to quality medical care and interventions, acute respiratory infections (excluding tuberculosis and pneumonia in HIV/AIDS infected people) are the number one cause of infectious burden of disease and death throughout the world (22). In low-income countries, pneumonia kills over 2 million children per year, more than any other infectious illness, more than AIDS, malaria, and measles combined and pneumococcal pneumoniae is thought to cause up to half (1 million) of these childhood deaths (23, 24). In wealthy countries, chronic illness kills more people than acute infections, however, lung infections remain the most common infectious disease with a burden of disease that has not changed much in over 80 years (25). Furthermore in high income countries mortality in adults due to pneumococcal pneumonia is approximately 20% and has remained unchanged over the last 60 years despite antibiotic use and intensive care (26).

Innate Mucosal Immunity

The upper respiratory tract (Figure 2) is one of the 5 mucosal sites of the mammalian body (the others being the lower respiratory tract, skin, gut, and reproductive tracts) and is in direct contact with the external environment. Due to this direct external exposure, mucosal surfaces are constantly exposed to innocuous environmental molecules (e.g. pollen, food particles), commensal microorganisms, as well as potentially pathogenic fungi, parasites, viruses, and bacteria. The mucosal immune system has developed an incredibly diverse and intricate system in order to maintain homeostasis in response to this constant exposure (27, 28). Parts of this system are the so-called host pattern recognition receptors (PRR) that recognize evolutionary conserved regions of microbes called pathogen-associated molecular motifs (PAMPs). Commensal bacteria colonize most mucosal surfaces, and as such the mucosal epithelium and immune system must balance tolerance to these commensals with inflammation and swift response to pathogens (27, 28). Evidence as to how the mucosal immune system accomplishes a healthy balance supports three complementary hypotheses: (i) PAMP's from commensals are biochemically altered and are not detected by PRR's (ii) symbiotic microbes express molecules that either antagonize pro-inflammatory pathways or actively induce tolerance (iii) in addition to generic PAMP's, pathogens also create a level of damage to the host that is perceived by additional host receptors as dangerous, termed danger signals (28).

The mucosal PRR's that specifically recognize *S. pneumoniae* are located on the surface as well as in the cytosol of both epithelial cells and phagocytes, and additionally in the endosomes of phagocytes [reviewed by (29, 30)] (Figure 3). The extracellular PRR's toll-like receptors (TLR) 1/2 recognize pneumococcal lipoteichoic acid (31-34). TLR4 is also involved, but the ligand is disputed (29). Cytosolic NOD-like receptor (NLR) NOD2 recognizes peptidoglycan (35) and NLRp3 inflammasome complex is activated by the pneumococcal toxin pneumolysin (36). Inside the phagosome of macrophages and neutrophils TLR9 recognizes pneumococcal DNA (37). The MyD88 adaptor protein is required for TLR signaling and activates the NF-κB complex (as does NOD2) (29, 30). Activation of NF-κB leads to release of mucins, and anti-microbial peptides from respiratory epithelial cells (30). Production of the pro-inflammatory cytokine IL-1β requires both the activation of NF-κB as well as inflammasome-mediated activation of caspase-1 (29). NOD2, antimicrobial peptide cathelicidin, and IL-1β can all mediate production of chemokine CCL2 (or macrophage chemotactic protein 1, MCP1) which leads to macrophage recruitment to the mucosa (29, 30).

Upon innate immune activation, neutrophils migrate to the URT first, however, they are generally not sufficient for clearance of pneumococci, probably due to the capsule (19, 33, 38). Monocyte/macrophages infiltrate into the URT later than neutrophils and are crucial for clearance of pneumococci (39). In the LRT, the order of infiltration is reversed since resident tissue macrophages (alveolar macrophages) are constitutively

present (40). Macrophages and especially neutrophils become more effective at clearing pneumococci with the help of adaptive immunity, specifically Th17 cells (41). The role of antibody-mediated clearance in the URT is less clear. In mice, antibodies are not required for effective URT clearance (42). Paradoxically, the new conjugate pneumococcal vaccines, which elicit a Th1-mediated antibody response, do reduce nasopharyngeal colonization with vaccine serotypes to a certain extent (43). Natural colonization is reported to be weakly immunogenic, and vaccine-mediated immunity is probably more immunogenic, however, recent models predict that the long-term effect of the vaccine on pneumococcal colonization will be small (17).

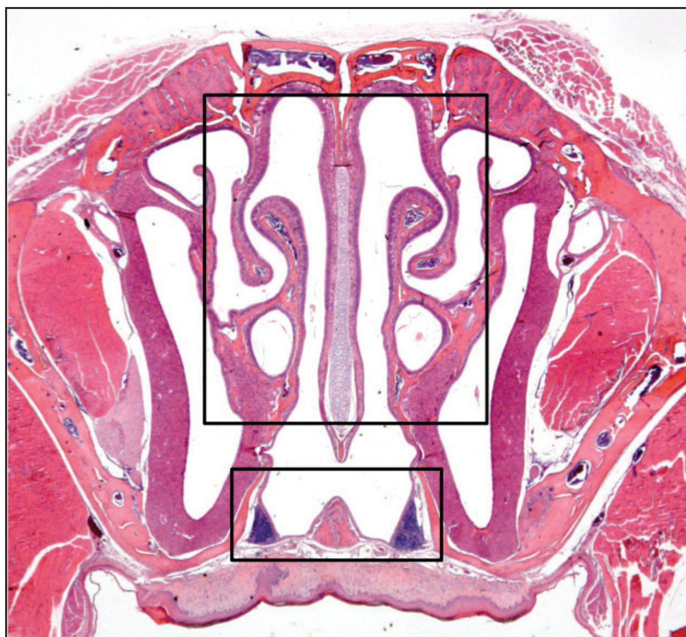
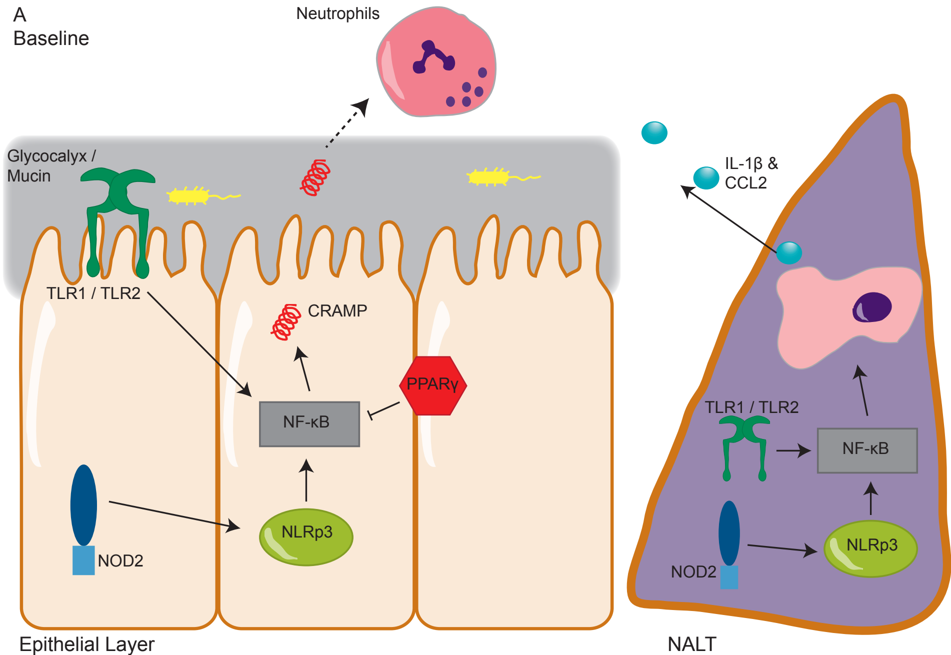


Figure 2: **Coronal section of the mouse upper respiratory tract**
Black boxes highlight the turbinates (top) that are covered in respiratory epithelial cells and the nasal associated lymphoid tissue (purple triangles in bottom box). Craniums were decalcified, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E). Image was taken on Nikon Eclipse E800 microscope with a 0.5x wide-angle lens.

Opposite page:
Figure 3: **Scheme of innate mucosal immunity before and during pneumococcal colonization**
Cartoon of respiratory epithelial layer and nasal associated lymphoid tissue (NALT) correspodning to Figure 2 (not to scale). A. Baseline activities or surveillance in the URT. Commensals populate the glycocalyx, low level of PRR's are expressed, and PPARγ negatively regulates activation of NF-κB. Low numbers of neutrophils are present in the URT cavity. In the NALT normal lymphoid architecture exists with some antigen presenting cells with low-to-no amounts of cytokine production. B. Colonization by the pathobiont *S. pneumoniae* induces changes in the URT. At the epithelium recognition is taking place with down-regulation of PPARγ and subsequent upregulation of PRR's and antimicrobial peptides such as CRAMP. Phagocytes infiltrate into the URT and traffic to NALT, where PRR and cytokine production is upregulated via inflammasome activation. This is a positive feedback loop with increasing amounts of phagocytes infiltrating into the URT until pneumococci have been cleared.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Mucin
IL-1β &
CCL2
TLR1 / TLR2
CRAMP
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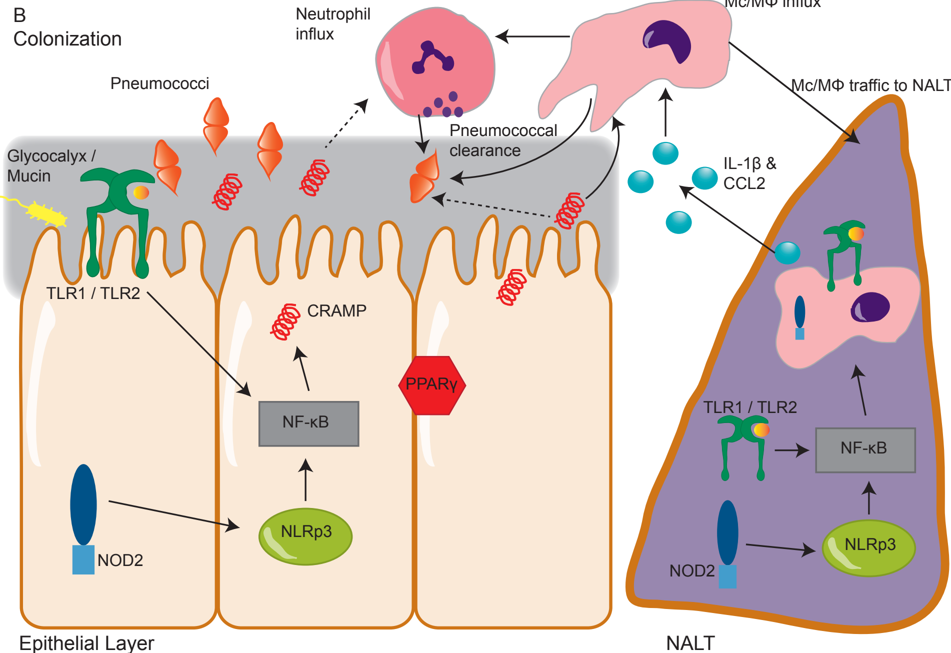
NALT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CCL2
TLR1 / TLR2
CRAMP
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NF-κB
TLR1 / TLR2
NF-κB
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NLRp3
NOD2
Epithelial Layer
NALT

Elderly

The elderly (defined as persons aged 65 and older) are more susceptible to infectious disease than their young-adult counterparts. It may seem obvious that as the body ages, so too does the defense system, however the exact mechanisms responsible for age-related disease susceptibility are only beginning to emerge. Furthermore, it is still debated if these defects due to ageing are universal and pre-determined by genetics, or instead determined by environment. Age-related defects of the immune system are termed immunosenescence. Concomitant with ageing and immunosenescence is a phenomena termed 'inflamm-aging,' where inflammation in the absence of sickness or symptoms is detected. This phenomena further complicates the debate regarding cause and effect; some regard it as the result of genetic programming that is beneficial only for the young (44), and some regard it as an underlying, undetectable viral infection (45). Understanding immunosenescence and the mechanisms involved could lead to longer, healthier lives due to better prevention and treatment of infections.

Although the introduction of the conjugate vaccine in children has led to herd effects on pneumococcal disease in adults, serotype replacement has resulted in a debatable effect on CAP in the elderly. In the UK replacement has led to zero net effect of CAP in the elderly (46, 47) whereas in the USA a decrease in pneumococcal pneumonia has been observed, but no decrease in all-cause pneumonia (48). Therefore, pneumococcal disease in elderly is still a large public health problem that needs urgent attention. Interestingly, though high rates of disease in children are reflected by high rates of pneumococcal URT carriage, in elderly there is a discrepancy between colonization and disease dynamics where conventional culture of the naso- and oropharynx are used.

Objectives and Outline of This Thesis

The focus of this thesis was to investigate pneumococcal – host interactions in the respiratory tract of the elderly. The culmination of medical advances in the 20th and 21st century has led to ever increasing life spans. As the global population ages, so does the demand on healthcare resources. The elderly are at particular risk for pneumonia, and specifically pneumococcal pneumonia, however, there is a paucity of information regarding the underlying mechanisms of disease in this risk group. In this theses we have addressed several issues related to ageing and susceptibility to pneumococcal disease in elderly.

The following hypotheses were formulated:

1. The discrepancy between the low to undetectable rate of pneumococcal colonization by conventional culture in elderly despite the high disease rate is a sampling bias, and carriage in the elderly is underestimated.
2. Immunosenescence in the elderly leads to aberrant innate mucosal immune responses, especially cellular responses, in the upper respiratory tract that have a direct impact on the response in elderly to pneumococcal colonization.
3. The microbiota profile of the upper respiratory tract in elderly is altered, possibly contributing to inflamm-aging and pathobiont behaviour compared to young-adults.
4. Molecular methods of pneumococcal detection in clinical samples of the elderly are superior to conventional culture based methods.
5. Alternative sampling sites, such as the oropharynx or saliva, are superior to the nasopharynx in the elderly.

A review of respiratory mucosal immunosenescence is presented in **Chapter 2**, along with evidence to support our argument that pneumococcal colonization is underestimated in the elderly. Furthermore, we developed an elderly mouse model of pneumococcal colonization and used the model to investigate mechanisms of immunosenescence in the URT. **Chapter 3** describes the innate mucosal immune deficits that we found in elderly mice that impact clearance of *S. pneumoniae* colonization. We also explored the effect of acquisition of *S. pneumoniae* on the URT microbiota profiles of young-adult and elderly mice, in **Chapter 4**. The URT microbiota of both young-adult and elderly mice is described before pneumococcal colonization, shortly after inoculation of pneumococci, and during the 28 days following. Age-related changes in the profiles, in connection with innate immune dysfunctions discussed in the previous chapter and the relation to susceptibility to respiratory disease are discussed.

Moving from mice to humans, **Chapter 5** presents the results of a cross-sectional observational study in elderly humans to compare detection of *S. pneumoniae* from three different sample types: trans-nasal nasopharyngeal swab, trans-oral swab, and saliva. Molecular, DNA based detection in the three niches is compared, as well as molecular detection compared to conventional culture. Our results from this study prompted further interest in saliva collection methods for use in pneumococcal surveillance studies. Currently, collected saliva is snap-frozen on dry ice at the point of collection, and transported to a -80°C freezer. However, this is cumbersome, expensive, and impractical for low-income countries. Therefore, we present in **Chapter 6** the results of a study on the feasibility of using dried saliva spots as an alternative to snap-frozen saliva for DNA-based pneumococcal detection in surveillance studies. In **Chapter 7** we summarize all of the findings and conclusions and in **Chapter 8** we discuss the implications of our findings and future perspectives.

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2

Chapter Two

Immunosenescence and pneumococcal disease: an imbalance in host-pathogen interaction

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Lancet Respiratory Medicine
Online 18 September 2013
http://dx.doi.org/10.1016/S2213-2600(13)70165-6

Summary

Respiratory infections are the most important cause of morbidity and mortality through infectious diseases worldwide. The most common causative bacterium, *Streptococcus pneumoniae*, frequently colonizes the upper respiratory tract, where it resides mostly asymptomatically. Occasionally, however, it may progress towards severe disease like pneumonia. Local host immunity is key to keeping colonizing pathogens at bay, preventing them from overgrowth, spread, and invasion. However, elderly are subject to age-related immune deficits, termed immunosenescence, which may contribute to the increased disease burden in elderly. Here, we review current knowledge regarding immunosenescence in the respiratory tract against Gram-positive bacteria, especially *S. pneumoniae*. We discuss the possible under detection of pneumococcal colonization in the elderly, and suggest changes to current surveillance methods to improve our understanding of the relation between colonization and disease in elderly. We conclude that current knowledge regarding immunosenescence altering host-pathogen interactions in the respiratory tract is limited, and needs further studies to allow for the design of better preventive measures.

Introduction

Respiratory infections transcend national boundaries and socioeconomic boundaries, and are the number one cause infectious disease burden throughout the world (1). Furthermore, despite advances in modern medicine, respiratory infections account for more than 6% of DALY losses (Disability-adjusted life year) (1, 2). While immature immunity is thought to be responsible for increased susceptibility to respiratory infections in young children, the factors behind the increased morbidity in the aging population are poorly understood. The increased risk of the elderly (defined as persons aged 65 or older) for community-acquired pneumonia (CAP) is independent of health status and co-morbidities (3). In high-income countries, mortality due to CAP in the elderly still ranges from 15-30% despite access to high quality health care, and can double among residents of nursing homes (1, 4, 5). Although the elderly are at increased risk for CAP, the microbial etiology is similar compared to younger adults (6). The major infectious etiology of pneumonia is *Streptococcus pneumoniae* (4-6) (the pneumococcus) a frequent but transient bacterial commensal of the human upper respiratory tract (URT), that may grow out and progress towards lower respiratory and invasive disease (Figure 1). *S. pneumoniae* is also the major etiologic cause of secondary bacterial pneumonia following influenza virus infection (7). With an increasing proportion of the population being of old age, the public health burden caused by pneumococcal disease will continue to rise in the future (1). Population growth alone is predicted to increase hospitalizations due to pneumococcal pneumonia by 100% between 2004 and 2040, and 87% of that increase will be attributable to the elderly (8). It demonstrates the need for effective strategies against pneumococcal disease in the aged population and underlines the importance of understanding the path leading to disease in the elderly.

It is well established that in order for bacteria to cause respiratory disease they must first colonize the upper respiratory tract (9, 10). In children, colonization by *S. pneumoniae* has been well documented with intensive carriage surveillance studies in many countries (11). High incidence of pneumococcal diseases in children younger than 5 years correlates with colonization rates of up to 90% (12) detected using the gold standard method (recovery of live *S. pneumoniae* colonies from solid-agar culture of deep trans-nasal nasopharyngeal (TN) swabs) (13) implying that in children, this method of carriage detection is appropriate. However, rates of colonization in adults and the elderly are currently contentious. Studies utilizing nasopharyngeal or oropharyngeal swabs and conventional culture to detect carriage report very low (<6%) (14-17) to no colonization (18) in the elderly. However, studies in adults or elderly utilizing either nasopharyngeal or oropharyngeal (TO) swabs (19-21) or saliva (22) and molecular methods of pathogen detection find substantially higher prevalence rates of up to 37% pneumococcal carriage in community dwelling elderly (22). Furthermore, surveillance studies performed in the pre-antibiotic era using saliva and a sensitive mouse inoculation method reported that 45-60% of adults in the community were colonized at any given time (23, 24).

Although specific ages of participants were often not stated in these early studies, the consensus was that at least half of all adults (including elderly) were asymptomatically colonized. This suggests that pneumococcal colonization may be underestimated in the elderly, and playing a larger role in disease dynamics than currently thought.

In addition to pneumococci, the human URT is often colonized by *Moraxella catarrhalis, Haemophilus influenzae, Neisseria meningitidis, Staphylococcus aureus*, various other potentially pathogenic bacteria, viruses, fungi, and a high variety of commensal microorganisms (25-29). They appear to form a dynamic and interactive microbiome that is thought to heavily interact with the host immune system. It is likely that immunosenescence has a direct impact on this interaction, tipping the balance towards higher susceptibility to respiratory disease in the elderly. This review describes the contribution of immunosenescence to *S. pneumoniae* infections in elderly paying special attention to factors known to be important during URT colonization. Furthermore, the dynamic microbiome was taken into consideration while reviewing the literature regarding factors contributing to susceptibility of the elderly to pneumococcal disease. Where possible, evidence from pneumococcal studies was used, and when pneumococcal-specific evidence was absent, studies on other bacterial pathogens were used, which will be stated accordingly.

Search strategy and selection criteria

We undertook a PubMed search using terms in varying combinations; for each search we combined a term of group A with a term of group B. Group A terms: aging (MeSH-term), immunosenescence, pneumococcus; group B terms (all MeSH-terms): *Streptococcus pneumoniae*, pneumococcal infections, bacterial infections, respiratory tract infections, Gram positive bacteria, bacterial pneumonia, innate immunity, innate immune response. In addition, we performed searches combining S. pneumoniae with the following terms: colonization, TLR, MARCO, antibodies (IgG or IgA), complement (C3 or C4) and virulence traits. Pre-antibiotic era references were identified via searches of articles published from 1880-1950 in the authors' personal files, and via PubMed, JSTOR, or Google Scholar using the terms pneumococcus and carrier or carriage. References for carriage prevalence in adults presented in table 3 were obtained from articles already cited in the review, and additionally bya PubMed search on streptococcus pneumoniae AND (carriage OR colonization) AND (adults OR elderly) AND healthy. Finally, references from all relevant articles were screened and where appropriate used for this review. Only papers in English were reviewed.

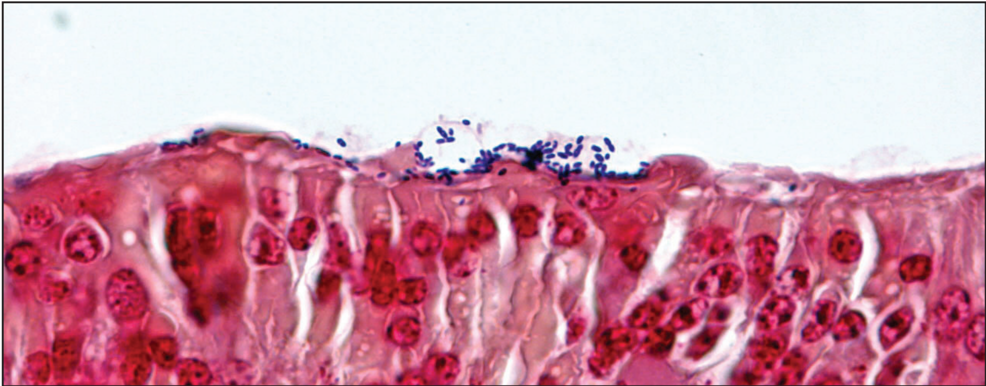

Figure 1: **Upper respiratory tract colonization by *Streptococcus pneumoniae* in an elderly mouse.**
Histology of the nasopharynx of an elderly (age 20 months) C57Bl/6 mouse at day three post-inoculation with a serotype 6B pneumococcal isolate. We observed micro-colonies of Gram-positive, lancet shaped diplococci, consistent with *S. pneumoniae*, associated with the glycocalyx layer above the epithelium, but not deeper within the epithelial layer. There was no obvious damage to the epithelial layers observed. The slide was Gram-stained and the picture was taken with magnification 60x under oil-immersion.

Mucosal Barrier

Due to the direct contact with the outside world, the mucosal surfaces of the respiratory tract are the first line of defense against pathogen invasion and employ a range of physiological mechanisms to aid the mucosal immune system. Respiratory epithelial cells are specialized cells that form a barrier covering the surface of the respiratory tract including alveoli. Goblet cells within this lining produce mucus, which is essential for mucociliary removal of bacteria from the lower respiratory tract (30, 31). It has been reported that aged adults have a diminished mucociliary clearance, (30) which may increase local pneumococcal load. In addition to this passive/mechanic antimicrobial mechanism, epithelial cells actively respond to pathogens with the release of various mediators (32, 33). Cytokines, chemokines, antimicrobial proteins, glycoproteins, enzymes, and other mediators secreted by respiratory epithelial cells trigger an inflammatory response and contribute to immune cell recruitment to the site of infection (31, 33, 34). Due to the polymicrobial nature of mucosal sites, the mucosal immune system must distinguish between commensal and pathogen and keep inflammation to a minimum. Also, an effective mucosal response is required to control and clear colonization by *S. pneumoniae* (9).

Immunosenescence of Innate Immunity

The innate immune system is indispensable for respiratory tract defense as it provides immediate and constant protection against microbial invasion. It is active both at the primary site of pneumococcal colonization, within the nasopharynx and oropharynx, and at the sites of infection, within the bronchioli and alveoli, as specialized

mucosa-associated lymphoid tissue (MALT). MALT forms distinctive nasal-associated (NALT) at the upper respiratory tract and bronchus-associated (BALT) tissue at the lower respiratory tract which are crucial for pathogen recognition and host responses. Unless specified, the mechanisms discussed below are applicable to both sites. In Table 1 data regarding immunosenescence of innate immunity are reviewed.

In general, aging seems to be associated with chronic innate immune activation and change in both surface receptors and function of immune cells and tissues (50). Evidence for chronic systemic inflammation in the absence of detectable infection in elderly, called 'inflamm-aging' has been reported (50-52). One example is higher baseline levels of innate immune activation markers CXCL10, neopterin, and sCD 163 in sera of aged individuals (35). It also includes elevated serum levels of CCL2, a chemokine that normally rises during infection in order to recruit macrophages to the site of infection (36). Evidence of inflammation has also been found in the lungs of naïve elderly mice, defined as significantly higher concentrations of IL-1β, IL-6, TNFα, and CXCL1 in tissue homogenates (38).

Recognition of Bacterial Pathogens

Recognition of bacterial pathogens will occur at the URT during colonization and, in the alveoli, after bacterial invasion into the lungs. Epithelial cells and resident alveolar macrophages can recognize *S. pneumoniae* by extracellular and intracellular pattern recognition receptors (PRRs), including Toll-like receptors (TLRs); whether these innate recognition pathways are affected by immunosenescence in elderly humans is still unclear. Due to the central role of TLRs in pathogen recognition, many have searched for potential changes in TLR expression and possible impaired pathways due to immunosenescence. Extracellular TLR1/2 recognizes lipoteichoic acid (a Gram-positive cell wall component) (53) and is crucial for clearance of pneumococcal colonization in mice (54). TLR1 was less present in lung tissue after pneumococcal infection in elderly compared to young adult mice, though no difference was observed at baseline in naïve mice (41). No differences have been found for TLR2 surface expression between elderly and young adult mice on murine alveolar macrophages after lung infection in either pneumococcal models (42) or tuberculosis models (43). In humans no difference was found in TLR protein content in peripheral blood mononuclear cells (PBMCs) of elderly compared to young adults measured by western blotting (39), but extracellular TLR expression measured via flow cytometry did show differences. Two studies found significant decreases in baseline TLR1 surface expression in elderly humans compared to

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Table 1: Immunosenescence of innate immunity regarding Gram-positive bacteria
*Key: 1 = Shown in 1 study; 2 = shown in 2 studies; 3 = similar trends shown in several studies or more than 1 species.

+ = Pneumococcal specific models.

Element of Innate Immunity		Site	Species	Level of Evidence*	Effect of Ageing
Innate immune activation (general biomarkers)	(35)	Systemic	Human	1	↑
Baseline inflammation (genreal)	(39)	Systemic	Human	3	↑
Baseline inflammation (respiratory)	(59, 40)	Lungs	Human and mouse	3	↑
Mucocilliary clearance	(59, 40)	URT	Human	1	↓
Recognition of Pathogens					
TLR1 surface expression	(46, 47)	Systemic	Human	2	↓
TLR1 expression during infection	(43)	Lungs	Mouse	1+ (serotype 2)	↓
TLR2 surface expression	(46)	Systemic	Human	1	=
TLR2 expression during infection	(44, 45)	Lungs	Mouse	1+ (serotype 2)	=
TLR4 surfae expression	(46)	Systemic	Human	1	↓
MARCO expression	(51)	Spleen	Mouse	1	↓
Moncyte / Macrophages					
Percentage of cells in BAL	(59, 60)	Lungs	Human	2	↓
Percentage of cells	(61)	Spleen	Mouse	1	↓
Percentage of non-classical monocytes	(38, 39, 69)	Systemic	Human	3	↑
Percentage of classical monocytes	(38, 39)	Systemic	Human	2	↓ / =
HLA-DR surface expression (MHCII)	(39)	Systemic	Human	1	↓
CX3CR1 surface expression	(39)	Systemic	Human	1	↓
CCR2 surface expression	(39)	Systemic	Human	1	=
Neutrophils					
Baseline number and percentage	(59, 60)	Lungs	Human	2	↑
Phagocytosis capacity opsonized antigens	(64)	Systemic	Human	1	↓
Antibody-dependent killing	(68)	Systemic	Human	1+ (serotype 6B, 14, 23F)	↓
Expression Fcγ and complement receptors	(68)	Systemic	Human	1+	=

young adults when measured on all PBMCs (39) or subsets of PBMCs (40); but TLR2 showed no difference (39). TLR4 appears important for *S. pneumoniae* clearance as well (55), although there is controversy regarding the pneumococcal ligand (56); TLR4 surface expression was significantly reduced in elderly humans as well (39). Intracellular TLR9, which recognizes unmethylated CpG DNA, appears to recognize pneumococci and plays a role in the early response to bacterial lung invasion (57). Only one study has investigated TLR9 in elderly mice and no differences were found for TLR9 surface expression on alveolar macrophages after tuberculosis infection (43). An important macrophage scavenger surface receptor, MARCO, which is able to bind pneumococcus (33) was reported to be reduced in naïve elderly mouse splenocytes (44). Furthermore during influenza infection, upregulated IFN-γ can downregulate MARCO, which is associated with increased mortality from pneumococcal pneumonia in mice (7). Care must be taken, however, when analysing these various studies, as heterogeneity of the monocyte/macrophage population both in different tissues and after different infectious stimuli has been observed (52, 58). Other PRR's found in both monocyte/macrophages and neutrophils that are crucial for responses to pneumococci in the URT such as NOD (59) and NOD-like receptors (60) appear not to have been studied in the elderly yet. Furthermore, pneumococcal toxin pneumolysin can directly activate innate immune cells through intracellular NLRp3 activation, and via this route dramatically amplify the production of pro-inflammatory cytokines IL-1β and Il-17A (56), which have been shown to be important for natural immunity to pneumococcus (61-63). Current knowledge hints towards impaired TLR and perhaps other PRR function with ageing, which may contribute to delayed or diminished pneumococcal recognition in elderly. However, information is limited, incomplete, and no clear mechanistic studies have been published yet.

Phagocytes

At the URT, colonization by pneumococci leads to an increase in neutrophil infiltration, followed by an increase in macrophage infiltration when the bacteria persist (62). In the lungs, alveolar macrophages are the first phagocytic defense against pneumococci with neutrophils infiltrating into the alveolar space in case of an acute infection (33). Macrophages are thought to become regulators of the immune response when neutrophils are infiltrating the site of infection, which results in a dampening of the immune response to reduce damage to the host (64). The role of phagocytes in host immunity at the URT is multifunctional; therefore numerous parameters can be tested as a proxy for function of these cells in the elderly. One parameter to investigate is the number and ratio of cells existing in a certain compartment. Broncho-alveolar lavage (BAL) fluid is representative of epithelial lining fluid from the lungs and has shown a change in presence of phagocytic cells with ageing (37, 45). BAL-fluid from elderly compared to young healthy adults showed a lower percentage of macrophages and a higher percentage of neutrophils (37, 45). Although not statistically significant, this relative decrease

in macrophage numbers, when considered a first defense mechanism, may increase the potential for pneumococci to colonize and invade successfully. The reports on the function of phagocytes in elderly in response to Gram-positive bacteria are conflicting. Many of the functional assays performed on mouse cells were (bone-marrow) derived cells, differentiated *ex vivo*, (46) or cells pre-screened for plastic adherence (44). Macrophages are known to behave differently in vivo than in vitro, and their phenotype can be easily changed during the differentiation process (58). Also cytokine production can vary depending on the site and type of macrophage isolated (65). Therefore it is not surprising that different conditions produce conflicting results (44, 46, 66). Work with human cells and phagocytosis has mostly been reported for neutrophils and there is a paucity of information on effectiveness of Gram-positive bacterial phagocytosis. One study stimulated peripheral blood neutrophils with heat-killed *S. aureus* and reported decreased phagocytosis by elderly neutrophils (48). Much more work has been done on phagocytosis with the Gram-negative bacteria *E. coli*, where elderly neutrophils consistently showed diminished phagocytosis of these bacteria as well; (48, 67, 68) however, the immune response to these two classes of bacteria, Gram-negative and Gram-positive, is quite different so we conclude that more data regarding possible impairment of phagocytosis of Gram-positive bacteria in elderly is needed.

Killing of bacteria by phagocytes is also an important functional parameter, but again there is a lack of data in this area for Gram-positive bacteria. Bone marrow-derived macrophages from elderly mice showed no difference in ability but did show reduced speed to kill *S. pyogenes* (46). Human peripheral blood neutrophils from the elderly also showed no difference in antibody-mediated killing of Group B Streptococcus (69) or the highly encapsulated serotype 3 pneumococcus, but did show significantly less killing for serotypes 6B, 14, and 23F pneumococci (49). Both studies also showed significantly less functional antibodies in the elderly sera for these bacteria, which could affect the outcome on killing in an antibody-dependant assay.

Surface expression of receptors on human PBMCs and peripheral blood neutrophils has also been investigated in the elderly. Receptors of importance for defense against pneumococcal infection include HLA-DR and CX3CR1, which are reported to be significantly decreased on elderly PBMCs (36). CCR2, (36) on the other hand, as well as Fcγ and complement receptors on neutrophils (49) were of equal quantity in young and elderly. It remains to be determined though if these receptors on elderly cells signal properly. Surface receptor expression is also used to classify human PBMCs into two classes or populations; first CD14++/CD16- monocytes, called classical monocytes that are generally CCR2+, CX3CR1+ corresponding to "inflammatory monocytes" in mice and usually comprise the majority of circulating monocytes in the young (36). Second, in contrast, non-classicalCD14+/CD16++ monocytes are CCR2-, and CX3CR1+ and correspond to resident tissue macrophages in mice (36). Interestingly, several studies have found that the majority of circulating monocytes in elderly humans are non-classical

(35, 36, 47); however, only one of these studies checked CCR2 expression and found no difference in amount of CCR2 on classical monocytes (36). These changes of the monocyte population in elderly compared to younger humans may contribute to decreased monocyte recruitment and delayed activation during pneumococcal colonization and infection, although this needs to be investigated in more detail.

Cytokines

Recognition of pneumococci results in a cascade of signaling with the goal of production of a broad range of both pro- and anti-inflammatory cytokines (33). TLR adaptor proteins such as MyD88 are crucial for an adequate response to pneumococci both in humans and mice (33). Furthermore, signaling machinery NF-κB (33) and inflammasome NLRp3 (56) are both crucial in production of key cytokines. Cytokines are small signaling proteins that regulate a well-orchestrated immune response; a dysregulation or imbalance of this complex response may induce harm to the host and contribute to infection in elderly. IL-1β is an early cytokine crucial for clearance of both colonization and pneumonia, TNFα stimulates phagocytosis, IL-6, IL-17, and IL-18 are important pro-inflammatory cytokines and IL-10 is an important anti-inflammatory cytokine that contributes to the recovery of the lungs after the damage inflicted during acute infection.

The majority of the research on cytokine responses in elderly mice has been obtained from *in vitro* rather than *in vivo* systems, with various kinds of mouse macrophages (35, 42, 66, 70-74), the pitfalls of which were discussed earlier. Furthermore, only a handful of studies have been performed using live pneumococci or Gram-positive bacteria or stimulations, the remaining used Gram-negative or purified TLR agonists. However, what is clear from these studies is that macrophages from elderly mice respond aberrantly in cytokine production. The few elderly mouse *in vivo* studies showed that specifically IL-1β, IL-6, TNFα (38), and Nf-κB (75) are significantly increased in the naïve lung. Shortly after pneumococcal infection however, there is less IL-1β and IL-6 in elderly mouse lungs, but no difference in TNFα amounts (42). In contrast with *in vitro* studies (70), IL-10 was not found in the lungs of mice after infection, but it was increased in the serum of these mice (42), which may be a further indication of differences in site-specific immune responses.

Research on the cytokine responses of human cells to Gram-positive or pneumococcal specific stimuli are also scarce. Several studies reported spontaneous production of cytokines by PBMC's of the elderly including TNFα (76) and IL-1β and IL-6 (47), supporting the 'inflamm-ageing' hypothesis. Studies using Gram-negative stimuli report either no change in production of cytokines by elderly PBMC (36, 47) or lower production after stimulation (77). One study that could shed light on pneumococcal specific responses is a study where human PBMCs were stimulated with purified pam3cys (a

TLR1/2 agonist); significantly less IL-6 and TNFα production was observed in cells from elderly compared to young counterparts (40). This same study also found that phosphorylation of another TLR adaptor protein MAPK1/3 was decreased in elderly cells and that this correlated with diminished cytokine production. Together these studies support the hypothesis of chronic baseline inflammation and lower responses upon specific infectious stimulation in elderly. Human clinical studies have also looked at circulating amounts of cytokines in the serum of young and elderly patients hospitalized for pneumonia, however the results were conflicting and not consistent with clinical outcome (77, 78).

Complement System

The complement system is a diverse set of proteins found in the blood that enhance both the innate and adaptive arms of the immune system; mainly aiding chemotaxis of phagocytes, opsonization of antigens, and pore formation in bacteria. Complement function is classified into three different pathways. The alternative and the mannose-binding lectin pathways can be activated directly by bacterial cell surface components and are considered as effectors of the innate immune response. The classical complement pathway can be activated by antibody-antigen complexes and is therefore considered an effector of the adaptive immune response. Both human and mouse studies have shown interaction of a key component of the classical and alternative pathways, C3, with pneumococci, (79, 80) suggesting an important role for complement in the defense against pneumococcal systemic infection. Very little is known about how ageing affects complement activation and activity in general, but some studies have begun to examine the interaction of complement with pneumococci in older persons to unravel its contribution to pneumococcal disease. Data regarding the effect of ageing on serum concentrations of C3/C4 is however inconsistent, though the more recent work suggests that complement doesn't appear to contribute to the impaired neutrophil function observed in the elderly (49, 81).

Immunosenescence of Adaptive Immunity

Lymphoid Tissue

Supporting the physiological barrier, the mucosal lymphoid tissue (NALT in URT, BALT in lungs, and GALT in gut) forms a second barrier against potential pathogens, and many immune cells are active in this tissue, including antigen-presenting cells, B- and T-cells. In aged mice it is suggested that immunosenecence occurs earlier at mucosal lymphoid tissue than systemic lymphoid tissue (e.g. spleen) (82), but when different mucosal surfaces are compared in vaccination studies, immunosenescence seems to be less apparent at the NALT at any given time than at the GALT (82-84). Choice of adjuvant (84), dose of vaccine/adjuvant (85), and frequency of vaccinations (85) are all

factors that seem to heavily influence the outcome of vaccination at the elderly mucosal site, with increased dose and frequency in elderly mice needed to attain the same protection as in young mice.

B-cells

As part of the lymphoid tissue B-cells contribute to immunity by producing antibodies after phagocytic cells present antigens to them, and eventually by developing into B-memory cells available for reactivation in the future. Table 2 gives an overview of data regarding immunosenescence of adaptive immunity. Concerning the pneumococcus, several mouse models have shown that antibodies have limited effect on clearance of colonization (9, 94-96), whereas for systemic clearance of pneumococci it appears antibodies are crucial (63). In humans, pneumococcal polysaccharide vaccines (PPV) which induce systemic antibodies, indeed have high efficacy on prevention of invasive pneumococcal disease (97) and are assumed to not affect pneumococcal nasopharyngeal colonization (98) though the latter was studied in children where PPV is known to be less immunogenic compared to adults.

The immune system often naturally develops antibodies to bacterial components. For mucosal infections, two types of antibodies appear relevant, secretory IgA (sIgA) and IgG. Both are present locally such as in the saliva, while IgG is also present systemically in the serum. Exposure to pneumococci, either naturally or through vaccination, results in production of both types of antibodies in children (99). To our knowledge, there is little data available on the impact of ageing on sIgA in humans, or on mucosal antibodies in general, although this seems highly relevant to study because of their function in the URT. In mice, sIgA has been studied after intransal vaccination. Middle-age mice (1 year old) produce similar amounts of sIgA in response to a weak antigen (OVA) and cholera toxin adjuvant compared with young-adult, but elderly mice (2 years old) have almost no response (84). Elderly mice do produce comparable amounts of sIgA as young mice after vaccination with pneumococcal surface protein and DNA adjuvants targeting TLR9 or dendritic cells, however, a dose 5-times larger was required in elderly (85).

Naturally acquired serum IgG against six pneumococcal serotypes, which commonly cause IPD, showed a tendency to decrease with ageing; elderly (≥ 65 years) had significantly lower IgG concentrations for serotypes 3 and 6B compared to younger adults (30-64 years) (49, 87, 88). Naturally acquired serum IgG against pneumococcal proteins decreased similarly with ageing (87, 88). This suggests a possible decrease of available antibodies against *S. pneumoniae* in the systemic circulation of elderly. In contrast, extensive research investigating PPV efficacy found no significant differences in IgG serum concentrations between elderly and younger adults after vaccination and this vaccine has not shown effective protection against mucosal colonization (89-92).

In addition to measuring the amount of antibodies, it is also important to test their functionality, notably the ability to opsonize pneumococci for phagocytosis. Serum from vaccinated adults had higher opsonophagocytic activity for all serotypes in younger adults than in elderly, which was independent of the assay method used (90, 92). A similar decrease was observed for naturally acquired serum IgG in elderly compared to young adults, with the exception of serotype 3 (49). According to the authors, this exception may be due to a generally lower detected functional activity for serotype 3 and a low percentage of people with opsonically active antibodies to serotype 3 (49). However, a study by Kolibab *et al* regarding serotype 4 and 14, found only lower opsonophagocytic antibody titers in the oldest subgroup of elderly (>77 years of age) compared to younger adults (89).

IgG must bind to *S. pneumoniae* in order to opsonize the bacterium. This binding strength, also called avidity, may be reduced in elderly although variable or conflicting data are reported (89-91). In summary, the elderly appear to respond with similar amounts of IgG to pneumococcal stimulation as younger adults, however, there may be some functional deficits of these antibodies in elderly, which may contribute to impairment of pneumococcal clearance.

Overall, it is unknown whether deficient quantitative or qualitative antipolysaccharide antibodies are the primary reason for high burden of invasive infections and pneumococcal pneumonia in elderly, who often suffer from comorbidity as well. However, high functional antibody levels upon vaccination may offer protection against pneumococcal disease in old age. A Cochrane meta-analysis of the efficacy of PPV reported that the vaccine prevents invasive disease in adults but due to lack of power the meta-analysis was inconclusive regarding prevention of pneumonia in risk groups with chronic disease or elderly. Furthermore, it found no effect of PPV on all-cause mortality (97).

T-cells

CD4+ T-cells play a central role in cell-mediated adaptive immunity against *S. pneumoniae*, especially the Th17 subset (61). To investigate the possible effect of ageing on T-cell immunity, Meyer *et al* compared BAL-fluid between healthy, non-smoking elderly (≥65 years) and younger adults (20-36 years) (86). The CD4+/CD8+ T-cell ratio in these BAL-fluids increased significantly as a function of age. This indicates a relative increase of CD4+ lymphocytes in elderly; however the study did not indicate the absolute values (86). In another study, CD4+T-cells isolated from human blood showed no difference between different age groups (mean ages of 30, 50 and 72 years old respectively) in the percentage of cytokine secreting T-cells (IFN-γ, IL-5, IL-17A) upon stimulation with three pneumococcal proteins (87). In contrast, a recent study showed a decrease in number of CD4+ regulatory T-lymphocytes in peripheral blood samples with ageing (100). So there may be a tendency towards less CD4+ regulatory cells in elderly

and more mature CD4+ effector cells, which may contribute to the 'inflamm-aging' phenomena or overwhelming inflammation during infections. Recent efforts to induce protective T-cell immunity in elderly mice via mucosal vaccination have shown effective CD4+ responses, correlating with clearance of colonization, however, increased dose of antigens, extra adjuvants, and extra boosters were all required to elicit similar responses in elderly as young mice (85). Therefore, it is of major importance to study the potential protective effect of the newer conjugate pneumococcal conjugate vaccines on protection against mucosal diseases in elderly. In infants conjugate vaccines have shown to induce both systemic and mucosal protection against acquisition, colonization and disease caused by vaccine serotype pneumococci (101). If this is true for elderly, conjugate vaccines may offer improved protection against vaccine serotype pneumococcal pneumonia in elderly over PPV; Metersky *et al* (102) and Musher *et al* (103) have recently covered this topic in depth. Currently, nation-wide vaccination of young children with the 7-valent pneumococcal colonization vaccine showed to reduce circulation of vaccine serotype pneumococci in the population including elderly (herd effect) as well. Unfortunately, all-cause pneumonia in elderly was not found to be reduced, largely due to serotype replacement (104, 105). If conjugate vaccination of elderly would not lead to a reduction in mucosal diseases, alternatively, vaccination during middle age could be considered as a strategy to induce protection before immunosenescence emerges.

In any case, potential implementation of PCV in adults and elderly raises the question whether the immunogenicity of PCV in adults is influenced by previous exposure to PPV (102). Many guidelines currently recommend pneumococcal polysaccharide vaccination for the elderly every five years (102). However, some evidence is available showing that PCV following PPV elicits diminishing antibody responses; this phenomenon, called immune hyporesponsiveness, is only observed when PCV vaccination follows PPV vaccination and does not apply to PCV followed by PCV or PCV followed by PPV (102). Furthermore the effectiveness of PCV compared to PPV in the elderly still needs to be evaluated (102, 103).

Colonization and the Path to Disease in Elderly

As mentioned earlier, the demand for healthcare resources for elderly with pneumococcal pneumonia is expected to significantly rise in the coming decades. Furthermore, reports from the UK indicate that disease and mortality in adults attributed to pneumococcal serotypes 1 and 3 seen in the 21st century are very similar to historical accounts from the early 20th century (106). These authors further highlight the pressing need to understand the path to disease for respiratory pathogens. A wealth of data indicates that carriage of potential pathogens in the URT is required for disease to develop (9, 10, 23, 24, 106). This is generally accepted for children, however due to the lack of elderly carriage data, and low carriage reported in the few studies in the last decades (Table 3), (14-18) some have questioned if the path to disease in the elderly includes a prerequisite

Table 2: **Immunosenescence of adaptive immunity regarding Gram-positive bacteria.**

Element of Adaptive Immunity		Site	Species	Level of Evidence*	Effect of Ageing
T-cells					
Baseline CD4+ / CD8+ ratio in BALF	(99)	Lung	Human	1	↑
IL-17 production	(93)	Systemic	Human	1+ (pneumococcal proteins)	=
B-cells					
Number of cells following challenge	(61)	Peritoneal	Mouse	1 (*S. pyogenes*)	↑
Secretory IgA concentration (response to vaccine)	(85)	Saliva	Mouse	1+ (5x dose vaccine and extra adjuvant used in elderly)	=
IgG concentration (naturally acquired)	(92)	Systemic	Human	1+ (pneumococcal proteins)	↓
IgG concentration (response to PPV vaccine)	(94-97)	Systemic	Human	3+ (serotype 4, 6B, 9V, 14, 18C, 19F, 23F)	=
IgG opsonic titers (response to PPV)	(68, 92, 94, 95, 97)	Systemic	Human	3+ (exception serotype 3)	↓
IgG avidity (response to PPV)	(94, 96, 97)	Systemic	Human	3+	↓
IgG required for 50% killing (naturally acquired)	(68)	Systemic	Human	1+ (serotype 6B, 14)	↑
IgG required for 50% killing (naturally acquired)	(68)	Systemic	Human	1+ (serotype 3, 23F)	=
IgM memory B-cell (response to PPV)	(114)	Systemic	Human	1+	↓
IgM concentration (natural or response to PPV)	(68, 92, 114)	Systemic	Human	2+ (serotypes 3, 4, 6B, 9V, 23F)	↓
IgM concentration (natural)	(68, 92)	Systemic	Human	1+ (serotype14)	=
IgM required for 50% killing	(92)	Systemic	Human	1+ (serotype 14)	↑

*Key: 1 = Shown in 1 study; 2 = shown in 2 studies; 3 = similar trends shown in several studies or more than 1 species. + = Pneumococcal specific models.

carriage state (118). However, looking at the evidence, the discrepancy between high incidence of IPD and low carriage in elderly has several possible explanations.

First, the upper respiratory tract is already a low-density niche for bacteria in children (26) it's possible that with increasing age and immune system maturation, the niche becomes even less dense. This is underlined by recent culture-based evidence showing that the density of pneumococcal colonization of the nasopharynx is indeed less dense in middle-aged adults compared to children (119). With decreasing density, the method considered the gold standard in pneumococcal carriage detection, namely the transnasal nasopharyngeal sample becomes less sensitive. Furthermore, it is known that using culture-independent methods increase the sensitivity of detection (120) but these methods are not yet widely used.

Second, the niche that pneumococci inhabit may change with age. Evidence has emerged that using alternate swabbing sites (trans-oral / oropharynx) coupled with molecular methods significantly increases carriage detection in asymptomatic adults (19). Looking back at historical pneumococcal surveillance studies, swabs were not commonly used for detection of pneumococci at the start of the 20th century (23, 24). As discussed earlier, saliva was instead collected from both children and adults to detect pneumococcal carriage. The prevailing opinion of experts at the time, based on numerous epidemiological studies, was that transnasal swabs were the least sensitive method, transoral swabs sufficiently sensitive, and saliva the most sensitive sample to use for colonization detection (24, 110). More recent studies testing a range of URT samples, albeit using conventional culture, showed carriage rates of 16% using saliva, 29% using transoral swabs, and 8% using transnasal swabs in adults with COPD or asthma (121). Indirect evidence for the role of saliva in transmitting pneumococci among adults in crowded settings has also emerged (122). Owing to the current understanding of the path to pneumococcal disease where URT carriage is a necessary prerequisite and pneumonia can result from micro-aspiration of pneumococci colonizing the URT, it would be wise to perform surveillance using the transoral swab or saliva samples together with molecular detection methods in elderly before concluding that pneumococcal carriage is rare.

Opposite page

Table 3: Carriage of *Streptococcus pneumoniae* in healthy elderly and adults

We have summarized carriage rates observed in elderly and adults according to isolation method and sample type. All colonization studies performed in elderly were included, whereas studies in adults or parents were included if the number of participants was above 100. Only data from healthy individuals was included. With respect to methodology, 'mouse' refers to recovery of live pneumococci from blood of mice injected with a human sample, 'culture' refers to isolation of live pneumococci directly from the sample, and 'molecular' refers to lytA gene detection in quantitative-real-time-PCR. *When year of execution of the study was not mentioned in the original publication, year of publication is reported. **(–) - not stated; ***OP - oropharyngeal or trans-oral nasopharyngeal swab; ****NP - trans-nasal nasopharyngeal swab.[#] only period prevalence available, [##] both authors report results for samples collected in the same surveillance study but pneumococcal carriage was either detected by culture method (Spijkerman *et al.*) or by molecular method (Trzcinski *et al.*).

Author, Place		Year*	Sample	Method	Study population	Subjects (N)	Prevalence
Stillman, New York, USA	(107)	1916	saliva	mouse	Adults	398	43
Stillman, New York, USA	(108)	1917	saliva	mouse	Adults	297	39
Rosenau, Massachusetts, USA	(129)	1923-24	saliva	mouse	Adults	130	48
Christie, Scotland	(109)	1932-33	saliva	mouse	Adults	100	55
Smillie, Massachusetts, USA	(130)	1933	nose/throat swab	mouse and culture	Adults	493	43
Viktorow, Soviet Union	(131)	1934	oral wash	mouse	Adults	308	54
Hendley, Virginia, USA	(111)	1973	throat swab	mouse	Elderly / institutionalized	85	22
Sailer, Georgia, USA	(132)	1917	nose/throat swab	culture	Adult / military	700	16
Christensen, Denmark	(24)	1923	—**	culture	Adult / military	1474	16
Putnam, California, USA	(112)	1994-95	OP*** swab	culture	Adult / military	915	1
Millar, Arizona, USA	(113)	1997-2002	NP**** swab	culture	Adults (18+)	1729	14
Millar, Arizona, USA	(113)	1997-2002	NP swab	culture	Elderly (65+)	70	13
Regev-Yochay, Israel	(14)	2001	NP swab	culture	Adults (18-65)	1105	4
Regev-Yochay, Israel	(14)	2001	NP swab	culture	Elderly (65+)	195	5
Palmu, Finland	(17)	2003	NP swab	culture	Elderly (65+)	592	5
Abdullahi, Kenya	(114)	2004	NP swab	culture	Adult (20-49)	195	6
Abdullahi, Kenya	(114)	2004	NP swab	culture	Adult (50+)	107	5
van Gils, The Netherlands	(101)	2005-6	NP/OP swab	culture	Parents	1878	22
Levine, Israel	(115)	2007	NP/OP swab	culture	Adult / military	742	6
Regev-Yochay, Gaza strip	(116)	2009	NP swab	culture	Parents	376	8
Spijkerman##, The Netherlands	(117)	2009-10	NP/OP swab	culture	Parents	324	19
Trzcinski##, The Netherlands	(19)	2009-10	NP/OP swab	molecular	Parents	268	39
Ansaldi, Italy	(21)	2012	NP swab	molecular	Elderly (60+)	283	20#
Suzuki, Japan	(22)	2006	saliva	molecular	Elderly (62+)	30	37

Last, there is the possibility that the path to disease in elderly is different than in any other age group, however, this possibility seems biologically less plausible than the reasons discussed above.

Additional risk factors for pneumococcal disease

Two additional clinical conditions greatly increase pneumococcal disease in the elderly. First, infection with respiratory viruses such as influenza virus and RSV greatly predisposes persons of all ages to secondary pneumococcal pneumonia (7, 123). It is interesting to note that influenza causes some of the same immune responses seen during immunosenescence, e.g., general inflammation and dysfunction of innate cells via down regulation of receptors such as MARCO. Since presence of those viruses is also positively associated with increased pneumococcal colonization in children (124), and inflammation caused by influenza virus increased pneumococcal colonization density in young mice, (125) it might be worthwhile studying whether the combined immune deficits caused by immunosenescence and viral presence have a synergistic effect in elderly on pneumococcal colonization or otherwise. In light of this knowledge, there is a strong indication for yearly influenza vaccination for prevention of secondary pneumococcal pneumonia in the elderly (7).

Second, the elderly are at greater risk for aspiration, especially chronic silent aspiration (5, 126, 127). Aspiration is thought to be responsible for 5-15% of CAP in the elderly (126). Furthermore, swallowing disorders in the elderly are a predictor of CAP mortality (126). Since increased oral bacterial density has been observed in the elderly (127), and the presence of potential pathogens in the oropharyngeal cavity is an additional risk factor for aspiration pneumonia in elderly (126), good oral hygiene seems additionally important for elderly to reduce bacterial colonization and reduce the risk for consecutive lung infections (5, 127).

Concluding Remarks

Immunosenescence is likely to contribute to increased susceptibility to *S. pneumoniae* infections in elderly, which is a major public health problem. Extensive research has been performed on immunosenescence in general, however, knowledge is still limited regarding the particular mechanisms of immunosenescence and their contribution to increased susceptibility to pneumococcal infections in elderly. Known contributions of immunosenescence in the lung are summarized in Figure 2. There seems to be a trend towards imbalanced immune responses in elderly resulting in an apparent lack of immune homeostasis. For example, cytokine secretion and macrophage population seem to differ in the elderly compared to younger adults. In addition, effects of immunosenescence may not be universal for all elderly, because of possible underlying factors and co-morbidities that affect each individual in different manners. With respect to innate

immunity, there seems to be parallel dysfunctions, such as a decrease in the presence of immune cells, a state of chronic inflammation, reduced immune activation upon stimulation, and impaired phagocytosis capacity with ageing. In contrast, humoral immunity seems to become mainly functionally impaired in the elderly whereas absolute concentrations of antibodies are relatively stable.

Further research is needed, since data are very limited and variable, and often derived from murine experiments with non-pneumococcal models. So far, investigations of humoral immunity were unable to reveal immune deficits clearly related to increased infection rate and decreased vaccine effectiveness in elderly. Therefore, future research may be performed directed towards a possible role for impaired innate immune function contributing to *S. pneumoniae* infections in elderly; this is of ultimate importance for the design of future preventative measures, especially the improvement of new vaccination strategies.

Lastly, due to the highly poly-microbial nature of URT samples, and the potential for bacterial-bacterial interactions we believe that a large proportion of pneumococcal carriage in adults and the elderly goes undetected in point-prevalence studies using swabs. This is a view shared by our predecessors in the early 20thcentury who conducted large, long-term carriage surveillance studies before the introduction of antimicrobials diminished interest in carriage. Both Heffron and White wrote comprehensive books reviewing all current literature on pneumonia or the pneumococcus, respectively, in the 1930's. At that time surveillance had been performed on thousands of adults, and sometimes sampling continued for up to 3 years, resulting in the general consensus that 45-60% of all adults (including elderly) carry pneumococcus at any given time, but that period-prevalence was a more accurate measure than point-prevalence (23, 24, 110, 128). It is entirely plausible to conclude that the path to disease likely has not changed in 100 years and we should utilize currently available molecular methods and epidemiological tools for better understanding of this pressing public health issue.

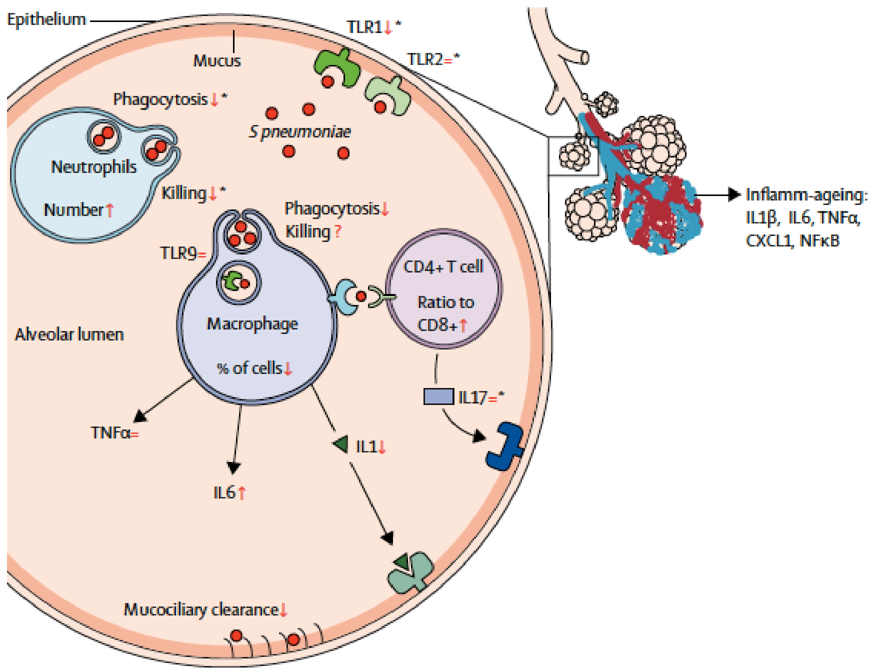

Figure 2: **Compilation of factors involved in immunosenescence of mucosal immunity at the alveoli which may contribute to increased *S. pneumoniae* susceptibility in the elderly.**
Red arrows indicate evidence suggesting an increase or decrease in elderly compared to young adults. **S. pneumoniae* specific evidence. Abbreviations: IL (interleukin); NK (natural killer cells); PRR (Pattern recognition receptor); TLR (Toll-like receptors); TNF (Tumor necrosis factor).

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3

Chapter Three

Impaired innate mucosal immunity in aged mice permits prolonged *Streptococcus pneumoniae* colonization

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Infection and Immunity; in revision

Abstract

Streptococcus pneumoniae is a frequent asymptomatic colonizer of the nasopharyngeal niche, and only occasionally progresses towards infection. The burden of pneumococcal disease is particularly high in the elderly, and the mechanisms behind this increased susceptibility are poorly understood. Here we used a mouse model of pneumococcal carriage to study immunosenescence in the upper respiratory tract. Nasal associated lymphoid tissue (NALT) showed increased expression of Toll-like receptor 1, interleukin-1ß, NLRp3 inflammasome, and CCL2 in naïve elderly compared to young animals. This suggests an increased pro-inflammatory expression profile in the NALT of aged mice at baseline. Simultaneously, we observed a more tolerogenic profile in respiratory epithelium of naïve elderly compared to young-adult mice with upregulation of the NF-κB pathway inhibitor peroxisome proliferator-activated receptor-γ (PPARγ). After nasal instillation of pneumococci, pneumococcal colonization was prolonged in elderly mice compared to young-adults. Delay in clearance was associated with absent or delayed up-regulation of pro-inflammatory mediator(s) in the NALT, diminished influx of macrophages into the URT niche, absent down-regulation of PPARγ in respiratory epithelium, accompanied by diminished expression of cathelicidin (CRAMP) at the site of colonization. These findings suggest that unresponsiveness to pneumococcal challenge due to altered mucosal immune regulation is the key to increased susceptibility to disease in elderly.

Introduction

Streptococcus pneumoniae is a frequent but transient commensal of the human URT, that may grow out and progress towards respiratory and invasive disease. Pneumococcal disease disproportionably affects the very young and the elderly; while immature immunity is thought to be responsible for increased susceptibility in young children, the factors behind the high susceptibility of the elderly are poorly understood. The increased risk of the elderly (defined as persons aged 65 or older) for community acquired pneumonia (CAP) is independent of health status and co-morbidities (1). Microbial etiology of CAP is similar in all adults with *S. pneumoniae* as the primary bacterial cause (2). In high-income countries, despite access to high quality health care, mortality due to CAP in the elderly still ranges from 15-30% and can double among residents of nursing homes (3-5). Continuous increases in life expectancy leading to a growing population of elderly people underlines the public health importance of understanding the basis of this major health problem (5).

The increased risk of infectious diseases in the elderly has been attributed to age associated deterioration of the immune system, called immunosenescence, however the exact nature of these defects in immune responses have not been fully elucidated and immunosenescence of the mucosal system of the upper respiratory tract (URT) in particular is still poorly explored (6). Hinojosa *et al* (7) have recently shown an association of decreased protein levels of TLR1 and 2 in lung homogenates and increased levels of pro-inflammatory cytokines at baseline in elderly mice with higher mortality in a model of invasive pneumococcal pneumonia. This data suggest that there are innate defects in the lungs of elderly mice that likely contribute to increased risk and severity of pneumonia. Furthermore TLR1/2 are crucial for clearance of pneumococcal colonization in mice, (8) but their contribution to immunosenesence in the URT is still unknown. Other groups have also sought to elucidate the mechanisms of immunosenescence of the innate compartment, however, most of these studies were performed in vitro (9-12) using primary cells known to change phenotype *ex vivo* (e.g. macrophages) (13) and/or used antigens such as LPS that are relevant for Gram-negative but not for Gram-positive bacteria like *S. pneumoniae*. Furthermore, *in vivo* infection models (7, 14, 15) and in vitro work using alveolar macrophages (14, 15) are not informative regarding the prerequisite colonization state before pneumonia develops.

The innate cellular effectors mediating clearance of pneumococcal colonization in young-adult mice have recently been described by Zhang *et al* (16); the results indicate a major role for monocyte/macrophages in initiation and progression of URT clearance, and provided further evidence that neutrophil influx alone is insufficient (17, 18). It has also been demonstrated that the inflammasome NLRp3 is activated by pneumococcal pneumolysin upon intranasal inoculation with *S. pneumoniae* leading to secretion of Il-1β by several cell types. Furthermore, NLRp3 is also required for bacterial clearance

in a mouse pneumonia model (19). In addition, NOD2 and other PAMPs synergizing with the inflammasome is crucial for IL-1β processing and secretion (20). Il-1β in turn can synergize with cathelicidin to induce the production of other cytokines such as CCL2, (21) which via NOD2 recognition of pneumococcal peptidoglycan has been identified as a key part of the recruitment of monocyte/macrophages to the URT of mice and therefore crucial for pneumococcal clearance (22). All of these components correspond to a healthy immune response to *S. pneumoniae* colonization. We therefore hypothesize that one or more of these components might be affected in elderly as part of immunosenescence, contributing to increased susceptibility to respiratory infections. Since asymptomatic colonization of the URT is an important prerequisite to pneumococcal disease (23), we studied local innate immune responses in elderly mice for possible defects that might specifically impair clearance of *S. pneumoniae* from the upper respiratory niche. As yet, this is the only *in vivo* study to address immunosenescence of the URT related to clearance of a respiratory pathogen and the information has the potential to further our understanding of this highly important, pre-disease carrier state in elderly.

Materials and Methods

Ethics statement / Animals

Animal experiments were performed in accordance with the Dutch Animal Experimentation Act and EU directives 86/609/CEE and 2010/63/EU related to the protection of vertebrate animals used for experimental or other scientific purposes. The experimental protocols were approved by the Committee on Animal Experiments of the University of Utrecht (DEC # 2009.II.10.107; 2010.II.07.125; 2011.II.11.175). C57BL/6 female specific pathogen free mice were purchased from Harlan (Venray, Netherlands). For the purpose of this study 3-4 month old mice were defined as young-adult and 18-23 month old mice as elderly. Animals with noticeable tumors were excluded from experiments and animals with neoplasm detected in post mortem examination were excluded from analysis. Mice were euthanized with 120mg/kg pentobarbital (Veterinary Department Pharmacy) administrated intra-peritoneally to induce respiratory arrest. Blood samples were taken via post-mortem cardiac puncture.

Bacterial strains

Streptococcus pneumoniae strain 603 is a serotype 6B clinical isolate that has been previously used in murine models of pneumococcal colonization (24). This strain was mouse-passaged and grown to mid-log phase in brain-heart infusion broth (BHI), aliquoted, and stored frozen in 10% glycerol at -80°C. Prior to animal inoculation, bacterial cells were thawed, washed twice with saline, re-suspended to 5×10^8 CFU/ml, and cell suspensions were tittered by plating tenfold dilution of inoculum on blood agar plates supplemented with gentamicin (SB7-Gent, Oxoid). An autolysin-negative variant of the un-encapsulated *S. pneumoniae* strain Rx1 (Rx1Δply) (24) and the unencapsulated

variant of strain 603 (603Δcps) (25) were used for functional assays.

Mouse colonization experiments

Mice were inoculated intranasally, as described previously, with 10 µl of bacterial suspension containing approximately 5×10^6 CFU (24). In colonization experiments that followed 5-10 mice were sacrificed per time-point. From each animal 500 µl of retro-tracheal nasal lavage were collected with saline followed by nasal tissue harvest. Tissue samples were crushed in 1 ml of PBS, stored on ice and processed within 3 hours. Samples were vortexed vigorously and plated in serial dilutions onto SB7-Gent medium in order to quantify *S. pneumoniae* presence in the URT. Per mouse, CFU counts from the lavage and nasal tissue were summed. Blood and lungs of all mice were cultured to check for presence of *S. pneumoniae.*

Cell preparation

Upper respiratory tract phagocytes were collected from mice as previously described, with some modifications (16). Briefly, a nasal lavage was performed with calcium- and magnesium-free PBS supplemented with 1% BSA (PBS+BSA), collecting the first 500 µl of fluid exiting the nares. Sixty µl of lavage fluid was used for pneumococcal CFU counts. The remaining fluid was centrifuged at 1200 RPM (~300G) for 10 minutes at 4°C. Supernatant was removed and frozen at -80°C. The cell pellet was resuspended in 100 µl of PBS+BSA. Single cell suspensions were obtained from NALTs or spleens as described previously (26). If necessary, red blood cells were lysed using ACK lysing buffer (BioWhittaker).

Histopathology

Crania for histopathological examination of nasopharynxes were prepared as described (8), and processed at our Pathology Department. Craniums were embedded in paraffin, sectioned sagitally or cranially, and slides were stained either with hematoxilyn and eosin (H&E) or by Gram staining method. Images were taken with a Nikon Eclipse E800 microscope with a Nikon DXM1200 digital camera using the Nikon ACT-1 software version 2.70 (Nikon Netherlands) at similar anatomical positions. Images were cropped using Adobe Photoshop CS3; NALT images were all cropped to the exact same size: 4x6 inches. For NALT area and infiltration quantification, see supplemental methods.

Flow cytometry

Nasal lavages were performed as described above and pooled from 2, 3, or 5 mice depending on cell counts. Cells were blocked with anti-mouse CD16/CD32 (Mouse Fc Block, BD Pharmingen). A 50 µl cocktail of fluorophore-conjugated antibodies recognizing different cell surface proteins was added, incubated in the dark for 30 minutes at 4°C (for details see supplemental methods). 200 µl of PBS+BSA was added to terminate the reaction. Pellets were collected and washed again with 200 µl buffer. Pellets were

resuspended in buffer + 0.5% formalin and subjected in full volume to flow cytometry analysis on a BD FACSCantoII flow cytometer (BD Biosciences). FlowJo version 7.6.3 software (Tree Star, Inc.) was used to analyze flow cytometry data. GR-1^{high} $CD11b^{+}$ $F4/80^{-}$ cells were considered to be neutrophils and GR-$1^{+/-}$$CD11b^{+}$$F4/80^{+}$ cells were considered to be monocytes/macrophages.

Quantitative real time RT-PCR

NALT tissue dissected from mice (26) was instantly placed into 1 ml of RNA Later (Ambion), and RNA extracted using the RNAqueous-4PCR kit (Ambion). Upper respiratory tract epithelium RNA was collected after URT retro-tracheal lavage with PBS+BSA (as described above) by flushing airways with 500 µl Lysis/binding buffer from RNAqueous-4PCR kit (adapted from (8)). URT samples were determined to be of epithelial origin by RT-PCR using primers specific for keratin 18. RNA quantity and quality for all samples was determined in NanoDrop spectrophotometer (260/280 =1.8-2 and 260/30 above 2) and by conventional agarose gel electrophoresis.

RNA was reverse transcribed using GeneAmp RNA PCR cDNA synthesis kit. cDNA was used with SYBR Green PCR master mix (Applied Biosystems) and primers at a concentration of 250 nM. Amplification was performed on a StepOnePlus (Applied Biosystems) machine. Data was analyzed using LinRegPCR software (www.hartfaalcentrum.nl) (27, 28). Transcript amount determined by LinRegPCR for 2 housekeeping genes for each sample was used for normalizing the data. The geometric mean of Ywhaz (29) and GAPDH (8) was used for housekeeping normalization. For details see supplemental methods. For primer sequences, see Table S1.

Neutrophil surface killing assay

Opsonin-independent neutrophil killing was measured via surface phagocytosis assays (26). For details on mouse bone marrow neutrophil isolation and preparation see supplemental methods. Briefly, neutrophils were resuspended at a concentration of $2.5x10^{6}$ cells/ml in HBSS+0.1%FCS. One hundred replicates of a 10 µl bacterial cell suspension containing on average 25 CFU of the 603Δcps strain were plated on SB7-Gent plates and allowed to air-dry on agar. 20 µl of neutrophil suspension was overlaid onto 50 spots of bacteria and allowed to air dry. Plates were incubated overnight at 37°C with 5% CO_2 after which colonies were counted in order to calculate the fraction of pneumococcal cells killed by neutrophils compared to control plates without neutrophil overlays.

Macrophage functional assays

For details on mouse bone-marrow-derived macrophage (BMDM) isolation and preparation see supplemental methods. Mouse BMDM were used to test their ability to phagocytose and kill live pneumococci in an assay modified from van Zoelen (30). Briefly, macrophages were thawed and adjusted to a concentration of $2.5x10^{6}$ cells/ml in

DMEM/F-12 with GlutaMAX and 10% FBS. 100 µl of cells were added to 1 ml media in a 12-well tissue culture plate. Cells were allowed to adhere overnight at 37°C with 5% CO_2. The assay was performed both with and without bacterial pre-incubation in autologous serum. For assays performed with serum, a mouse serum pool was made with serum from 2-6 naïve mice, and aliquots frozen at -20°C. Young-adult mouse serum was used with cells from young-adult mice, and elderly mouse serum for cells deriving from elderly mice. Serum and bacteria were incubated on ice for 10 minutes prior to infection with final volumes of 50 µl serum and 100 µl of pneumococcal cells suspension per well. Adherent monolayers were infected with pneumococcal strain Rx1Δply at a multiplicity of infection (MOI) of 50, centrifuged at 400G for 5 minutes, and placed at 37°C for 10-15 minutes allowing phagocytosis. After this time, wells were washed 3 times with ice cold PBS to remove extracellular bacteria and 1 ml of sterile water was added to half of the wells to lyse macrophages. Lysates were diluted in DPBS and plated onto SB7-Gent plated to determine the CFU of bacteria inside the macrophages at baseline. 1 ml of culture media was added to remaining wells and placed back at 37°C for 90 minutes, after which time media was removed, cells were lysed, and bacteria quantified as above. Macrophage killing was determined based on the reduction in CFUs after 90 minutes compared to CFU at baseline.

For stimulation assays, mouse BMDM cells were thawed, seeded onto 12-well plates as described above, allowed to adhere for 24-48 hours and stimulated for either 2.5 or 3.5 hours with 100 µl of media or pneumococcal whole-cell antigen (WCA). WCA was made of ethanol killed cells of strain Rx1Δply prepared as described by Malley *et al* (24). RNA was harvested from macrophage monolayers (pool of 3 wells each) using RNAqueous-4PCR kit (Ambion). qPCR was performed as described above.

Statistical analysis

Statistical analysis was performed using the graphing software GraphPad Prism version 5.0. Data were tested for normality and non-parametric tests were used accordingly. Log-rank test was used to compare survival-like data of duration of carriage. Paired t-test was used to compare in vitro pneumococcal killing by phagocytes. Kruskall-Wallis tests or two-way ANOVA were used to compare group dynamics, and either Dunn's post-tests or Bonferroni's correction were used to control for multiple comparisons. Planned single comparisons were tested with Mann-Whitney U-tests (with Bonferroni correction as needed); e.g. naïve young compared to naive elderly.

Results

Delayed clearance of pneumococcal colonization in elderly mice

To investigate whether aging is related to prolonged colonization of the mouse nasopharynx, we studied colonization through time in elderly and young-adult mice using a

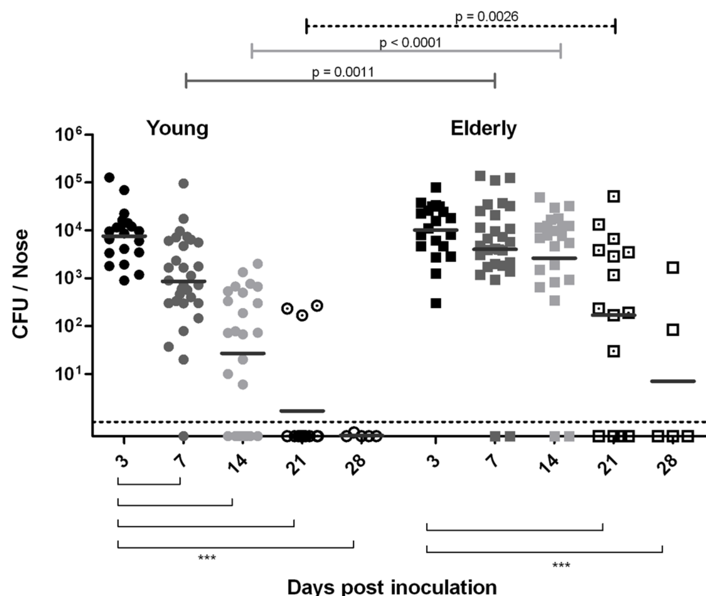

Figure 1: **Effect of age on duration and density of pneumococcal colonization**
Elderly and young-adult mice were colonized with serotype 6B pneumococcal strain and sacrificed after 3, 7, 14, 21, or 28 days. Circles represent young-adult mice, squares are elderly mice; solid lines at medians and the dotted line represents the lowest level of detection. The differences in density between the young-adult and elderly mice are statistically significant at all times except at days 3 and 28 post-inoculation (Mann-Whitney with Bonferroni correction $p<0.002$ significant). In young-adult mice, density of colonization is statistically lower at days 7, 14, 21, and 28 compared to day 3. In elderly mice, density of colonization is not statistically lower until day 21 compared to day 3. This figure represents 5 independent experiments with a combined N=15-20 at each time-point except day 28 (N=5).

live sampling method allowing continuous monitoring of an individual animal for the presence of *S. pneumoniae* in the URT. Young-adult mice began to clear pneumococci by day 14 post-inoculation and by day 21 the presence of pneumococci was undetectable (Figure S1). In contrast, elderly mice did not begin to clear pneumococci until day 17, and 50% of the elderly animals remained colonized at the termination of the experiment on day 28. Elderly mice were colonized for a statistically longer period of time than young-adult mice (Log-rank test; $p=0.0025$), with a median time to clearance of 26 days versus 19 days for young-adult mice.

Next, we tested if either higher initial density of pneumococcal presence or a delay in *S. pneumoniae* clearance contributed to the extended carriage duration seen in elderly mice. Mice were intranasally inoculated with *S. pneumoniae* and density of pneumococcal colonization was assessed in URT lavages and harvests of nasal tissue collected post mortem. As shown in Figure 1, both groups of mice had equal densities of colonization 3 days post-inoculation. Young-adult mice had a significant drop in density of colonization from day 7 on ($p<0.002$ Mann-Whitney), whereas elderly mice did not have a significant drop in density pneumococcal density until day 21 ($p<0.002$). Two out of

5 elderly mice compared to none out of five young-adult mice were still colonized 28 days post-inoculation (Figure 1). The spread in colonization density within the elderly group could not be attributed or correlated to age (ranging from 18 to 23 months, data not shown). The aim of our model was to establish asymptomatic colonization in elderly mice, and indeed, no mice showed symptoms of pneumonia nor were pneumococci recovered from blood or lungs of any mouse.

Cellular Infiltration Dynamics in the URT in response to pneumococcal colonization

Previous studies on innate immune responses to pneumococcal colonization in murine models showed that a switch to a high proportion of monocyte/macrophages infiltrating into the URT around day 7 was crucial for clearance of pneumococci from the airways (16). In concordance with those results, we observed in young-adult mice a significant monocyte/macrophage influx around day 7 (Figure 2A) in two independent experiments (ANOVA with Bonferroni post-test p<0.05). However in elderly mice the majority of cells infiltrating into the NALT were neutrophils until day 28, when eventually a substantial increase in presence of monocyte/macrophages was detected, resulting in a significant difference in monocyte/macrophage ratio between young-adult and elderly mice at day 7 (ANOVA with Bonferroni post-test p<0.05). Similar results were obtained

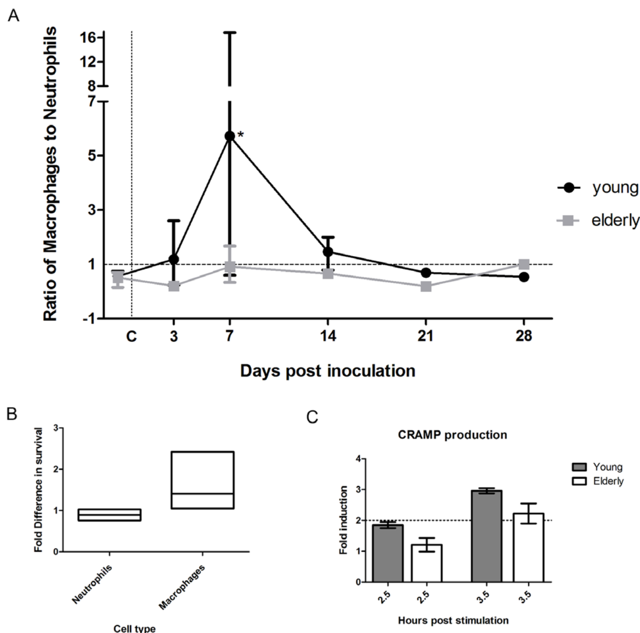

***Figure 2:* Age related differences in phagocyte infiltration and function.**
A. Elderly and young-adult mice were colonized with serotype 6B and sampled post-mortem. Depicted is the ratio of infiltrating macrophages to neutrophils quantified by flow cytometry (mean and range). For days 0, 3, 7 and 14 the figure is a compilation of results from two independent experiments (10 animals per time-point in total) and for days 21 and 28 the figure shows result of a single experiment (5 animals per time-point). (* ANOVA with Bonferroni's post test p<0.05). B. Pneumococcal killing by phagocytes. Phagocytes were challenged with live, un-encapsulated pneumococci. Shown is the fold difference in pneumococci surviving between young-adult and elderly mice for each assay, i.e. the percentage bacteria surviving killing by elderly phagocytes divided by the percentage surviving young phagocytes. No defect in killing is found in elderly neutrophils. BMDM of elderly, however, show on average 1.5 fold more pneumococci surviving than young (paired t-test, p=0.051). C. Fold-induction of CRAMP production by BMDM from young-adult and elderly mice after stimulation with pneumococcal WCA for either 2.5 or 3.5 hours. Expression in macrophages was measured with qRT-PCR. Macrophages from young-adult mice showed an increase in CRAMP production after 2.5 and 3.5 hours of stimulation, which was less apparent in macrophages from elderly (Student's t-test of 2.5 hours expression, p=0.059).

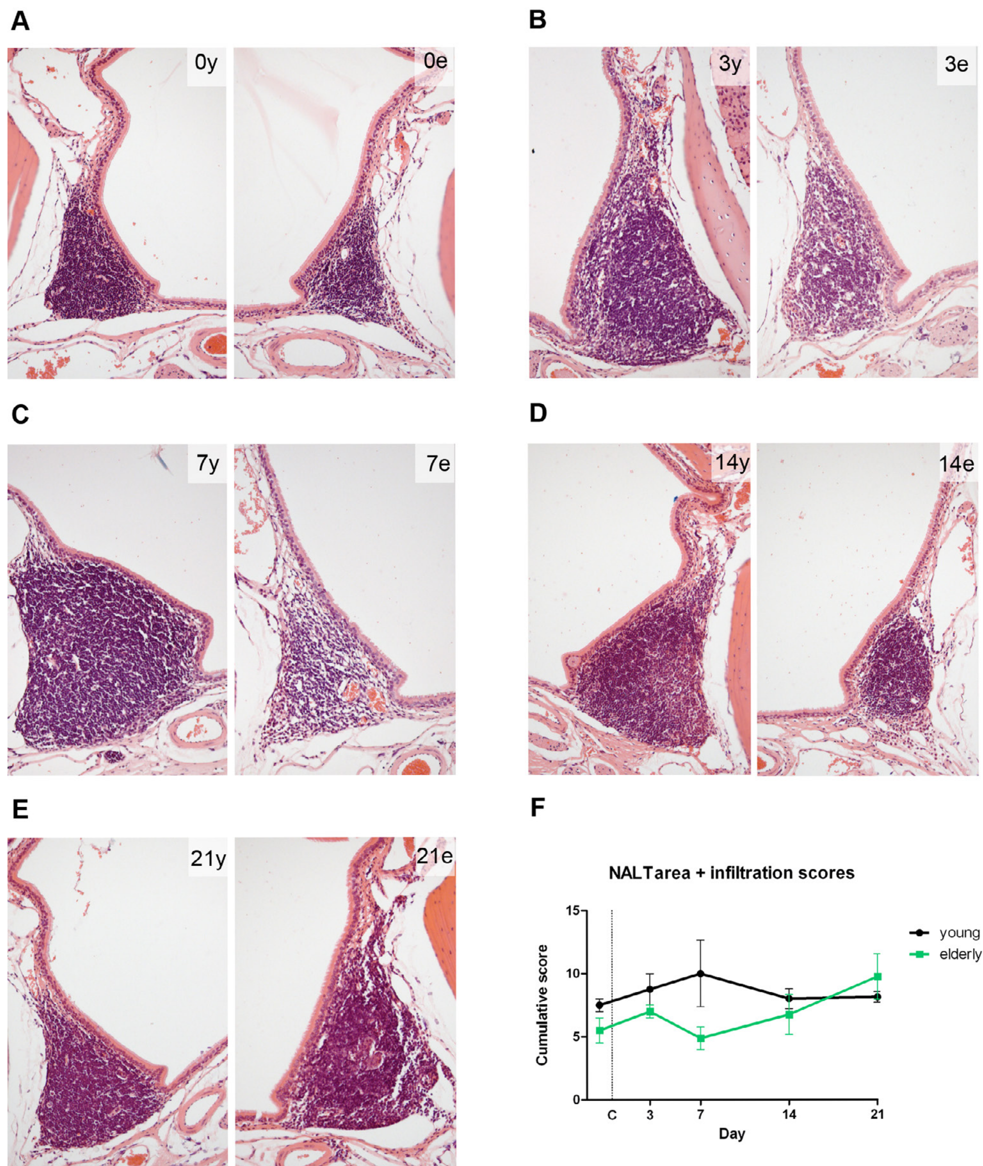

Figure 3: **Response of the nasal associated lymphoid tissue (NALT) of young-adult and elderly mice to pneumococcal colonization**

Coronal sections were taken of paraffin embedded young-adult (y) and elderly (e) mouse craniums and stained with H&E before (A), and 3, 7, 14, and 21 days post-inoculation (B-E) with pneumococcal serotype 6B. Pictures are representative of 1-2 mice per group per time (2-4 NALTs). A clear response to challenge was observed in all young-adult mice, whereas more heterogeneity was observed in elderly mice showing both responders and non-responders. F. NALT responses were quantified using a combined score for NALT size (area) and infiltration.

by studying the morphology of cells obtained in pooled nasal lavages from 5 mice each that were stained with Diff-Quik after Cytospin preparation (data not shown).

NALT dynamics in response to colonization

Due to the apparent different dynamics of cell-infiltration in the nasopharynx between young-adult and elderly mice upon pneumococcal colonization, we evaluated NALT tissue morphology in naïve and colonized animals. As expected, histological examination of NALT tissue showed predominantly lymphocytes with some visible macrophage/ dendritic cells. There was a trend towards reduced NALT sizes and cellular density in naïve elderly compared to naïve young-adult mice. After colonization, NALT size (area) and cell density reached its peak at day 7 post-challenge in young-adult mice (Figure 3A-E) whereas elderly mice responded to pneumococcal colonization with little to no increase in NALT size or cell density until days 14 to 21 (Figure 3A-E). NALT area and cellular infiltration were quantified by a 1-10 scoring method, and the combined scores are presented in Figure 3F, clearly showing age associated differences, although no significance was reached. In both groups, increases in area and infiltration correlated with pneumococcal colonization clearance.

Gene expression in the NALT in response to colonization

Having shown that in elderly mice duration of colonization was prolonged, and correlated with diminished macrophage influx and delayed NALT infiltration responses, we hypothesized that innate inflammatory mediators, including pattern recognition receptor (PRR) expression, would be aberrant already in naïve elderly mice or that upregulation might be affected. We therefore studied mRNA expression in the NALT of naïve and colonized mice and focused on immune pathways known to be involved in both clearance of pneumococci or other Gram-positive bacteria from the respiratory tract and potentially involved in immunosenescence. TLR1 (31), TLR2 (8, 18, 32), NOD2 (22), NLRp3 (19), IL-1ß (33), CCL2 (16) have all been demonstrated as crucial in the innate response to pneumococci. Furthermore, TLR1 (7), TLR2 (7, 34), IL-1ß (34), M-CSF (35) have been reported to be altered in elderly mouse models of candidiasis and streptococcal infections. At baseline we observed significantly higher expression levels for TLR1, NLRp3, IL-1ß, in naïve elderly mice when compared to naïve young-adult mice, but similar expression levels of TLR2, and NOD2 (Figure 4). In young-adult animals, we observed significnt upregulation of TLR1, NLRp3, IL-1ß at day 3 and of TLR2 and NOD2 at day 7, plus a strong trend in increased CCL2 expression following colonization (Figure 4). However, in elderly mice no significant upregulation of expression in response to colonization with *S. pneumoniae* was found; although, there was a trend towards a delayed upregulation of NOD2 by day 14 (Figure 4). In addition, we observed a negative correlation between IL-1ß induction (measured by fold change over baseline) and pneumococcal density 3 days post colonization, and between CCL2 induction and pneumococcal colonization density at day 7 post-inoculation (Figure

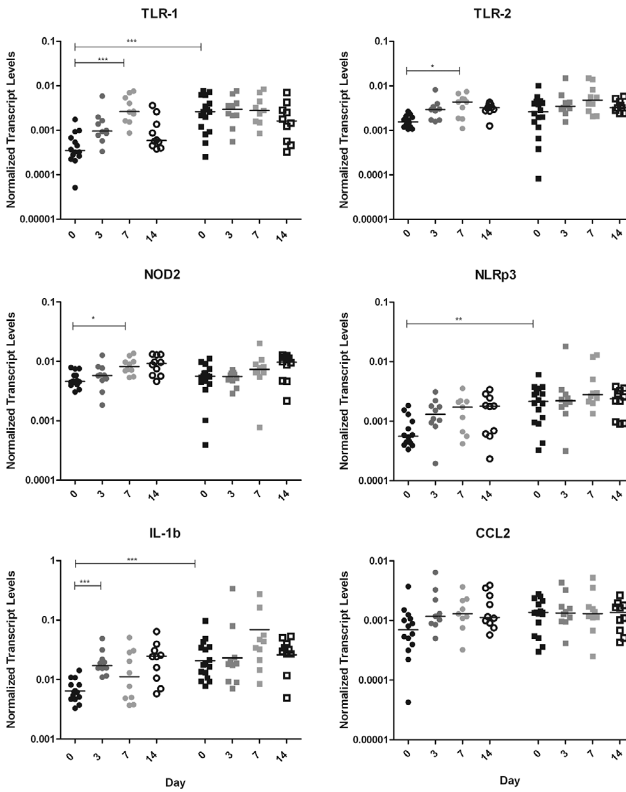

Figure 4: **Gene expression in the NALT in response to colonization with pneumococcal serotype 6B.**
Circles represent individual young-adult mice, squares individual elderly mice; lines are at median. The dynamics of expression are significantly different for TLR1 ($p<0.0001$), TLR2 ($p=0.004$), NOD2 ($p=0.002$), NLRp3 ($p=0.0002$), and IL-1ß ($p=0.0001$) by Kruskall-Wallace test. Individual planned comparisons were tested by Mann-Whitney with a significant p value of <0.0036 as per Bonferroni's correction. Baseline expression levels of TLR1, NLRp3, and IL-1ß are significantly higher in elderly naïve compared to young-adult naïve mice ($p=0.0002$, 0.002, and <0.0001). After pneumococcal inoculation, young-adult mice significantly upregulate 5 genes (trend for CCL2 at day 3 $p=0.054$), but elderly mice do not (trend for NOD2 at day 14 $p=0.0425$). * $P \leq 0.0036$, ** $P \leq 0.002$, *** $P \leq 0.0002$. Raw expression data per mouse was normalized to two housekeeping genes, Ywhaz and GAPDH**.**

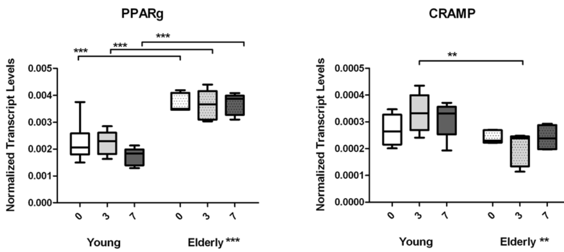

Figure 5: **Transcriptional regulation in the URT epithelium**
There is a significant effect of age on transcription of the NF-κB inhibitor PPARγ (two-way ANOVA; $p<0.0001$) and the murine antimicrobial peptide CRAMP (two-way ANOVA; $p=0.0013$) at the URT epithelium. At all times tested, elderly had significantly higher levels of PPARγ (Bonferroni post-test; $p<0.001$) and at day 3 post inoculation young-adult mice expressed significantly more CRAMP (Bonferroni post-test; $p<0.01$). There is also a trend of decreased expression of PPARγ and increased expression of CRAMP following pneumococcal colonization in young-adult mice, which are absent in elderly mice. Raw expression data per mouse was normalized to two housekeeping genes, Ywhaz and GAPDH.

S2). There was no difference in expression of GM-CSF between naïve and colonized young-adult and elderly mice (data not shown).

Gene expression in URT epithelium in response to colonization

Mucosal surfaces are continually colonized by a wide variety of bacteria and homeostasis in these areas is maintained via pathways unique to the epithelial surface (36, 37). Therefore, we additionally investigated possible age associated differences in response to URT pneumococcal colonization by studying expression of signaling and effector molecules at the local mucosa. First we assessed expression of the regulatory genes IRAK3, PPARγ, and SIGIRR. Expression of IRAK3 and SIGIRR did not differ between age groups at baseline, or following colonization, (Figure S3). For PPARγ, however, we observed significantly higher expression at baseline and after colonization in elderly compared to young-adult mice ($p<0.0001$ by ANOVA) (Figure 5). Moreover, we observed a trend towards down-regulation of PPARγ over the first 7 days post colonization in young-adult but not elderly mice. In epithelial cells, important downstream products inhibited by PPARγ include anti-microbial peptides such as cathelicidin; higher levels of PPARγ should result in lower levels of transcription of cathelicidin (21, 37). Indeed, we observed decreased expression of mouse cathelicidin CRAMP in the epithelium of elderly mice compared to young-adult mice at all time-points ($p=0.001$ ANOVA). Upon pneumococcal colonization, a strong trend towards CRAMP upregulation was observed in young-adult but not elderly mice (Figure 5). There were no statistical differences seen in gene expression of PRRs in URT epithelium between young-adult and elderly mice, however, a trend was observed for young-adult mice expressing more NOD2, TLR1, and TLR2 at baseline and more NOD2 after pneumococcal inoculation (Figure S3).

Phagocyte function

Neutrophils and monocytes present in the URT are critical effector cells for pneumococcal colonization clearance in mice (16). We therefore studied age-related differences in their function. Neutrophils isolated from bone marrow of elderly mice responded at an equal speed and with an equal amount of ROS as neutrophils isolated from young-adult mice to pneumococcal stimulation (Figure S4). Moreover, antibody-independent pneumococcal killing demonstrated by surface phagocytosis (26) did not differ between neutrophils from young-adult and elderly mice (Figure 2B). We also tested bacterial killing by bone-marrow derived macrophages (BMDM), and observed no differences in phagocytosis capacity (data not shown) though approx. 20% lower capacity of elderly BMDM to kill pneumococci compared to young-adult macrophages which equaled a 1.5-fold greater amount of bacteria surviving killing by elderly BMDM than young-adult BMDM ($p=0.051$) (Figure 2B). Finally, we measured expression of CRAMP in BMDM stimulated with whole cell antigen (WCA). BMDM from young-adult mice clearly upregulated expression of CRAMP in response to pneumococcal stimulation at either time point whereas there was a delay in response of BMDM from elderly mice

(Figure 2C). Induction of CRAMP expression was lower in elderly compared to young-adult macrophages (p=0.059).

Discussion

In this study we show an impaired innate mucosal immune response to *Streptococcus pneumoniae* colonization in the upper respiratory tract of elderly mice, which was associated with prolonged duration of carriage. Our data suggests that monocyte/macrophage influx is delayed in elderly mice. Moreover, macrophages from elderly mice were less bactericidal and produced less CRAMP than young-adult mice both at baseline and in response to pneumococcal whole-cell antigen. Furthermore, CRAMP expression was significantly lower in the URT of aged mice, which was in line with our in vitro data in BMDM. The dynamics of neutrophil influx and neutrophil function were similar between age groups. The local lymphoid tissue responses were aberrant in aged mice with inflammation in the URT already present in naïve elderly mice, specifically high expression of TLR1, NLRp3, IL-1β, and CCL2. In addition, elderly mice failed to upregulate TLR1, TLR2, NOD2, NLRp3, and IL-1β in response to colonization. Baseline inflammation in aged mice and failure to upregulate innate response genes could impede signaling for monocyte/macrophage influx and thus explain delayed clearance. Lastly, we found evidence that mediators of tolerance to commensals in the URT were aberrantly expressed in elderly mice with significant over-expression of PPARγ that persisted throughout pneumococcal colonization.

There is growing evidence that infiltration of monocyte/macrophages into the URT is the key to innate pneumococcal clearance in mice (16) with neutrophils playing a secondary role (17, 18). Our observation of delayed monocyte/macrophage influx correlating with delayed clearance in elderly mice is in agreement with those earlier studies. Because the products of activated neutrophils can be chemotactic for monocyte/macrophages we also tested the function of the effector cells, after confirming that neutrophil influx by itself seemed adequate. (38). We found no functional defects in neutrophils of elderly mice, but BMDM of elderly mice showed lower killing activity against *S. pneumoniae* and macrophages produced less transcripts of the mouse cathelicidin CRAMP, both at baseline and in response to pneumococcal antigen. CRAMP, a mouse analog of human cathelicidin, plays a significant role in mucosal clearance of bacteria, stimulation of the immune system (21), and was recently shown to be relevant for natural clearance of pneumococci (39). However, it has been shown that macrophages can change phenotype and function when cultured in vitro (13) and *ex viv*o adaptations can occur when monocytes from elderly mice are given sufficient quantities of cytokines for macrophage differentiation in vitro (40). Therefore, we continued our studies on innate immune function using the NALT and URT mucosal epithelium of mice directly.

The first step of innate immune responses is recognition of pathogens. TLR1/2 recognize

cell wall associated polysaccharide of pneumococcus (lipoteichoic acid) (31, 32) and NOD2 recognizes peptidoglycan (22); both types of recognition upregulate inflammatory components leading to macrophage influx and pathogen clearance. Furthermore, it has been reported in a mouse model of invasive pneumonia that protein levels of TLR1 and -2 are significantly decreased in aged mice compared to young-adult following infection (7). We investigated the expression of these PRRs in the URT of aged mice before and during colonization and found no statistical differences between age groups both before and during colonization in the epithelium. However, we did observe differences in expression in the NALT. In contrast to studies using splenic (9) or peritoneal (12) macrophages we observed significantly higher expression of TLR1, and a trend in TLR2 in elderly NALT compared to young. However, the failure of elderly mice to upregulate these genes in response to colonization is in line with previous studies showing aberrant responses of TLR's to infection (7). Also delayed upregulation of NOD2 could contribute to the delayed clearance of pneumococci in elderly mice, a finding unique to this study. Since factors other than recognition of danger signals are involved in monocyte/macrophage influx, we investigated expression of other genes downstream of PRRs involved in clearance of pneumococci (16, 19, 33). Again, increased baseline expression of inflammatory components such as inflammasome NLRp3, IL-1β, and CCL2 were observed in elderly mice, with delayed or no upregulation of those factors upon colonization compared to a marked response in young-adult mice. This supports our general observation of (relative) unresponsiveness of elderly mice to pneumococcal challenge. The question remains as to how the increased pro-inflammatory state at baseline is connected to the absence or delay in response to pneumococcal challenge.

At mucosal sites the local lymph nodes are not the only source of immune signaling; the epithelium is also involved in recognition of microbes, signaling, and some responses such as production of anti-microbial peptides (36, 37). Mucosal surfaces of the upper airways are colonized by a variety of bacteria, forming the normal flora of these sites; therefore the epithelial layer must discriminate between symbiotic colonizers and true pathogens and maintain homeostasis (36). SIGIRR and IRAK3 which are negative regulators of mucosal homeostasis (36) are reported to be increased in elderly mouse models after Gram-negative stimulus (10, 12); whereas PPARγ is reported to be similarly expressed in elderly as in young macrophages (12). In contrast to the studies performed in vitro with other stimuli, we found no differences in expression of SIGIRR or IRAK3 in vivo following pneumococcal colonization. Furthermore, we observed significantly higher expression of PPARγ in elderly mice. On one hand evidence exists that URT commensals specifically use PPARγ to promote tolerance and homeostasis (41, 42), but on the other hand there is also evidence that PPARγ is controlled by genetic programming to promote longevity in mice (43) and genetics has also been cited as a possible cause for the observed phenomena of background inflammation in elderly, called 'inflamm-aging' (44). So, it is difficult to ascertain if this increased expression of PPARγ is the result of lifetime host-pathogen interactions resulting in increased

tolerance at the mucosal surface, or if this might be the result of counterbalancing the chronic inflammatory mucosal status, or even the result of specific genetic programming. However overexpression of PPARγ might, at least partially, explain the lack of signaling and upregulation of pro-inflammatory responses after pneumococcal danger signals are present, as we observed in the NALT.

We also tested a key downstream product of PPARγ regulation: CRAMP (45). CRAMP expression was indeed significantly lower in aged mice, which was in line with our in vitro data in BMDM. Since the function of antimicrobial peptides such as CRAMP is not only to mediate killing of bacteria but also to act as direct chemoattractants to monocyte/macrophages (46) it supports our cytological findings of diminished monocyte/macrophage influx in the upper respiratory tract of elderly mice following pneumococcal challenge.

Since high colonization density is linked with pneumonia in humans, we investigated differences in density of colonization between young-adult and aged mice (47). Recently Shivshankar *et al* suggested that increased pneumococcal ligand expression in lung tissue plays a role in severity of pneumococcal pneumonia in the elderly (14). However, if this mechanism were involved in pneumococcal colonization, it would affect colonization density in our model, but we did not observe any differences in density of pneumococcal colonization following intranasal challenge between age groups. In humans, duration of pneumococcal carriage is also associated with incidence of pneumococcal disease (48, 49). Accelerating pneumococcal clearance is therefore also a goal of new vaccines (50), however success of such prevention strategies in the elderly will require intact innate signaling including epithelial responses, lymphoid tissue function, and phagocyte function. So far, there is no clear evidence of longer duration of human carriage in elderly compared to young-adults, although this might be a consequence of scarce longitudinal colonization data in adults and elderly or relatively low sensitivity of conventional detection methods (51-53).

Our data indicate that two major processes seem to be driving immunosenescence in aged mice. First, generalized inflammation that is reported by many to be present systemically in both elderly humans and mice, called 'inflamm-aging'(40, 44, 54) is present in the URT as well. Our data supports the hypothesis that this background inflammation might, at least partially, be responsible for the lack in recognition of danger signals and therefore lack in up-regulation of many important mediators necessary for pneumococcal colonization clearance (54). Increased duration of colonization is the same phenotype seen in studies where monocyte/macrophage populations are depleted (16), matching the absent/delayed influx of monocyte/macrophages in our elderly mouse model as well. Second, tolerance to commensal bacteria is an important part of human survival. It appears that mediators of tolerance at the URT mucosa are indeed increased in aged mice, specifically PPARγ, which will also contribute to absent

or delayed upregulation of pro-inflammatory processes upon pneumococcal challenge. The impairments we observe in the aged mice are relatively mild, but consistent within the required network of immune signaling, and therefore together they might add up to the eventual clear phenomenon observed of prolonged pneumococcal colonization in elderly mice. Despite being generally asymptomatic, lack of clearance of potential pathogens from the URT may lead to increased susceptibility to disease in elderly individuals. Although the data we present here is correlative in nature, this is the first in vivo study to investigate age-related defects in the URT related to clearance of a potential pathogen. We focused on the innate responses to colonization for this first study, however, it seems highly relevant for future studies to also investigate adaptive immunity, especially the Th17 pathway in the URT as activation of this pathway seems especially critical for pneumococcal colonization clearance. Further studies on colonization duration in relation to immunosenescence and susceptibility to respiratory disease in elderly are warranted and necessary for deliberate preventive strategies.

Acknowledgments

The authors would like to thank Cindy Fevre for advice on flow cytometry analysis, Marc Jansen for advice on qPCR expression analysis, Kok van Kessel and Andras Spaan for assistance with neutrophil isolation and functional assays, Natalie ter Hoeve for assistance with histopathology, Belinda van 't Land for advice on cytokine responses and Richard Malley for sharing with us the pneumolysin-negative variant of *S. pneumoniae* strain Rx1.

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Supplementary Material

Methods

Live sampling

Repeated live sampling was performed twice a week for four weeks following inoculation. Briefly, mice were restrained in a 50 ml conical tube with the end cut off, allowing access to the nose, and held inverted over a sterile Petri dish. Ten microliter volumes of ice-cold saline were applied to each nostril and droplets sneezed out by the animal onto the dish were recovered by washing the plate with 500 μl saline. Samples were stored on ice and cultured on SB7-Gent plates to detect the presence of pneumococci.

NALT quantification

Before sections were taken for quantification experiments, we took multiple series of coronal sections from 2 mice to determine the general shape of the NALT and the optimal distance from the eyes to take sections. Once this was determined, all sections were taken at the same distance from the eyes and several sections were taken to ensure reproducibility. To quantify morphological changes within the NALT in response to pneumococcal colonization, pictures of individual NALTs were taken under identical magnification and microscope settings. 1-2 mice per time-point were used, each with a left and a right NALT that were treated as individual NALTs. Pictures were reviewed with FIJI open source software (http://fiji.sc/Fiji). The area of the NALT was measured with FIJI and converted to a 1-10 score. FIJI area measurements ranged from 32,000 μm2 –120,000 μm2; surface areas were coded by assigning 0.5 points to each 5,000 μm2 above 30,000 μm2 with a maximum of 9 points (115,000-120,000 μm2). To measure NALT cellular infiltration, pictures of individual NALTs were randomized and scored in a blinded manner by two independent observers using a 1-10 score scale. Naïve young-adult mice NALTs, being the control, were set at a score of 5 points for infiltration. Remaining pictures of NALTs were blinded and put in groups of visual comparable infiltration. These groups were ordered from least to most infiltrated and

given a score from 1-10 using only whole numbers. Both area and infiltration scores were added per NALT and the sums compared with scores observed in naïve mice of corresponding age.

Flow cytometry

Cells from the spleen of 1 mouse were used for single-stained and un-stained controls. Anti-mouse CD16/CD32 (Mouse Fc Block, BD Pharmingen) was diluted 1/100 and all wells were blocked with 50 µl for 20 minutes at 4°C. A 50 µl cocktail of fluorophore-conjugated antibodies recognizing different cell surface proteins was added, incubated in the dark for 30 minutes at 4°C. Typical final dilutions of the antibodies included 1:200 for F4/80-APC or FITC antibodies, and 1:400 for GR-1-APC or PE, CD11b-PerCP-Cy5.5, MHC-II-FITC, CD45-Pacific Blue antibodies, and 1:800 for MHC-II-APC antibody. 200ul of PBS+BSA was added to terminate the reaction. Pellets were collected and washed again with 200 µl buffer. Pellets were resuspended in buffer + 0.5% formalin and subjected in full volume to flow cytometry analysis on a BD FACSCantoII flow cytometer (BD Biosciences).

Quantitative real time RT-PCR

Mouse universal total RNA (Qiagen) was used as internal control of PCR. For NALT and epithelial cells 1µg of total RNA was reverse transcribed, for macrophage stimulation assays 500ng of total RNA was reverse transcribed using GeneAmp RNA PCR cDNA synthesis kit (Applied Biosystems) applying random hexamers. Absence of genomic DNA in the samples was determined by omitting reverse transcriptase enzyme in the cDNA reaction and including these samples in the RT-PCR. cDNA was diluted 25x for NALT samples and 5x for epithelial samples and 10.5 µl was used as template in 25 µl mixture with SYBR Green PCR master mix (Applied Biosystems) and 0.5 µl primers at a concentration of 250 nM. Amplification was performed on a StepOnePlus (Applied Biosystems) machine under the following conditions: 95 °C/10min; 95 °C/15 seconds; 60 °C/1min; return to step 2 for 44 more times. Primers used in this study are listed in Table S1.

Specificity of the PCR products was assessed by melt curves analysis and running reactions without templates. Amplification efficiency was not equal for all primers, so efficiency per amplicon group was used in determining the amount of transcripts using LinRegPCR software (www.hartfaalcentrum.nl) (1, 2). Transcript amount determined by LinRegPCR for 2 housekeeping genes for each sample was used for normalizing the data. The geometric mean of Ywhaz (3) and GAPDH (4) was used for housekeeping normalization.

Cell preparation

Bone marrow cell isolation was performed as described in (5). In short, the femur

and tibia were prepared post mortem and flushed with a 25g needle and MACS buffer through a 70-µm cell strainer (BD Biosciences). Marrow plugs were dispersed on the strainer and cells were collected via centrifugation. Bone-marrow neutrophils were obtained by discontinuous Percoll® (Amersham Biosciences) gradient centrifugation, modified from Boxio 2004. Briefly, bone marrow cells were resuspended in 2 ml PBS+BSA. This suspension was layered over 72%, 64%, and 52% Percoll solution in PBS+BSA, in a 10 ml polystyrene tube and centrifuged without brake at 1500 RPM (~480 G) for 30 minutes at room temperature. The band between 64% and 72% layers was collected; cells were washed and quantified by morphology using Cytospin (details below).

Macrophages were derived from the total bone marrow cell population according to previously described procedures (6) with minor modifications. In short, bone marrow cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 with GlutaMAX (Gibco), 10% fetal bovine serum (FBS), and 10-20% L-cell-conditioned medium (LCM) for 7 days of differentiation. Plastic adherent cells were collected on day 7 with 5% EDTA trypsin (Life Technologies) and cells were 98% F4/80+ and 97% CD11b+ by flow cytometry. Macrophages were frozen in liquid nitrogen in aliquots of $3x10^6$ cells in FBS with 10% DMSO.

Differential cell quantification

Murine cells recovered from the nasal lavages as described above were applied to a glass slide in the Shandon Cytospin 3 cytocentrifuge run at 500 RPM for 5 minutes. The samples were air dried and subjected to 3-step differential staining using Diff-Quik (Dade Behring) staining kit. Quantification of differential cell types was based on standard morphologic criteria at a magnification of 40x. Absolute numbers were used for data analysis.

Phagocyte functional tests

Reactive oxygen species (ROS) production by neutrophils was measured via luminol based chemiluminescence assays. Mouse bone marrow neutrophils were resuspended at a concentration of $2.5x10^6$ cells/ml in HBSS+0.1%FCS. Luminol balanced salt solution (LBSS) was made with HBSS (Lonza) + 0.33µM luminol (Sigma) + 0.05% human serum albumin (Sanquin) and was pre-warmed to 37°C. 100 µl of warm LBSS was added to each well of a white 96-well microplate (Thermo Scientific). 50 µl of stimulents were added to respective wells, then 50 µl of cells. Chemiluminosity was measured immediately and every 10 seconds for 3000 seconds at 37oC in a Berthold Technologies Centro LB960 luminometer. Stimulants used: pneumococcal WCA final concentration 1/100 or PMA 12.5 ng/ml for positive control.

Table S1: Sequences and sources of the qPCR primers used in this study.

Name		Sequence	Source
GAPDH	Forward	TGT GTC CGT CGT GGA TCT GA	(4)
	Reverse	CCT GCT TCA CCA CCT TCT TGA	
Ywhaz	Forward	CAC AGC CTC CCC TCA TCC T	(3)
	Reverse	GGG AGA CGG TGA CAG ACC AT	
Krt18	Forward	ATT GCC AGC TCT GGA TTG AC	(7)
	Reverse	GTC TCA GCG TCC CTG ATT TC	
CCL2	Forward	AGC TCT CTC TTC CTC CAC CAC	(8)
	Reverse	AGC TCT CTC TTC CTC CAC CAC	
CRAMP	Forward	CTT CAA CCA GCA GTC CCT AGA CA	(9)
	Reverse	TCC AGG TCC AGG AGA CGG TA	
IL-1β	Forward	GAA GAA GAG CCC ATC CTC TG	(10)
	Reverse	TCA TCT CGG AGC CTG TAG TG	
IRAK3	Forward	TGA GCA ACG GGA CGC TTT	(11)
	Reverse	GAT TCG AAC GTG CCA GGA A	
NOD2	Forward	CCG CTT TCT ACT TGG CTG TC	This study
	Reverse	AGC CCT GGA TAC ACA GGT TG	
NLRp3	Forward	CTC CCG CAT CTC CAT TTG T	(12)
	Reverse	GCG TGT AGC GAC TGT TGA	
PPARγ	Forward	GCC TGC GGA AGC CCT TTG GT	This study
	Reverse	AAG CCT GGG CGG TCT CCA CT	
SIGIRR	Forward	AGT GTC GGC TGA ACA TGC TG	This study
	Reverse	GAC ACG TAG GCA TCG TAT AAC TTC	
TLR1	Forward	GGA CCT ACC CTT GCA AAC AA	(13)
	Reverse	TAT CAG GAC CCT CAG CTT GG	This study
TLR2	Forward	AAG AGG AAG CCC AAG AAA GC	(13)
	Reverse	CGA TGG AAT CGA TGA TGT TG	

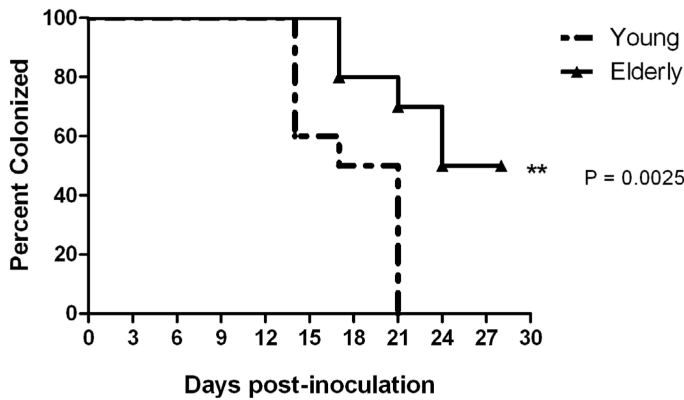

Figure S1: Effect of age on duration of pneumococcal colonization with serotype 6B in mice.
Elderly and young-adult mice (n=10 each) were colonized with pneumococcal strain 603 and live sampled twice a week for 4 weeks. Mice were scored based on presence or absence of pneumococcus in the sample. The median time to clearance for the young-adult mice was 19 days compared to 26 days for the elderly mice (Log-rank test: p = 0.0025)). Also, 50% of the elderly mice remained colonized at 28 days.

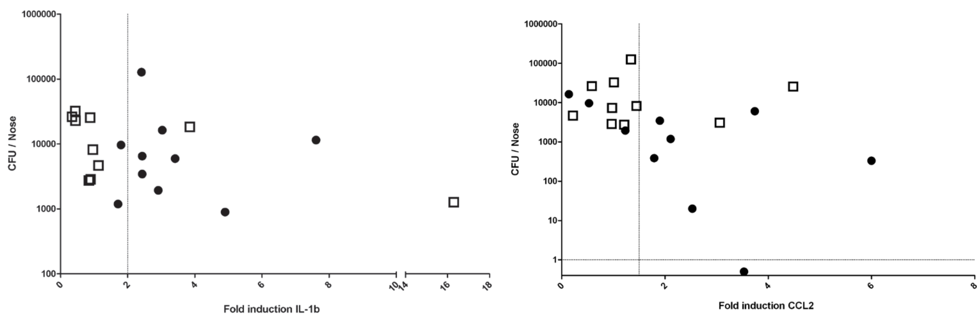

Figure S2: Correlation between expression of innate mediators and clearance of pneumococci.
Circles represent individual young-adult mice, squares individual elderly mice. Fold changes in expression of IL-1ß 3 days post-inoculation and CCL2 7 days post-inoculation (when compared to baseline) are inversely correlated with colonization density. (IL-1ß induction; Spearman r =-0.3889 and P=0.0902. CCL2 induction; Spearman r =-0.466 and P=0.0484). Raw expression data per mouse was normalized to two housekeeping genes, Ywhaz and GAPDH.

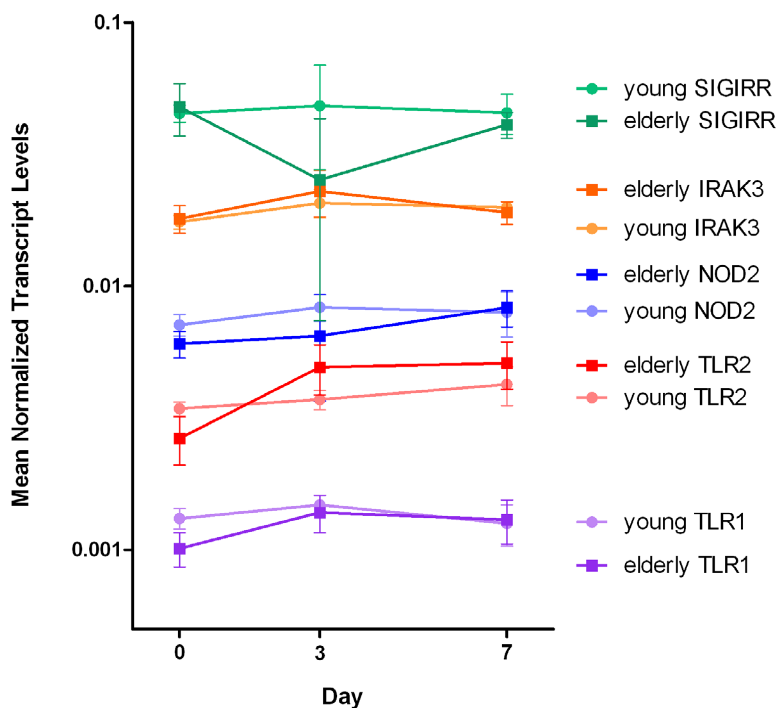

Figure S3: Gene expression in URT epithelial cells.
Expression of transcriptional regulators and PRR tested in URT epithelial cells before and after pneumococcal inoculation. No significant differences between young and elderly were detected (two-way ANOVA age factor: SIGIRR p=0.079; IRAK3 p=0.759; NOD2 p=0.481; TLR2 p=0.471; TLR1 p=0.445). Raw expression data per mouse was normalized to two housekeeping genes, Ywhaz and GAPDH.

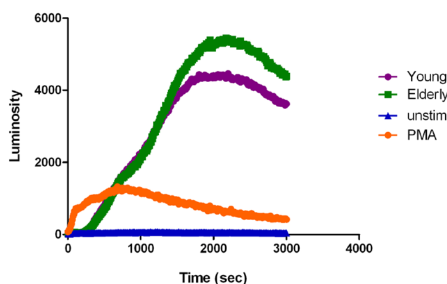

Figure S4: In vitro neurtophil function.
Bone marrow neutrophils from young-adult and elderly mice were incubated with pneumococcal WCA and luminol; chemiluminescence created by formation of ROS was measured by a Berthold Centro LB960 luminometer. Pooled cells of young-adult and elderly were used for negative (HBSS) and positive (PMA) stimulation controls. Figure is a mean of 6 wells each condition; similar results were obtained in 2 additional independent experiments, total 3 mice each age tested.

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4

Chapter Four

Respiratory microbiota dynamics following *Streptococcus pneumoniae* acquisition in young and elderly mice

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Abstract

The upper respiratory tract (URT) is a distinct microbial niche of low-density bacterial communities and also a portal of entry for many potential pathogens, including *S. pneumoniae*. Thus far, animal models have been used to study dynamics of and interactions between limited numbers of different species in the URT. Here we applied a deep sequencing approach to explore, for the first time, the impact of *S. pneumoniae* acquisition on the URT microbiota in a mouse model and potential age-dependent effects. Young-adult and elderly mice were inoculated intranasally with *S. pneumoniae*, and nasal lavages were collected for up to 28 days post-colonization. Bacterial DNA extracted from lavages was subjected to barcoded pyrosequencing of the V5-V7 hypervariable region of the small-subunit ribosomal RNA gene. We observed highly diverse microbial profiles with the overall presence of 15 phyla and approximately 645 OTUs. We noted differences in microbiota composition between young and elderly mice, with significantly higher abundance of Bacteroidetes in the young mice. The introduction of *S. pneumoniae* into the URT led to a temporary dominance of pneumococci in the microbiota of all mice, accompanied by a significant decrease in microbial diversity. As mice gradually cleared colonization, diversity returned to baseline levels. Diversification was accompanied by an early expansion of Bacteroidetes, *Staphylococcus spp.* and Lachnospiraceae. Moreover, Bacteroidetes expansion was significantly higher in young-adult compared to elderly mice. In conclusion, we observed differences in URT microbiota composition between naïve young-adult and elderly mice, which were associated with differences in pneumococcal clearance in time.

Introduction

The bacteria indigenous to the mammalian body are a diverse and important component of a healthy existence, even outnumbering the cells of the host. New techniques in sequencing have allowed a greater understanding of the breadth and diversity of these microbial populations while experimental animal models have elucidated mechanisms of commensal-host-pathogen interactions including beneficial immune stimulation and species specific pathogen protection (1, 2). The upper respiratory tract (URT) is a distinct microbial niche of low-density bacterial communities (3) and also a portal of entry for many pathogens, including *S. pneumoniae*, which is a frequent cause of pneumonia worldwide and a major cause of death in particular in the elderly population (4). Furthermore, pneumococcal colonization of the URT is a prerequisite state before disease develops (5, 6). Due to the key role that colonization plays in both the survival of the bacteria in the upper respiratory tract of the human host and the development of disease, studies on the relation between the bacterial community composition of the URT and pneumococcal acquisition and clearance are of great importance (3).

Despite being a frequent, asymptomatic component of the URT microbiota, pneumococcus can cause serious diseases such as pneumonia, septicemia, and meningitis (5). Young children and the elderly are especially at risk for pneumococcal disease and while immature immunity causes the increased risk in children, age-related defects in the immune system, termed immunosenescence, are thought to contribute to the increased disease risk in the elderly (7). In a previous study, we determined that baseline inflammation and delayed cellular responses were correlated with delayed pneumococcal colonization clearance in elderly mice compared to young-adult mice (8). We observed that a higher pro-inflammatory status in the nasal-associated lymphoid tissue (NALT) of elderly mice occurred simultaneously with the increased expression of tolerance pathways in the nasal epithelium, suggesting that homeostasis in the URT of elderly mice is altered. We hypothesized that URT microbiota of elderly mice might be different from younger mice and might be contributing to this phenomenon.

Thus far, animal models have been used to study interactions in the URT for a limited number of species at a time. Here we applied a deep sequencing approach to explore, for the first time, the correlation between URT microbiota and dynamics of *S. pneumoniae* acquisition in a mouse model of pneumococcal colonization. We used the model to investigate age-dependent differences in microbiota responses to pneumococcal carriage in the context of immunosenescence.

Materials and Methods

Ethics statement / Animals

Animal experiments were performed in accordance with the Dutch Animal Experimentation Act and EU directives 86/609/CEE and 2010/63/EU related to the protection of vertebrate animals used for experimental or other scientific purposes. The experimental protocols were approved by the Committee on Animal Experiments of the University of Utrecht (DEC # 2009.II.10.107; 2010.II.07.125; 2011.II.11.175). C57BL/6 female specific pathogen free mice were purchased from Harlan (Venray, Netherlands). For the purpose of this study 3-4 month-old mice were defined as young-adult and 18-23 month- old mice as elderly. Animals with noticeable tumors were excluded from experiments and animals with neoplasms detected in post mortem examinations were excluded from analysis. Mice were euthanized with 120mg/kg pentobarbital (Veterinary Department Pharmacy) administrated intra-peritoneally to induce a respiratory arrest.

Bacterial strains

Streptococcus pneumoniae serotype 6B strain 603 is a clinical isolate that has been previously used in murine models of pneumococcal colonization (9). This strain was mouse-passaged and grown to mid-log phase in brain-heart infusion broth (BHI), aliquoted, and stored frozen in 10% glycerol at -80°C. Prior to animal inoculation, bacterial cells were thawed, washed twice with saline, re-suspended to 5×10^{8} CFU/ml, and cell suspensions were tittered by plating tenfold dilutions of inoculum on blood agar plates supplemented with gentamicin (SB7-Gent, Oxoid).

Mouse colonization experiments

All samples analyzed here were randomly selected from samples collected in the study on effects of immunosenescence on colonization with *S. pneumoniae* in the mouse model of pneumococcal carriage (8). In short, mice were inoculated intranasally with 10 µl of bacterial suspension containing approximately 5×10^{6} CFU. At days -1, 3, 7, and 14 post-inoculation 5 mice of each age were sacrificed, and at day 28 post-inoculation 3 mice of each age were sacrificed. From each animal 500 µl of retro-tracheal nasal lavage were collected with calcium- and magnesium-free PBS supplemented with 1% BSA (PBS+BSA) followed by the nasal tissue harvest. Tissue samples were crushed in 1 ml of PBS, stored on ice and processed within 3 hours. Samples were vortexed vigorously and 60 µl of lavage fluid and all volume of tissue wash were plated in serial dilutions onto SB7-Gent medium in order to quantify *S. pneumoniae* presence in the URT. Per mouse, CFU counts from the lavage and nasal tissue were summed. The remaining volume of the lavage fluid was centrifuged at 1200 RPM (~300G) for 10 minutes at 4°C to remove mouse cells. Supernatant (bacterial fraction) was removed and frozen at -80°C until DNA extraction.

Bacterial DNA isolation

DNA was isolated from 100 ul of the URT lavage with phenol/bead-beating and magnetic bead separation using Agowa reagents as described by Biesbroek *et al* (10). Samples chosen to go through to 454-pyrosequencing were randomly selected and representative of the overall population of mice used for all experiments (8).

Deep sequencing analysis of URT samples

Microbiota composition was determined using 454 barcoded pyrosequencing of the 16Sr DNA gene as previously described (10). The total bacterial load was analyzed by quantitative real-time PCR (qPCR), using a universal primers-probe set targeting this gene (3). Amplicon library preparation and sequence processing was performed as described by Biesbroek *et al* (10). In short, we amplified the V5-V7 hypervariable region of the 16S rDNA gene. Aplicons were size checked, quantified, and equimolar pooled after which the library was unidirectional sequenced in the 454 GS-FLX-Titanium Sequencer (Life Sciences (Roche), Branford, CT). Sequences were processed using the modules implemented in Mothur v. 1.20.0 software platform (11); they were first de-noised and checked for quality and chimers (using Chimera Slayer) (12) resulting in 80,739 sequences available for downstream analysis. The remaining high quality aligned sequences were classified using the RDP-II naïve Bayesian Classifier and clustered into operational taxonomic units (OTUs, defined by 97% similarity). For all samples rarefaction curves were plotted and community diversity indices (Shannon diversity and Simpson's index) calculated. Sequence data were subjected to unweighted UniFrac analysis using the UniFrac module implemented in Mothur (13). The UniFrac algorithm calculates the distance between microbial communities based on the phylogenetic lineages in each sample.

Prior to analysis OTU tables were filtered: any OTU accounting for 3 or less sequences in the entire data set was removed. Relative abundance was calculated as the proportion of sequences assigned to a specific OTU divided by the overall number of sequences obtained per sample (3) and absolute abundance was calculated by multiplying relative abundance by the obtained bacterial load per sample measured by qPCR.

Data analysis

Culture data, qPCR, and diversity indices were graphed and analyzed using GraphPad Prism software. Kruskal-Wallis with Dunn's post-test was used for non-parametric data, and otherwise One-way ANOVA with Bonferroni post-tests were used to detect statistical differences. Relative abundance sequence data was graphed using Adobe Illustrator CS6. Significance analysis of microarrays (SAM) (14) on sequence data was performed using TIGR MeV software (http://www.tm4.org/mev/) (15) using Pearson correlation with complete linkage clustering and an FDR of 19% to study differences between groups on OTU and family level.

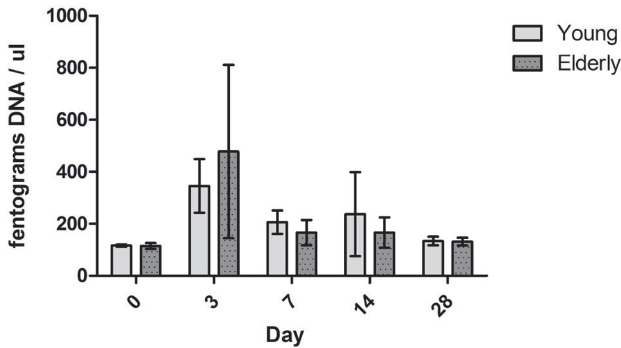

Figure 1: **Quantity of DNA in nasal lavages following pneumococcal challenge**
Quantity of DNA isolated from nasal washes from the mice (mean of 5 mice per group per time-point, except for day 28 (3 mice per group). No differences were found in the quantity of DNA isolated between young-adult and elderly mice. Introduction of *S. pneumoniae* into the niche increased on the quantity of DNA isolated at day 3 post-inoculation, p<0.001 (Kruskall-Wallis with Dunn's post-test). Shown are means and SD.

Results

Upper respiratory tract bacterial density is correlated with introduction of a pathogen

In our previous study, we showed that elderly mice have a prolonged duration of pneumococcal colonization compared with young-adult mice and that this was associated with aberrant innate immune. For the microbiota analyses, a subset of mice was selected randomly from the previous study and we confirmed that the pneumococcal density in this subset did not differ significantly from the mean density dynamics presented in the original study. We isolated total bacterial DNA from upper respiratory tract lavages of young-adult and elderly mice and quantified DNA by 16S qPCR. As seen in Figure 1, introduction of *S. pneumoniae* into the URT niche does have a significant effect on the total bacterial density at 3 days post-inoculation as suggested by a 3-fold increase in bacterial DNA recovered from young-adult and a 4 fold increase in bacterial DNA recovered from elderly mice (Kruskal-Wallis with Dunn's post-test p<0.001).

Sequence characteristics

A total of 80,739 sequences were obtained from all mice (n=46), on average this corresponded to 1793 sequences/sample, standard deviation 946 sequences, which corresponded to 1259 unique OTUs. After filtering out OTUs that occurred ≤3 times in the complete data set, a total of 79,757 sequences corresponding to 645 unique OTUs remained. The overwhelming majority of the sequences came from 15 bacterial phyla. Only a very small proportion of the sequences (n=189, 0.2%) were unclassifiable to the phyla level. Sequence coverage calculated from the rarefaction curves for all samples was high; mean 0.9779, median 0.9845, range 0.9081-0.9982.

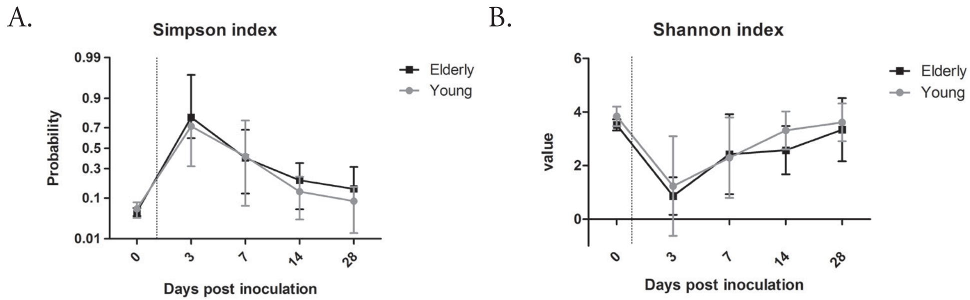

Figure 2: **Alpha diversity of bacterial communities in respiratory lavages following pneumococcal challenge**
Both the Simpson and the Shannon indices show a high amount of diversity before the introduction of *S. pneumoniae* into the URT niche, and a subsequent loss of diversity during pneumococcal colonization. The mean value of each index with the standard deviation is shown. Five mice each were sequenced, except for day 28 where 3 mice each were sequenced. Introduction of S. pneumoniae significantly reduces diversity; p<0.0001 at day 3 for Simpson index and p<0.001 at day 3 for Shannon index (ANOVA with Bonferroni post-test).

Bacterial diversity

Before the introduction of *S. pneumoniae* in to the URT, we observed a high diversity in both age groups, with mean Simpson index values of 0.059 for young and 0.051 for elderly (Figure 2). Although young-adult mice had slightly higher Shannon indices (mean 3.83) compared to elderly (mean 3.52), these differences were not significant. After introduction of *S. pneumoniae* into the niche, diversity was temporarily diminished with higher inter-individual variety in index scores than before inoculation. The decrease in diversity was significant at day 3 post inoculation compared to naïve mice (p<0.001 for both Simpson index and Shannon index, ANOVA with Bonferroni post-test). Starting from 7 days post-inoculation diversity gradually returned to baseline in both young-adult and elderly mice, as indicated in their Simpson and Shannon index scores (Figure 2). Although in general young-adult mice clear pneumococcal colonization significantly more rapidly than elderly mice (8) there was no significant difference in diversity dynamics in young-adult compared to elderly mice over time.

Respiratory microbiota composition in young and elderly naïve mice

Sequences resulting from 454-pyrosequencing of URT samples were classified in Mothur according to 97% similarity and analyzed taxonomically at the Phyla, Family, and OTU level. At the phyla level, microbiota of both young-adult and elderly mice contained approximately 50% Gram-positive and 50% Gram-negative bacteria, with Firmicutes, Bacteroidetes and Proteobacteria being the most predominant phyla in the URT of both age groups (Figure 3). However, in young-adult mice, there were consistently more Bacteroidetes observed compared to elderly mice. Furthermore, the young-adult and elderly naïve mice cluster in different quadrants when plotted using principle coordinate analysis (PCoA) of unweighted UniFac distances (Figure 4), suggesting that the phylogenetic composition at baseline is different.

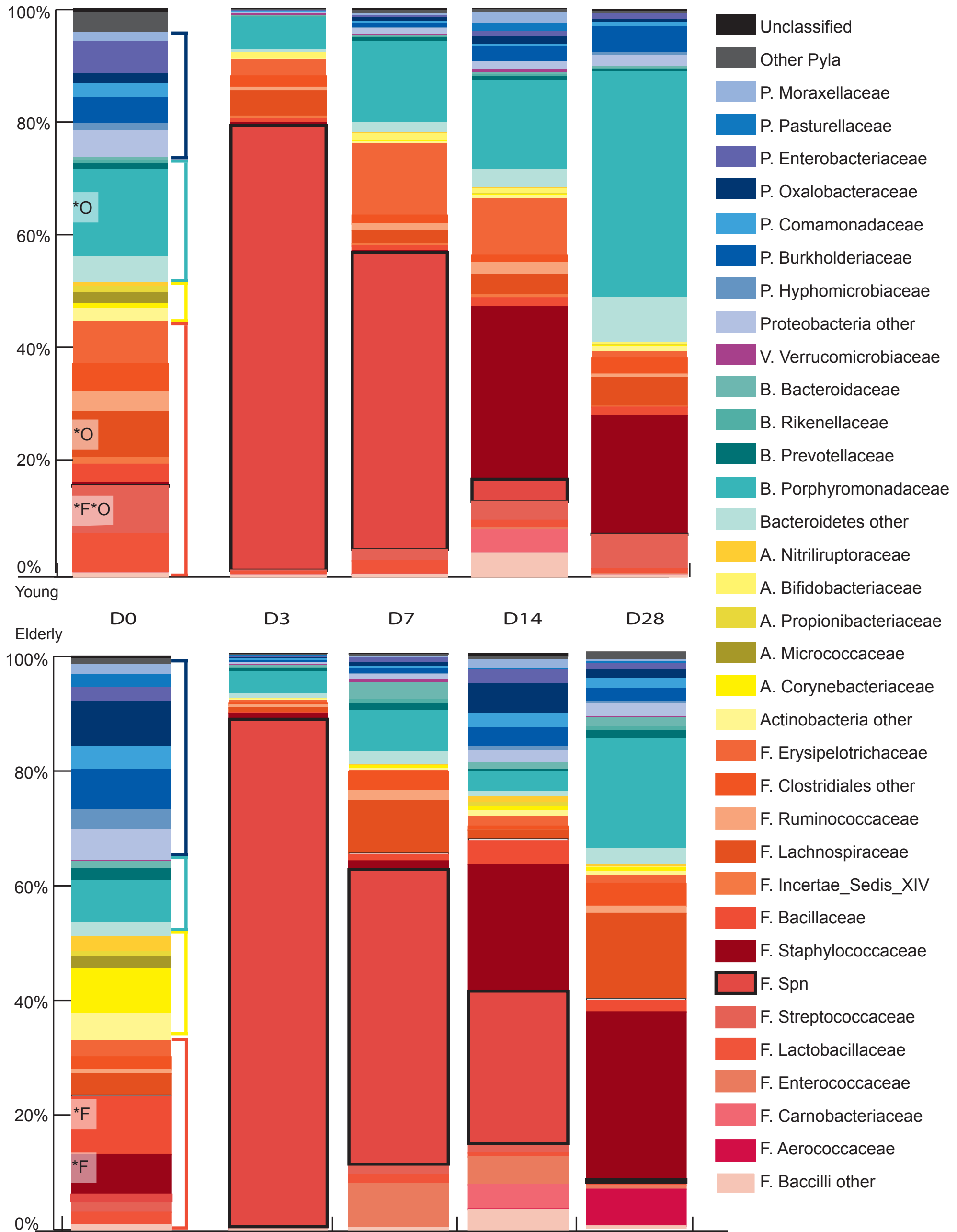

Figure 3: **Microbiota profiles of the URT after introduction of *S. pneumoniae* into niche**
Mean relative abundance of bacteria observed in the URT of young and elderly mice, represented at the family level (only the top 30 most abundant families are shown). Young mice are represented in the top 5 bars and elderly mice in the bottom. Gram-positive phyla are warm colors (orange bracket Firmicutes, yellow bracket Actinobacteria) and Gram-negative phyla are cool colors (teal bracket Bacteroidetes, dark-blue bracket Proteobacteria). The OTU correlating with *S. pneumoniae* observed by culture is indicated by the pink box outlined in black. Shown are bacteria significantly different between young and elderly mice indicated at family level (*F) and OTU level (*O). Initials of family names refer back to phyla: P for proteobacteria, F for firmicutes, A for actinobacteria, B for Bacteroidetes.

Looking deeper at the family level, the profile of young-adult mice shows a higher relative abundance of symbionts such as the Bacteroides family Porphyromonadaceae and the Firmicutes family Lachnospiraceae. In contrast, elderly mice have a higher abundance of Staphylococcaceae (significant in SAM). At the OTU level, three Barnesiella OTU's, three OTU's belonging to the Porphyromonadaceae and a single Lachnospiraceae OTU were significantly higher in young-adult compared to elderly mice before nasal inoculation with *S. pneumoniae*.

Niche composition during *S. pneumoniae* colonization

At both the Phyla level and the Family level (Figure 3) the most abundant bacteria in the URT niche during pneumococcal colonization were Firmicutes and Streptococcaceae respectively, specifically the streptococcal OTU matching our pneumococcal culture data (median relative abundance at day 3 post challenge was 0.97 in young-adult mice and 0.90 in elderly mice). This was also apparent in the PCoA, where colonized mice grouped farther away from their naïve counterparts (Figure 4). Although greatly reduced, all other major phyla (Bacteroidetes, Proteobacteria, and Actinobacteria) were still present in the niche of both age groups during the temporary dominance of pneumococci. Although both the culture and the sequence data indicate that the young-adult mice cleared pneumococci faster, the microbiota composition and diversity shift between days 7 and 14 was similar in both age groups and the distance between colonized mice and naïve counterparts became less at these timepoints. However, the microbiota composition did not return to the profiles present before introduction of pneumococci in the

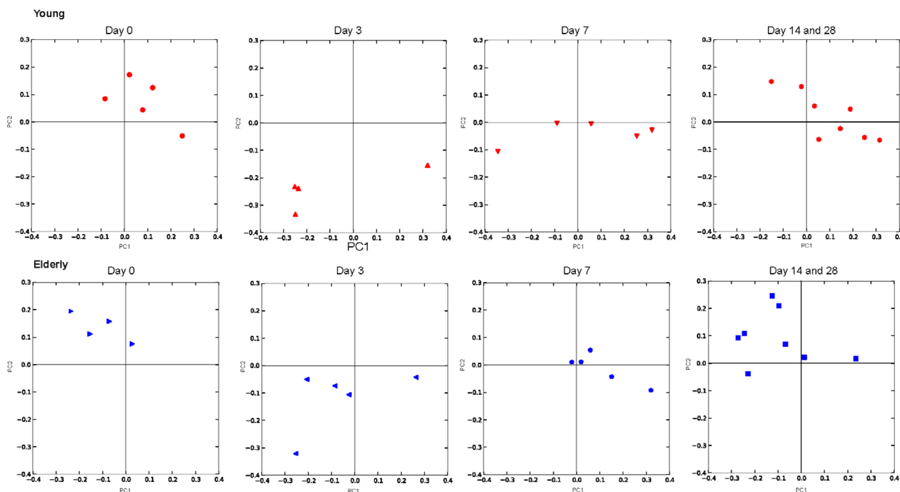

Figure 4: **Principle coordinate analysis of the Unweighted UniFrac distances**
Shown are coordinates 1 and 2 for all mice. Young mice (top boxes) are red, elderly mice (bottom boxes) are blue. Days 0, 3, and 7 (5 mice each) are plotted separate from days 14 and 28 (8 mice total). Young and elderly naïve mice cluster apart from each other, and both groups of naïve mice cluster apart from colonized mice. Over time, the mice begin to cluster closer to where they began as the pneumococcal colonization is cleared.

URT niche within the 28 days of observation. For example, Staphylococcaceae were the first bacteria to re-establish in both age groups, with a tendency to grow out more abundant when compared to before pneumococcal challenge (Figure 5). Two commensal bacterial families that maintained a relatively high presence throughout pneumococcal colonization were Porphyromonadaceae and Lachnospiraceae (Figure 3). Furthermore, Porphyromonadaceae (*Barnesiella* in particular) were the first commensals to re-establish it's abundance in the niche to pre-inoculation levels, even gaining slightly higher abundance levels in young-adult mice at day 28 compared to naïve (Figure 5).

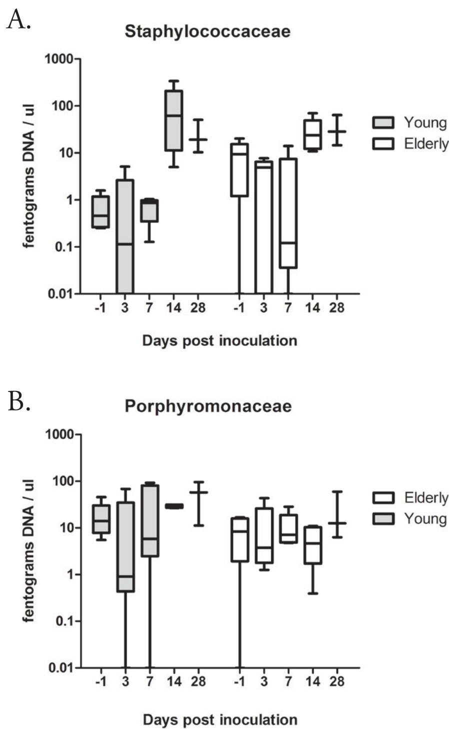

***Figure 5:* Absolute abundance of Stapylococcaceae and Porphyromonadaceae over time**
Absolute abundance of Stapylococcaceae (A) and Porphyromonadaceae (B) in young-adult and elderly mice pre and post-colonization with *S. pneumoniae*. Shown is the 5-95th percentiles with the median marked by the black bar. Where Porphyromonadaceae colonization stays fairly stable over time, Staphylococcaceae tend to grow out simultaneous to pneumococcal clearance, especially in young mice.

Discussion

To the best of our knowledge, this is the first study to 1. describe the URT microbiota composition in mice, 2. show the community dynamics in response to the acquisition of a potential pathogen i.e. *S. pneumoniae*, 3. study differences between young-adult and elderly mice. We found differences between young and elderly mice in the microbiota composition already present in naïve mice, with consistently more Bacteroidetes in young mice compared to elderly mice. Introduction of *S. pneumoniae* into the niche led to a temporary dominance of pneumococci and a loss of diversity. During the dominance of pneumococci in the niche, all four major phyla observed before were still detected in the mice, albeit at very low abundance. The mice gradually clear pneumococci, and during

this process microbial diversity gradually returns to baseline, with Porphyromondaceae and Lachnospiraceae among the first to re-establish. However, return to baseline was not complete within 28 days. We observed that certain families of bacteria that were in low abundance prior to introduction of the pneumococcus, were able to grow out in parallel to clearance of pneumococci; staphylococci are one example.

Before introduction of the pathobiont *S. pneumoniae* into the URT niche, diversity in the mouse URT was high with Simpson index scores comparable to nostril and oropharyngeal scores in humans (16) and the mouse gut (17), indicating that although microbiota of the URT are less dense than the gut (3, 10) there is a large variety of species present. The Shannon index scores for the URT of the mice in this study, however, are about half of what is found in the mouse gut (17), which is probably caused by the general predominance of certain bacterial species in the URT which was also observed for humans as well (3, 10). In the URT of naïve mice, young-adult mice had high abundance of normal commensals such as Streptococcaceae, Porphyromonadaceae, and Lachnospiraceae generally considered beneficial for the host in the niches they inhabit (1, 18-20). Interestingly, elderly mice had consistently lower abundance of anaerobic bacteria such as Porphyromonadaceae, specifically *Barnesiella*. This bacterium has previously been found to contribute to clearance of intestinal pathogens such as antibiotic resistant Enterococci in mice (20), and was therefore suggested to contribute to microbiota homeostasis.

In line with culture data from previous mouse colonization studies (21), the relative and absolute abundance of pneumococci peaked at day 3. This correlated with an increase in total bacterial DNA isolated at day 3 suggesting a temporary overgrowth of the pathobiont. Furthermore there was a significant reduction in diversity, both in richness and evenness, as measured by alpha-diversity indices, accompanying the dominance of pneumococci in the URT. This is the first study to show that pneumococci are rapidly overtaking the niche and becoming dominant within the microbial community. Moreover, we observed a significant increase in total bacterial DNA isolated at day 3, and this suggests a temporary overgrowth of this species in the URT.

In the period between pneumococcal dominance and full clearance, mice appear to gradually clear pneumococci and return to baseline levels of diversity. During this period of time, the commensal anaerobes, Porphyromonadaceae, and Lachnospiraceae are among the first to re-establish the niche. Both classes of bacteria, Bacteroidales and Clostridia, have been identified as key modulators of homeostasis in the gut, including immune maturation and modulation (18), and they may therefore be functioning similarly in the URT as well. Since especially Bacteroidetes are less present in the URT of elderly mice, we hypothesize this might relate to the increased baseline inflammation that we observed previously in the URT of elderly mice (8), and therefore this deserves further studies.

Clearance of pneumococci in the URT of mice over time seems a less homogenous process, with high variability in the amount of time needed for clearance of this potential pathogen. Since in our previous studies the majority of young-adult mice have cleared pneumococcci by 21 days, we hypothesized that the microbiota should have returned back to baseline by day 28. While the diversity indices did return to the same level as naïve young-adult mice, we were surprised to find that the composition of the microbiota had not returned to baseline. Even more surprising was that in both young-adult and elderly mice, bacteria that were low abundant in naïve mice were enriched in challenged mice that were mostly clear of their pneumococci. In our study the bacteria that grew out to the highest abundance were staphylococci; interestingly there is epidemiological evidence for a competitive relationship between *Streptococcus* (*S. pneumoniae*) and *Staphylococcus* (*S. aureus*) in humans as well, suggesting that complex bacterial-bacterial and host-bacterial interactions are involved in microbiota dynamics (22-24). This was especially observed in children, where a temporary increase in *S. aureus* colonization and disease was observed in individuals vaccinated with a pneumococcal conjugate vaccine versus non-immunized children, both in a randomized controlled trial setting as well as in surveillance studies in the population (24, 25).

It is difficult to determine if the bacteria that re-establish the niche are taking advantage of the immune system clearing out pneumococci, or if they are directly competing with the pneumococci and aiding in clearance. Further studies should be undertaken to investigate these potential interactions in more detail. A limitation of this study is that we studied colonization dynamics up to 28 days post-inoculation, and did not observe the microbiota return to baseline. Future studies therefore should consider follow-up for at least another 1-2 weeks.

In conclusion, this is the first in depth, culture-independent survey of microbiota in the URT of young-adult and elderly mice. We show significant changes in microbiota composition in the elderly that are correlated with dysregulated mucosal homeostasis as was previously observed (8) and the decreased presence of lactic acid anaerobes Bacteroidetes in the URT of elderly mice is in agreement with human studies of elderly gut dysbiosis (26). Furthermore, we show that acquisition of the pathobiont *S. pneumoniae* into the URT niche causes temporary pathogen overgrowth, a temporary loss of diversity, and dysbiosis that lasts beyond the point of pathobiont clearance from the URT. The dynamic changes in microbiota composition reported here can serve as the foundation for future research in host-pathogen-commensal interactions in the polymicrobial mucosal surfaces of the upper respiratory tract.

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5

Chapter Five

High rates of *Streptococcus pneumoniae* carriage in saliva of elderly

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Manuscript in preparation

Abstract

Background: Pneumococcal disease disproportionally affects the very young and the elderly. Unlike in children, in the elderly pneumococcal colonization is rarely detected by conventional culture of nasopharyngeal swabs. Here, we tested both nasopharyngeal and saliva samples for pneumococcal carriage in the elderly by molecular methods.

Methods: Trans-nasal (n=286), trans-oral (n=287) nasopharyngeal samples and saliva (n=291) samples were obtained from 146 persons aged 60-89 during an episode of influenza-like-illness (ILI), and 6-8 weeks later during recovery. All samples were tested for *S. pneumoniae* by conventional culture. Following, all visible culture plate growth (enriched samples) as well as raw saliva were further processed for DNA extraction. Pneumococcal presence was detected by quantitative-PCR (qPCR) targeting two *S. pneumoniae*-specific genes, *lytA* and *piaA*.

Results: *S. pneumoniae* was cultured from 6 of 286 (2%) trans-nasal, 9 of 287 (3%) trans-oral and 6 of 291 (2%) saliva samples from in total 16 of 146 elderly. Ten (3.5%) trans-nasal, 30 (11%) trans-oral and 97 (33%) saliva samples were positive for pneumococcus by qPCR. The sensitivity of carriage detection was highest by qPCR in culture-enriched saliva samples (89 of 108, 82%), followed by raw saliva samples (50 of 108, 46%) and culture-enriched nasopharyngeal samples combined (35 of 108, 32%). In total, seventy-three of 146 (50%) individuals were positive for *S. pneumoniae* at least once; 56 (38%) during an ILI, 52 (36%) after recovery and 35 (24%) at both sampling events.

Conclusions: Using culture-enriched saliva for the molecular detection of *S. pneumoniae* greatly increases the sensitivity of pneumococcal carriage detection in the elderly.

Introduction

Streptococcus pneumoniae is a frequent but transient commensal of the human upper respiratory tract (URT), that may grow out and progress towards respiratory and invasive pneumococcal disease (IPD) (1). The disease disproportionally affects the very young and the elderly (2). Since carriage of *S. pneumoniae* in the URT is considered prerequisite for infection, the impact on carriage is an important part of conjugate pneumococcal vaccine trials and pre- and post-marketing surveillance (3, 4). Surveillance provides insight into the serotypes circulating in the population before and after pneumococcal conjugate vaccine introduction and assesses both direct and herd effects of vaccine implementation.

Currently, the gold standard for pneumococcal carriage detection in children is collecting deep trans-nasal nasopharyngeal swabs followed by conventional culture and isolation of live *S. pneumoniae* (5). In adults, the addition of a trans-orally obtained pharyngeal swabs significantly increases the detection rate of carriage (6-8). Furthermore, switching from culture-dependent to culture-independent diagnostic methods largely improved the sensitivity of *S. pneumoniae* detection in nasopharyngeal samples from children (9-11) and from adults (6, 12). Interestingly, *S. pneumoniae* was discovered by Sternberg and Pasteur simultaneously in 1881 in human saliva samples that were injected in rabbits (13, 14). Following this discovery, epidemiological studies were performed by injecting mice with saliva obtained from healthy individuals and isolating the pneumococcus from the blood of sick animals. In the early 1900s the consensus was that the pneumococcus was carried in the saliva of 45-60% of all healthy asymptomatic people (13, 14). During the time between the dawn of the antibiotic era in the mid-20th-century and the introduction of the conjugated polysaccharide pneumococcal vaccines in the present millennium, carriage studies in adults and elderly were scarcely performed (15). Furthermore, the method of detecting pneumococcus in the upper respiratory tract moved from saliva to nasopharyngeal swabs, due in part to the highly polymicrobial nature of saliva making detection of *S. pneumoniae* by isolation of live bacteria from cultures very difficult. In the scarce recent studies among elderly however, the use of swabs generally resulted in carriage rates of under 5% (16-20). Recent advances in molecular detection methods prompted us to revisit saliva as diagnostic specimen in epidemiological studies to see whether we could improve detection of pneumococcal carriage in this risk group.

In this study, we compared both culture-based and molecular methods on both nasopharyngeal and saliva samples to detect *S. pneumoniae* in the elderly. We provide evidence for the superiority of saliva for pneumococcal carriage detection in aged humans and conclude that the current rates of pneumococcal carriage in the elderly might be largely underestimated.

Materials and Methods

Influenza-like-illness in elderly study

To assess the incidence and cause of influenza-like illness (ILI) among elderly in The Netherlands, an open cohort observational study was performed among adults aged 60-89 during the 2011/2012 autumn/winter season. In total 21,000 elderly were contacted and 2120 consented to participate in the study. Of these, 146 participants reported ILI symptoms and were eligible for first sampling. Written informed consent was obtained from all individuals and the procedures followed were in accordance with European Statements for Good Clinical Practice and the Declaration of Helsinki of the World Medical Association. This study was approved by an acknowledged Dutch National Ethics Committee (NDR 3386). Demographic information was collected from study participants at the first visit.

Trans-nasally collected nasopharyngeal swabs, trans-oral nasopharyngeal swabs, and saliva samples were simultaneously collected from 146 adults between December 2011 to 1 June 2012. Persons were sampled twice: first at the onset of influenza-like-illness (ILI) (rhinitis, cough, fever >38°C, sore throat) and 6-8 weeks later, after recovery. Trained personnel collected all samples during home visits. Deep trans-nasal samples were obtained using flexible sterile swabs according to the World Health Organization (WHO) standard procedure (5). Trans-oral nasopharyngeal samples were collected with rigid sterile swabs under direct observation of the posterior pharynx (8). Nasopharyngeal swabs were individually placed in Amies medium (Copan) and transported at room temperature to the diagnostic lab. Saliva samples were collected with Oracol Saliva Collection System (Malvern Medical Developments Limited, Worcester, UK). Immediately after collection saliva was transferred to tubes pre-filled with glycerol (final concentration of 10%) and placed on dry ice for transport. All samples were transferred within 8 hours to the Regional Laboratory of Public Health in Haarlem.

Culture of samples

On arrival 10 ul of the trans-oral samples were cultured on trypticase soy agar supplemented with 7% defibrinated sheep blood and gentimicin 5mg/l (SB7-Gent, Oxoid, Badhoevedorp, Netherlands) and processed for *S. pneumoniae* presence by a conventional diagnostic approach (21). All bacterial growth was harvested from all SB7-Gent plates as previously described (6) and stored frozen at -80°C. The trans-nasal Amies medium samples were supplemented with 10% glycerol and together with raw supplemented saliva samples stored at -80°C until further analysis. These samples were later thawed and 10 ml of trans-nasal sample and 100 ml of saliva sample were used to inoculate SB7-Gent plates and processed similarly as the transoral cultures where all visible bacterial growth was harvested and stored frozen at -80°C. We hereafter consider these harvests from culture plates as culture-enriched samples (6). Finally, culture-enriched

trans-nasal, trans-oral, and saliva samples were re-cultured to search for pneumococci after molecular methods identified samples as positive for *S. pneumoniae*. Saliva samples were used for culture enrichment before DNA was isolated from raw saliva, samples that did not have enough for both processes were analyzed by culture enrichment only.

Isolation of bacterial DNA

Bacterial DNA was extracted from 100 ml of glycerol-supplemented raw saliva samples, and from 200 ml of culture-enriched saliva and from nasopharyngeal samples using a high recovery phenol/bead beating method in combination with the Agowa Mag mini DNA extraction kit as describes previously (22). DNA was eluted into 50 ml template.

Quantitative PCR

S. pneumoniae-specific DNA was detected via quantitative PCR (qPCR) targeting the pneumococcal genes *lytA* (23) and *piaA* (6). Samples were considered positive for *S. pneumoniae* if the lytA specific signal alone was <35 C_T (6, 9) or when samples gave a signal for both genes of <40C_T. Statistical differences were detected using two-way Fisher's exact tests.

Results

For this study 21,000 elderly were contacted and 2120 consented to participate in the study. Of these 146 participants reported ILI symptoms and were eligible for first sampling. The mean age at the time of first sample collection was 69, and the percentage of female participants was 56.8%. Three of 146 participants refused to participate in the second sample collection (following recovery) and two persons were sampled at 2 consecutive reported ILI episodes (3 total sets of samples, twice during symptoms and once during recovery). This resulted in samples taken from 146 persons at the first sample time, 2 persons with a second ILI, and 143 persons at the recovery phase (Figure 1). At the first sampling moment 148 saliva samples, 147 trans-oral, and 147 trans-nasal samples were collected (including 2 individuals with a second ILI). At the second sampling moment 143 saliva samples 140 trans-oral, and 140 trans-nasal samples were collected. In total, 291 saliva samples, 287 trans-oral, and 286 trans-nasal samples were collected and processed for DNA extraction and tested in qPCR for the presence of *S. pneumoniae*.

Isolation of *S. pneumoniae* from cultures of trans-oral, trans-nasal, and saliva samples

Live *S. pneumoniae* were isolated from 3 of 287 (1%) trans-oral samples by conventional culture (per-protocol analysis). Additional *S. pneumoniae* were isolated from 7 of 287 trans-oral, 6 of 286 (2%) trans-nasal, and 6 of 291 (2%) saliva samples upon reculture of samples stored frozen in glycerol (Figure 2A). In total, all cultures combined identified

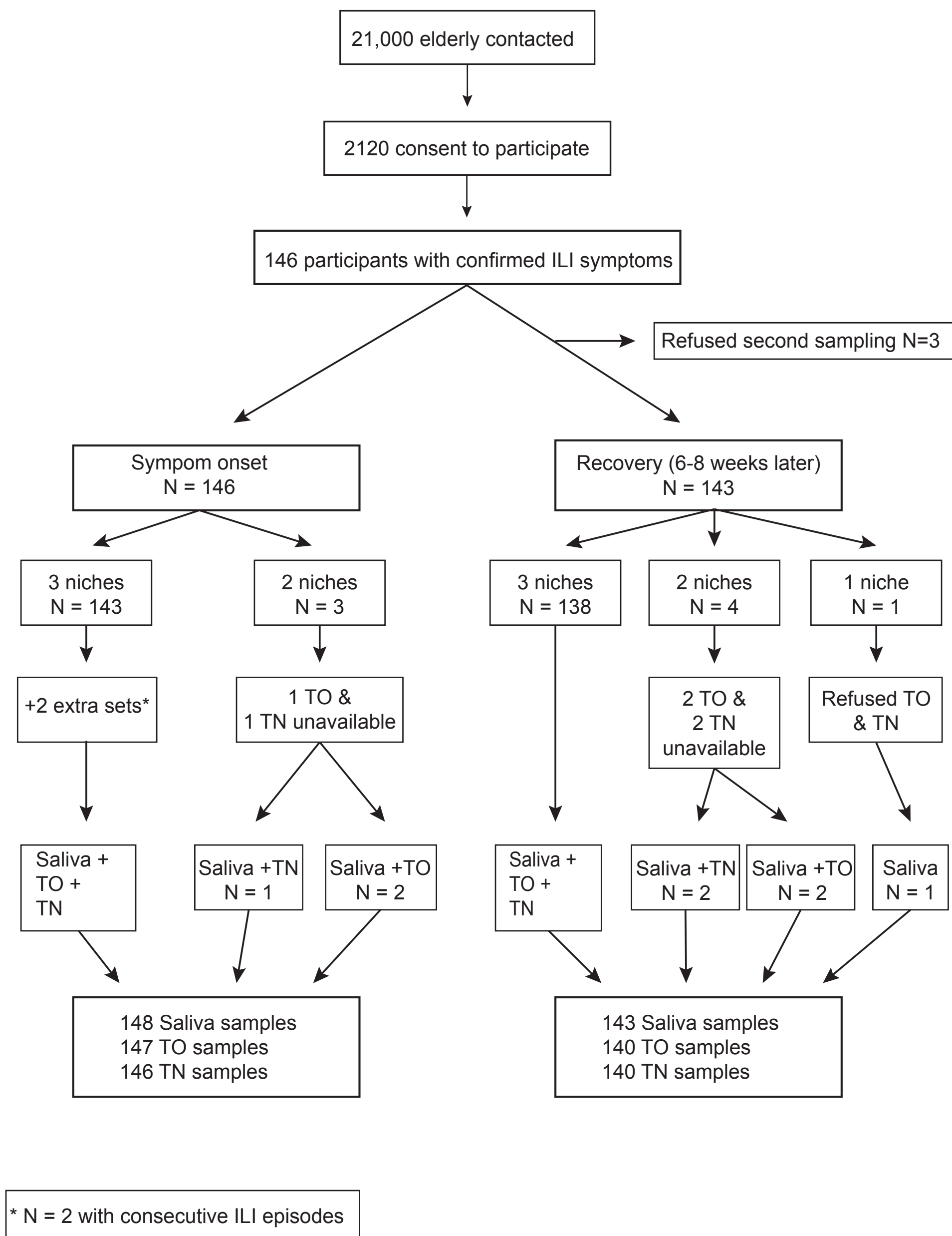

Figure 1: **Enrollment and samples in this study.**
Saliva total N = 291; OP total N = 287; NP total N = 286. In total 864 samples were analyzed.

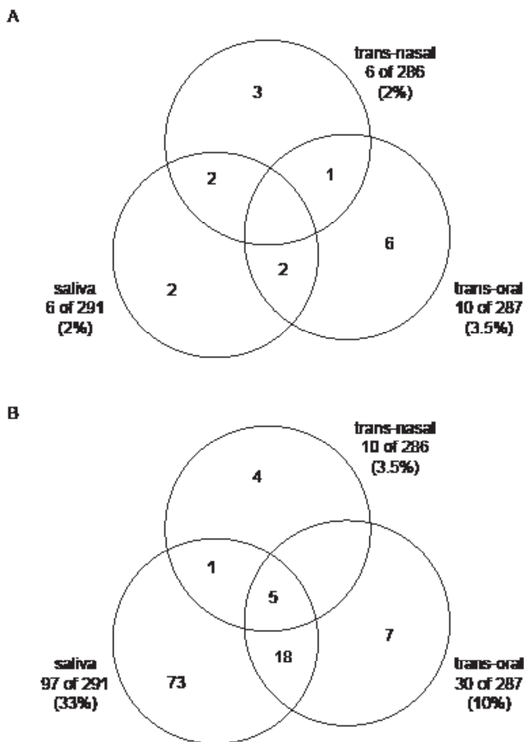

Figure 2: **Detection of *Streptococcus pneumoniae* in all samples collected in this study**
A. Detection of carriage based on culture results and isolation of live *S. pneumoniae* from samples.
B. Detection of *S. pneumoniae* by qPCR.

16 (11%) elderly with pneumococcal carriage, nine during ILI and 7 following recovery. One of these 16 participants was identified as carrier at both sampling moments and there was no significant difference in the number of samples culture-positive for *S. pneumoniae* during an ILI episode or after recovery in the different sampling methods.

Molecular detection of *S. pneumoniae* in culture-enriched samples

Ten of 286 (3.5%) trans-nasal samples, 30 of 287 (10%) trans-oral samples, and 89 of 291 (31%) saliva samples were qPCR-positive for *S. pneumoniae* after culture-enrichment (Figure 2B). These differences were all significant ($p<0.002$). All samples culture-positive for *S. pneumoniae* were also identified as positive for pneumococcus by qPCR.

The process of culture enrichment for *S. pneumoniae* has been shown to increase pneumococcal carriage detection in nasopharyngeal samples from children (9) and adults (6). In this study we tested the impact of culture-enrichment on the sensitivity of *S. pneumoniae* detection in saliva samples from elderly. There was a significantly lower number of carriers (50 of 285 or 17%) detected by qPCR in raw saliva compared to the number of carriers detected by qPCR of culture-enriched saliva samples (89 of 291 or 31%), showing that the use of culture enrichment considerably increases the sensitivity of

S. pneumoniae detection by molecular methods in saliva samples from elderly (p <0.001). Overall, 97 of 291 (33%) saliva samples were positive by qPCR in DNA extracted either from raw or culture-enriched samples, with 8 individuals positive only in raw samples, 47 only in DNA extracted from culture-enriched samples and 42 in both extraction methods. Culture enrichment also increased the signal strength of the genes we tested,

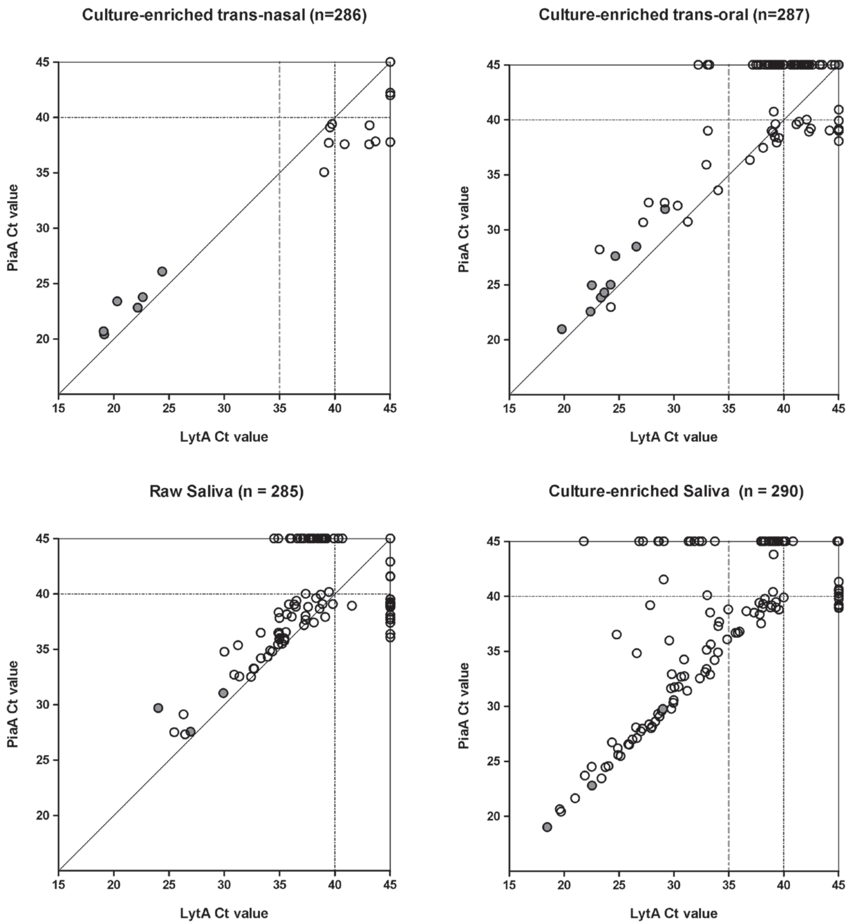

Figure 3: **PCR based detection of *Streptococcus pneumoniae* versus isolation of live pneumococci from trans-nasal nasopharyngeal, trans-oral nasopharyngeal and saliva samples analysed in the study.**
Figure depicts results of qPCR-based detection of *S. pneumoniae*-specific genes *lytA* and *piaA* and results of live *S. pneumoniae* isolation from 286 culture-enriched trans-nasal nasopharyngeal samples (upper left graph), 287 culture-enriched trans-oral nasopharyngeal samples (lower right graph), 285 raw saliva samples (lower left graph) and 291 matching culture-enriched saliva samples (lower right graph) from 146 elderly. Each dot represents an individual sample. Position of the dot corresponds to C_T values for *lytA*- and *piaA*-specific signals as marked on corresponding axes. Gray dots represent samples from which live pneumococci were isolated by conventional culture or re-culture. Dashed lines mark the threshold assigned to discriminate between positive and negative samples based alone on *lytA* specific signal below C_T 35, dotted lines mark the threshold of sample positivity based on presence of signals for both *lytA* and *piaA* below C_T 40 and the continuous lines represent total number of 45 cycles in each qPCR reaction.

with a maximum increase of 5.55 C_T and an average increase of 4.16 C_T values for the *lytA* gene.

Optimal niche for detection of pneumococcal carriage in elderly

Altogether, carriage of *S. pneumoniae* was detected by culture in 17 (6%) and by molecular method in 108 (37%) of 291 sampling moments (Figure 3). The number of trans-nasal samples positive for *S. pneumoniae* by qPCR (10 of 286) was not significantly higher compared to the number of trans-nasal samples positive by culture (6 of 286; p=0.45). The number of samples identified as positive for *S. pneumoniae* by qPCR was substantially higher for both trans-oral (30 vs. 9 of 287; p=0.0007) and saliva (97 vs. 6 of 291; p<0.0001) when compared to culture. Overall, the qPCR-based detection of *S. pneumoniae* in culture-enriched samples of saliva was the most sensitive method of pneumococcal carriage detection in this study (Table 1). Processing only saliva instead of nasopharyngeal samples would lower the number of detected carriage events from 37% to 34%.

Point and period prevalence of pneumococcal carriage in elderly

In this study, we collected samples of individuals at two time points, at the onset of

Table 1: Sensitiviy of methods used to detect *Streptococcus pneumoniae* carriage in this study

Method of *S. pneumoniae* detection	Number (%)[a] of detected carriage events	Sensitivity of method (%)[b]
Culture (either conventional or re-culture)		
trans-nasal	6 (2)	5.5
trans-oral	10 (3.5)	9
saliva	6 (2)	5.5
Molecular detection		
culture-enriched trans-nasal	10 (3.5)	9
culture-enriched trans-oral	30 (10.5)	28
culture-enriched trans-nasal or culture-enriched trans-oral	35 (12)	32
raw saliva	50 (17.5)	46
culture-enriched saliva	89 (31)	82
either raw or culture-enriched saliva	97 (33)	90

[a]Fraction of all samples of a particular type processed in the study. Trans-nasal N = 286; trans-oral N = 287; raw saliva N = 285; saliva CE N = 291.
[b]Fraction of 108 *S. pneumoniae* carriage events identified by any method in the study.

Table 2: Characteristics of the elderly relating to pneumococcal carriage detection

		Number persons	Persons positive (%)	Persons sampled twice	Persons double positive (%)	Number samples	Samples positive
Sex	Female	83	38 (46)	82	17 (21)	167	55 (33)
	Male	63	35 (55.5)	61	18 (29.5)	124	53 (43)
Age (years)	81-90	13	6 (46)	11	2 (18)	24	8 (33)
	71-80	35	13 (37)	35	5 (14)	71	18 (25)
	60-70	98	54 (55)	97	28 (29)	196	82 (42)
	Total	146	73 (50)	143	35 (24.5)	291	108 (37)

ILI symptoms and during recovery (6-8 weeks later). Based on the combined results of pneumococcal carriage detection by any method in the study, the carriage prevalence for the first time point was 57 of 146 (39%) and for the second time point was 51 of 143 (36%) (p=0.8). A total of 73 people were identified as carriers of *S. pneumoniae* in any sample collected during either sampling moments in the study, resulting in a period prevalence of 50% in this population of elderly (Table 2). Thirty-five (24%) elderly were identified as positive for pneumococcal carriage at both study time points.

Carriage of *S. pneumoniae* by age category and by sex

We stratified all positive samples by age and sex to determine if either factor significantly effects detection of *S. pneumoniae.* We found no differences in the distribution of carriers by age (Table 2). Furthermore, there were no significant differences between males and females.

Discussion

The purpose of this study was to investigate the utility of saliva sampling and culture enrichment combined with molecular methods for detecting *S. pneumoniae* carriage in the elderly. The major finding of our study is the high rate of pneumococcal carriage in aged adults. We identified 50% of the elderly as pneumococcal carriers at least once during the study with half of these positive at both time points and 35% of elderly as carrier at either of the two study time-points. We found that in elderly using saliva as a sample source significantly increases detection of *S. pneumoniae* compared to trans-nasal and trans-oral nasopharyngeal samples. Our data suggest that saliva can be used as the sole specimen for pneumococcal carriage detection. Additionally, we found that

culture enrichment increases both the number of saliva samples identified as positive for *S. pneumoniae* as well as the overall abundance of pathogen DNA present in positive samples.

Our present data on the relative high rate of pneumococcal presence in oral fluids in elderly are in agreement with historical reports where saliva was collected from asymptomatic adults and was tested with the sensitive mice inoculation method (14). Although currently, the use of nasopharyngeal or oropharyngeal swabs combined with conventional culture is considered the standard method for *S. pneumoniae* carriage detection in adults (7, 8, 24), this method might be suboptimal in detecting carriage in elderly adults. Possible reasons why the culture method might be of low sensitivity in adults and in elderly may the be lower density of nasopharyngeal carriage at adulthood as compared to younger children, where conventional cultures of the nasopharynx identify carriage in up to 40-90%. Alternatively, changes in anatomy of the upper respiratory tract in adults and elderly may cause difficulty in accessing the nasopharyngeal niche. We now show that the use of swabs alone, even when combined with molecular methods and culture enrichment, may still greatly underestimate pneumococcal carriage in the elderly.

We also demonstrated that conventional cultures find significantly less pneumococci than molecular-based methods when applied to any niche sampled by us. This finding agrees with surveillance studies performed in the early 1900's where direct culture of swabs either in broth or on blood-agar yielded the lowest rates of detection (sensitivity of 47%) when compared directly to the inoculation of swab storage medium into mice (sensitivity of 93%) (25). Finally, our study shows the additive advantage of applying a qPCR-based method on culture-enriched samples, which is in accordance with other studies in younger adults (6) and in children (9).

A possible limitation of our study is that live *S. pneumoniae* could not be isolated from the vast majority of samples that were qPCR positive. We attribute this to the highly polymicrobial nature of the samples, in particular the saliva samples, that show abundant growth on culture plates making *S. pneumoniae* hardly detectable. The *lytA*-specific qPCR that we employed is described to be a highly sensitive and highly specific method for pneumococcal DNA detection (9, 11, 26). Currently the consensus on the criteria for assigning samples as positive for *S. pneumoniae* is a *lytA*–specific signal $<35\ C_T$ for culture-enriched samples and any signal $<45\ C_T$ for DNA obtained directly from respiratory samples. We further increased the specificity of this molecular method by also testing for a second pneumococcal gene, coding for the PiaA protein, and considered both raw and culture-enriched samples as positive when the *lytA*-specific signal was $<35\ C_T$ or when both genes were detected at C_T values <40. In line with findings of our previous study on carriage in adults, we observed an overall concordance between the quantity of *lytA* and *piaA* detected by qPCR (6). There were however several samples qPCR-positive for *lytA* but not for *piaA*. Since we and others have described *S.*

pneumoniae strains negative for the presence of the *piaA* gene previously, we felt confident about classifying those samples as positive for *S. pneumoniae* as long as a strong *lytA*-specific <35 C_T was present. When a similar study protocol was previously applied by us to trans-oral nasopharyngeal samples from parents of young children we were able to culture *S. pneumoniae* from three quarters of samples identified as positive by qPCR (6).

Although culture-enrichment of saliva did significantly increase the overall number of saliva samples that were identified as positive for *S. pneumoniae* from 50 to 97, 8 out those 97 (8%) were found positive in DNA extracted from raw but not from culture-enriched saliva samples. There might be a number of reasons why this occurred, including an inhibitory effect of gentamicin at the concentration used in the culture-enrichment step for some pneumococcal strains, suppression of *S. pneumoniae* growth by other microorganisms present in saliva in culture, the effect of freezing and thawing prior to the culture step or discordant results for samples with low numbers of pneumococcal cells and different volumes used. It may also be a consequence of residual pneumococcal DNA from dead or uncultivable *S. pneumoniae* cells present in raw but not in culture-enriched samples of saliva. Nevertheless, considering the small discordance between detection of pneumococci in raw versus culture-enriched saliva samples the use both methods in parallel could be considered in surveillance studies.

Another limitation of this study was the enrollment of elderly with ILI symptoms at the first sampling time. This might induce a selection bias, possibly affecting the overall pneumococcal colonization rates observed while experiencing a viral infection. However, since there was no statistical difference between the pneumococcal carriage rates observed during ILI and following recovery, the bias might be limited. However, follow-up studies in healthy elderly should be performed to confirm the high carriage rates observed in this pilot study. Another limitation is the limited sampling moments. We already found that two sampling moments significantly increases colonization detection and adding more time point may further increase detection rates (12, 14, 27).

In general, we observed a point prevalence of pneumococcal carriage of over 35% and a period prevalence of 50% in the elderly, which are a stark contrast to many studies performed in the last 15 years, showing point prevalences of 0-5% (16-20). However, our findings seem to be in line with results of early epidemiological studies from the pre-antibiotic era reporting that approximately half of all adults asymptomatically carry *S. pneumoniae* when saliva was tested (14). For us, it seems more plausible to link a disappearance of the *S. pneumoniae* reservoir in elderly carriage in the past half-century to changes in diagnostic procedures rather than attribute it to the universal improvement of living conditions and public health, especially since pneumococcal pneumonia is still a major disease burden in elderly (2). Furthermore, the elderly are at greater risk of silent aspiration (2, 28), where aspiration is thought to be responsible for 5-15% of

community acquired pneumonia (CAP) (28). The presence of potential pathogens in the oral cavity is considered a risk factor for developing aspiration pneumonia (28), therefore our finding seem highly related link to the epidemiology of CAP in its totality.

In conclusion, we found a high prevalence of pneumococcal carriage in elderly when molecular-based methods are used. Furthermore, use of saliva significantly increases detection in the elderly. Collection of saliva is easy and minimally invasive, therefore future carriage studies in the elderly should consider using these methods.

Acknowledgements

We gratefully acknowledge the participating elderly for their time and commitment to the study. We thank all members of the research team of the RIVM, the research team of the Linnaeus Institute of the Spaarne Hospital in Hoofddorp, the Netherlands, the laboratory staff of Regional Laboratory of Public Health, Haarlem, the Netherlands and the cooperating institutes for their dedication and work which made this project possible. The study was funded by the Dutch Ministry of Health. Grant support for microbiology was provided by Pfizer, the Netherlands, through a research grant to EAMS and KT.

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6

Chapter Six

Dried saliva spots are a novel, robust method for detecting *Streptococcus pneumoniae* carriage

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Submitted for publication

Abstract

The earliest studies in the late 19th century on *Streptococcus pneumoniae* carriage used saliva as the primary specimen. However, interest in saliva declined after the mouse inoculation method was replaced by conventional culture, which made isolation of pneumococci from the highly polymicrobial oral cavity virtually impossible. The high sensitivity of molecular methods prompted us to revisit saliva as a diagnostic specimen. Here, we tested the feasibility of using dried saliva spots (DSS) for studies on pneumococcal carriage. Saliva from healthy adult volunteers was spiked with *S. pneumoniae* cells, applied to Whatman 903 Protein Saver cards, dried and stored at various temperatures for up to 35 days. DNA extracted from DSS was tested with quantitative-PCR (qPCR) targeting genes specific for *S. pneumoniae*. In addition, pure saliva samples from children were evaluated. The quantity of pneumococcal DNA detected in spiked DSS from adults processed immediately after drying matched that in freshly spiked raw saliva. Pneumococcal DNA in DSS was stable for 7 days at 37°C, and for up to 35 days at -20°C, 4°C, room temperature, and 30°C. There were no differences in the results for various clinical pneumococcal strains used to spike saliva samples. Similar results were obtained for unspiked samples form children. Pneumococcal DNA is stable in DSS stored with desiccant for up to one month over a broad range of temperatures. Since collection of saliva does not require trained personnel and DSS allow robust transport conditions, the method could be particularly useful in surveillance studies conducted in remote settings.

Introduction

Streptococcus pneumoniae is an inhabitant of the human respiratory tract and frequently carried asymptomatically. On rare occasions it breaches the host's immune barrier and becomes a pathogen causing a range of diseases; including otitis media, pneumonia, bacteremia, and meningitis (1, 2). In general, pneumococcal colonization is considered a necessary precursor to disease (3) with a direct link between strains circulating in carriage and disease (4, 5). This link is commonly exploited by epidemiological surveillance studies targeting asymptomatic colonization in order to assess the community effects of therapeutic and preventive strategies (6-8).

For the past several decades, the nasopharynx was considered the optimal sampling niche for detection of *S. pneumoniae* colonization (6-9). In contrast, historically saliva was the diagnostic specimen of choice for detecting asymptomatic pneumococcal carriage (10). Both Pasteur and Sternberg independently discovered *S. pneumoniae* in 1881 by infecting animals with human saliva and for more than half a century testing saliva in mice was considered the optimal method for carriage detection (10, 11). When applied in cross-sectional studies conducted in the pre-antibiotic era, 45% to 60% of adults (10, 11) and 50% to 80% of schoolchildren were identified as asymptomatic carriers (12). With the rise of antibiotic use, and the progress in culture-based diagnostic methods, interest in the use of saliva for epidemiological surveillance declined. The sensitivity of conventional cultures however, was low when used for recovering live pneumococci from highly polymicrobial saliva samples (10), contributing further to the downfall of oral fluids as diagnostic specimens in surveillance studies on pneumococcal carriage. However, recent progress in culture-independent molecular methods has led to a dramatic increase in sensitivity and specificity of pathogen detection (9, 13, 14), and prompted our interest in re-visiting saliva as a specimen for epidemiological studies on *S. pneumoniae* carriage.

Saliva is an easily accessible, easy to collect body fluid secreted into the oral cavity at the crossover of the digestive und respiratory tracts. It is presumed that in order to preserve the saliva sample, it should be stored and transported at low temperatures, preferably snap frozen and shipped on dry ice (15, 16). The requirement for a cold chain does complicate the use of saliva as diagnostic specimen for pneumococcal detection. However, historical studies provide alternative solutions for the storage and preservation of saliva samples: the first to report on drying pneumococci for later use was Nissen in 1891 (17). By the 1930's dehydration was considered "an excellent physical method for the preservation of cultures of Pneumococcus, particularly… [for] long period[s] of time [that] require that the characters of the strain be held uniform and constant" (11).

The dried spot method is widely used to collect and preserve blood and other body fluids for a range of diagnostic purposes (18-22). In this study we evaluated dried saliva

spots stored at various temperatures as a method to detect pneumococcal presence in saliva of asymptomatic individuals.

Materials and Methods

Bacterial strains

The *S. pneumoniae* strains used in this study are listed in Table 1. Pneumococci were grown to mid-log phase in brain-heart infusion broth (BHI, Oxoid) and aliquots were frozen in 10% glycerol at -80°C. Prior to use, bacterial cells were thawed, washed twice with phosphate buffered saline (PBS), tittered by culturing tenfold dilutions on blood agar supplemented with gentamicin (SB7-Gent, Oxoid), and re-suspended in PBS to reach a particular CFU concentration. Experiments were performed with serotype 19F strain ATCC6319, unless specified otherwise.

Mock saliva experiments

Saliva samples were collected from nine healthy volunteer donors aged 21-49 years, 8 female and 1 male. Individuals were asked to spit saliva into a 50 ml polypropylene tube (Sarstedt), which was kept on ice. Samples were vortexed vigorously for 10 seconds and 100 ul of un-spiked saliva stored for determination of baseline presence of *S. pneumoniae*. Remaining saliva was spiked with *S. pneumoniae* to a final concentration of approximately 10^6 CFU/ml and vortexed again.

Dried saliva spots

Immediately after spiking, 100 μl of saliva was used to inoculate the spot on a diagnostic filter paper card (Whatman 903 Protein Saver Card, VWR International) and dried at room temperature (RT) for 2 hours; day zero samples were processed for *S. pneumoniae* detection when spots were fully dry. Unless specified otherwise, all DSS were stored sealed in a plastic zipper bag with a Minipax absorbent packet (Sigma-Aldrich) as desiccant, and placed in darkness to limit light exposure. DSS were processed in duplicate whenever possible. Samples were stored at the following temperatures: minus 20°C, 4°C, ~19°C (room temperature, RT), 30°C, and 37°C.

Clinical study

A small selection of samples (N = 12) were used from a study already conducted. Saliva was collected from healthy children aged 4-10 years; children were asked to spit saliva into a 15 ml polypropylene tube (Sarstedt) and samples were in parallel snap frozen and processed through DSS as described above. One DSS sample at day zero was lost due to technical difficulties. Description of the study: the study was conducted on a single day in June 2012 at a rural school of 190 students, in the Utrecht province. Students attending two different classes took part in the study. No demographic data were collected. Study was conducted in line with Ethical Committee Guidelines. Informed consent was obtained from the parents of each child.

Table 1: *Streptococcus pneumoniae* strains used in this study

Strain	Serotype	Description	Reference or source
GA07694	1	Invasive clinical isolate	(29)
GA03901	2	Invasive clinical isolate	(30)
GA07650	3	Invasive clinical isolate	(30)
TIGR4	4	Invasive clinical isolate	(31)
603	6B	Invasive clinical isolate	(32)
603J	NT	Acapsular variant	(29)
1101	11A	Invasive clinical isolate	(33)
SJD86	19A	Invasive clinical isolate	(34)
ATCC 6319	19F		Gift from D. Diavatopoulous

Isolation of bacterial DNA

DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen). DSS were cut out of the card and supplemented with 180 µl of 20mM Tris-Cl, 2 mM EDTA; incubated for 15 minutes at 95°C to inactivate DNAses; supplemented with equal volume (180 µl) of 2.4% Triton X-100, 80 mg/ml lysozyme in 20 mM Tris-Cl, 2mM EDTA, vortexed and incubated at 37°C for 30 minutes. The liquid phase was separated from the filter paper by pipetting, mixed with ethanol and processed according to the kit's original protocol. DNA was eluted with 200 µl of elution buffer and stored at +4°C.

Real-time quantitative PCR targeting *S. pneumoniae*

Detection of *S. pneumonia*e-specific DNA was conducted by real-time quantitative PCR (qPCR) using primers and probes specific for the gene coding for the major *S. pneumoniae* autolysin LytA (23) and for the iron uptake ABC transporter lipoprotein PiaA (13). Genomic DNA of *S. pneumonia*e 19F was used as a positive control in qPCR. and unless stated otherwise, 2.5 µl of DNA template was tested in 25 µl PCR volume. It corresponded to approximately 1.25×10^3 CFU of the artificially spiked saliva samples. Clinical samples were classified as positive for *S. pneumoniae* when C_T values for both targeted genes were below 45 (24). Serotype specific genes were quantified using qPCR protocol published by Azzari *et al*. (14).

Lower limit of *S. pneumoniae* detection

We spiked saliva with a range of tenfold PBS dilutions of pneumococcal cells from 10^5 to 100 CFUs per 100 µl volume of saliva and quantified *S. pneumoniae* presence in DNA extracted from DSS inoculated with these samples. Cell suspensions in PBS containing corresponding numbers of *S. pneumoniae* CFUs were processed as reference curve.

Statistical analysis

Results were analyzed using GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Due to small sample sizes we assumed the data was normally distributed and used the students t-test. For multiple comparisons either one- or two-way ANOVA with Bonferroni post-tests were used as indicated.

Results

Optimization of dried saliva spots

To examine the efficiency of recovery of *S. pneumoniae*-specific DNA from saliva samples collected with DSS, we compared raw saliva to DSS. Individual samples from five donors were used to examine the effect of drying saliva on filter paper on recovery of pneumococcal DNA. We observed no significant difference in quantity of the *S. pneumoniae*-specific gene *lytA* detected by qPCR in DSS versus fresh samples spiked with live *S. pneumoniae* (p=0.450) and for un-spiked samples (p=0.753) (Figure 1).

We studied the effect of humidity on sample quality since it had been reported for dried blood spots that storing with desiccant improves DNA stability over time (25, 26). Indeed we observed a clear benefit of storing DSS in the presence of desiccant with an increase of 2-3 C_T values of *S. pneumoniae*-specific signal at day 7 in samples stored with compared to without desiccant (data not shown); therefore we continued all further experiments with desiccant. We also tested survival of *S. pneumoniae* in DSS and found no live pneumococci recovered from freshly dried DSS, with few oral commensals surviving (~10 CFU). This killing effect was equally present when spots were inoculated with cells suspended in PBS but not when desiccated on plastic Petri dishes, indicating a strong bactericidal effect of the filter paper itself.

In consideration of DSS being an un-explored method for pneumococcal detection, we assessed the lower limit of *S. pneumoniae* detection in DSS. We first tested the sensitivity

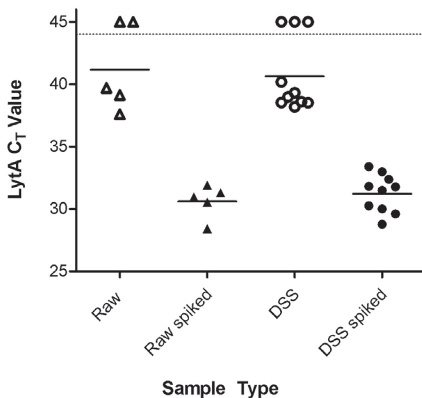

***Figure 1*: *S. pneumoniae* detection in raw saliva versus DSS**

Saliva samples from 5 donors were spiked with a serotype 19F strain of *S. pneumoniae*. Both spiked and un-spiked raw saliva and DSS specimens were processed for DNA isolation and qPCR-based pathogen detection. Each dot represents an individual sample (DSS were performed in duplicate). The dotted line marks the lower limit of detection of qPCR. Differences between un-spiked raw and DSS specimens as well as spiked raw and DSS specimens samples are not significant.

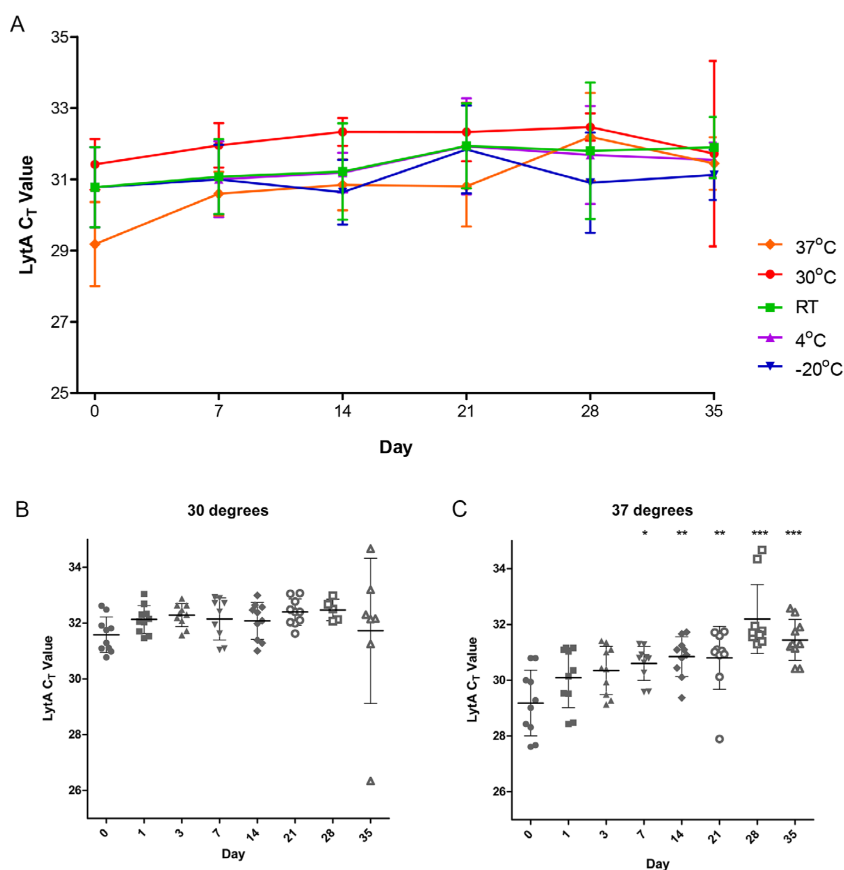

***Figure 2:* Pneumococcal signal in DSS is stable over long periods of time**
Mock DSS specimens from saliva (N=5 donors per experiment) spiked with *S. pneumoniae* serotype 19F. A. DNA was isolated immediately after drying (day 0) and after storing the DSS at the indicated temperatures for up to 35 days. Shown are the mean and SD of all C_T values. For 30°C, room temperature, 4°C, or minus 20°C, no significant differences were found for C_T values of DSS stored over time compared to C_T values at day zero. B. Graph of individual DSS stored at 30°C; C. Graph of individual DSS stored at 37°C for up to 35 days: a gradual loss of signal was observed at 37°C but not at 30°C (1-way ANOVA with Bonferroni post-test * $p<0.05$, ** $p<0.001$, *** $p<0.0001$).

of the molecular method by quantifying *lytA* presence in DNA extracted from a serial dilution of bacteria in PBS. In the absence of saliva components that could potentially interfere with pathogen detection, the qPCR method itself showed reproducible quantification in samples containing the equivalent of approximately 10 CFU per qPCR reaction (data not shown). Then we tested the DSS method, spiking the saliva with serially diluted pneumococci. The results for DSS were also highly reproducible and precise for samples with $\geq 10^2$ CFU per qPCR reaction, equivalent to approximately 10^4 CFU per spot.

Temperature stability

DSS specimens inoculated with saliva of five individual donors spiked with pneumococcal cells were stored for up to five weeks at five different temperature conditions ranging from -20ºC to 37ºC and tested weekly for pneumococcal-specific DNA by qPCR. Whenever possible, duplicates of DSS were tested throughout the course of time. Although we observed variation in the signal strength over time, there was no evidence of a significant decline of pneumococcal-specific signal in DSS specimens stored for up to one month at temperatures ≤30ºC (Figure 2A and 2B) or stored for up to 7 days at 37ºC (Figure 2C). The effect was similar for samples of saliva collected from various donors in the study.

Short-term DNA stability of DSS of different strains of *S. pneumoniae*

A previous study has shown that *S. pneumoniae* tolerance to desiccation is not strain specific, although some clinical strains were more robust than others (27). We wondered if the same was true of pneumococcal DNA in DSS. To study this, we tested eight clinical isolates, in addition to serotype 19F, of serotypes 1, 2, 3, 4, 6B, 11A, 19A, and an acapsular strain constructed in the lab (Table 1). Aliquots of saliva samples collected from three donors were individually spiked with cells of pneumococcal strains, spotted onto filter paper, and stored. Overall, pneumococcal DNA was stable for up to 7 days in all strains tested (Figure 3). In concordance with the above described results for serotype 19F, the qPCR C_T values were robust and showed only minimal variation over time, and we observed slight inter-strain variation in the quantity of *S. pneumoniae*-specific

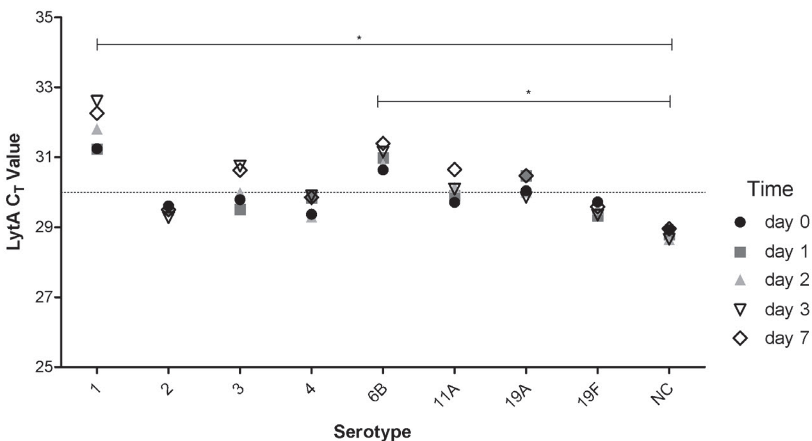

Figure 3: **Effect of saliva on various strains**
Saliva of 1 donor was aliquoted and spiked with different serotype strains, after which DSS were generated. DSS were analyzed for pneumococcal-specific signal at day 0, 1, 2, 3, and 7. Dots are the mean C_T of 3 experiments (different donor each experiment). DSS specimens were kept at RT for the duration of the experiment. NC: no capsule, acapsular variant of 6B. Stable C_T values are observed for each individual serotype/strain. Comparing all serotypes, only two are significantly different: serotype 1 compared to NC and serotype 6B compared to NC (2-way ANOVA, Bonferroni post-test $p<0.05$).

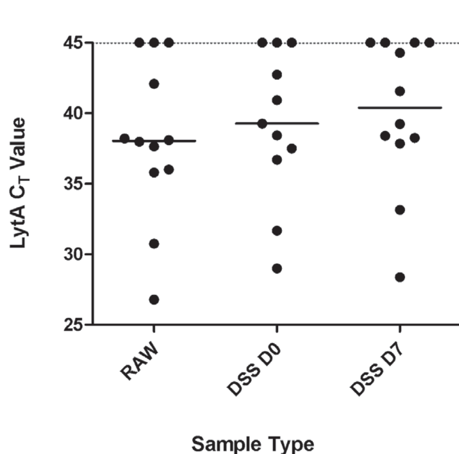

Figure 4: **Detection of pneumococci in DSS of clinical samples**
Saliva was collected from 12 school children (ages 4-10 years) and either frozen or processed for DSS immediately. DNA isolation was performed from raw saliva and DSS specimens from the same donors simultaneously. Samples are represented by dots with the median Ct value depicted per condition. Pneumococcal-specific qPCR identified 8 out of 12 children positive in both raw saliva and DSS at day zero. At 7 days at RT 7 out of 12 children were positive for pneumococcus-specific signal, so there was a loss of 1 positive individual.

signal detected at time zero. When comparing all strains, results for serotypes 1 and 6B were significantly different compared to the acapsular strain with mean 2.3 C_T and 1.7 C_T difference respectively (Two-way ANOVA with Bonferroni post-test, $p<0.05$), despite matching number of cells used to spike samples. It suggested to us variation in strains' sensitivity to lysis on contact with human saliva. Each sample was also run in the corresponding serotype- and *piaA*-specific qPCRs. These signals were equally robust as the signal for the *lytA* gene (data not shown), indicating that the pneumococcal DNA in DSS will be suitable for molecular determination of the serotype composition of the sample as well.

Detection of pneumococcal presence in DSS specimens from clinical saliva samples

In order to test the DSS method in a clinical setting, we tested saliva samples collected from 12 school children. DNA was extracted from raw saliva and matching DSS which were stored for up to 7 days. When we used our strict criteria to assign positivity to a sample (presence of signals <45 C_T for both *lytA* and *piaA* in qPCR), samples from 8/12 children were positive both in raw saliva and in DSS samples at day zero (Figure 4) and from 7/12 children at day 7. In general we observed a minimal decline in the signal detected in DSS compared to raw saliva samples (mean decline 0.88 C_T, range 0.22-2.9 C_T). Additionally we tested the DSS and raw clinical samples for the presence of pneumococcal serotypes using serotype-specific qPCRs (data not shown). Differences in the C_T values in serotype-specific qPCR for raw saliva compared to DSS were not significant.

Discussion

Our interest in saliva as a diagnostic specimen to study pneumococcal carriage was triggered by historical records of high carriage rates detected in the saliva of asymptomatic individuals tested with a sensitive mouse inoculation method. It was further strengthened by the outcome of our recent study on pneumococcal carriage (13), where we found trans-oral sampling to be superior to trans-nasal nasopharyngeal sampling for *S. pneumoniae* molecular detection in adults. Furthermore, two studies recently used dried spots for molecular detection of pneumococci in cerebral spinal fluid (22, 28). We hypothesized that the dried saliva spot (DSS) method could also be utilized for molecular detection of *S. pneumoniae* in highly polymicrobial saliva samples in order to simplify sampling methodologies for studies on pneumococcal carriage. To our knowledge, this is the first attempt to explore the possibility of using DSS as a diagnostic tool for *S. pneumoniae* carriage detection.

We found that despite processing through filter paper and desiccation, pneumococcal DNA was stable in DSS stored for up to one month and over a broad range of temperatures. This is in line with results reported by Peltola *et al.* on pneumococcal DNA being detectable in cerebrospinal fluid from patients diagnosed with meningitis applied to dried spots and stored at room temperature for up to 8 months (22). The robustness of the DSS method generates the opportunity for un-assisted sample collection. Saliva can be applied to DSS by individuals at home or in remote study centers and sent via regular mail to the laboratorial facility for processing. This would be of particular advantage in surveillance studies on pneumococcal carriage conducted in resource poor countries or remote areas.

Interestingly, at day zero we already saw limited but significant inter-individual variation in the quantity of *S. pneumoniae*-specific signal detected in saliva samples spiked with an equal number of pneumococcal cells. Given that the C_T value obtained for raw spiked saliva and for fresh or stored DSS was nearly constant for any one individual tested suggests our observation is not the effect of drying, handling, or storing the saliva but the intrinsic variability in saliva composition between individuals that accounts for the variation of C_T values obtained from different donors. This may be due to differences in composition of saliva itself (e.g. bactericidal molecules and enzymes produced by human), the competition among microorganisms present in the oral cavity and/or mediated by bacterial and fungal products and bacteriophages.

We also observed slight variation in quantity of *S. pneumoniae*–specific signal detected by qPCR in saliva from single donors spiked with different pneumococcal strains. It suggests to us the presence of inter-strain variation in sensitivity to bactericidal effects of saliva. We believe these limits can be easily addressed within the method. Since each qPCR reaction used only a fraction of the isolated DNA, increasing template volume or

concentrating the template could further increase the sensitivity of the DSS method, or any molecular based-method. Since saliva can be easily collected from a donor, sample volume shouldn't be an important limiting factor.

The limitation of this study is the relative small sample size of saliva samples tested in the clinical part of the study. Another limitation is the inability to culture live pneumococci from the filter paper, which does not seem possible due to the apparent bactericidal effect of the paper used. Therefore, the DSS should be considered useful only for studies not requiring isolation of live *S. pneumoniae* as the primary endpoint.

Further studies are needed to determine the diagnostic value of DSS compared to the gold standards, i.e. conventional culture or molecular detection of pneumococci in nasopharyngeal swabs. Since this is the first description of this methodology, further validation on larger data sets, plus side-by-side comparison with detection of pneumococcal carriage in other types of respiratory samples (nasal, nasopharyngeal or oropharynegeal swabs, nasal washes) will be necessary. Future research should aim to gain insight whether DSS has the potential to be used as a molecular quantitative method. For this, testing DSS samples from patients with pneumococcal disease or at risk of pneumococcal infection would be particularly informative. For use of this method in young or disabled individuals, we suggest to use diagnostic kits designed for saliva collection before applying saliva on the filter paper. Further studies are needed to validate this additional step to the protocol.

In conclusion, DSS is an easy, novel, and robust saliva sampling method that shows promises as a tool for pneumococcal surveillances in remote areas and resource-poor countries. For example it could be performed at home by study participants themselves and sent by mail to the nearest reference laboratory. Pneumococcal DNA is stable in DSS stored with desiccant in a wide range of temperatures for up to one week. Furthermore, long-term storage of DSS is possible at -70 and consequently it has great potential for diagnostic purposes. Therefore, DSS may be considered as an attractive alternative to nasopharyngeal or oropharyngeal swab samples in surveillance studies on pneumococcal carriage when molecular, culture-free methods are used for *S. pneumoniae* detection.

Acknowledgements

We thank the children and adult volunteer donors for saliva samples used and tested in the study, and the members of the Respiratory Infections Group at the UMC Utrecht for helpful comments and suggestions during the course of this work. The study was supported by internal funds from Wilhelmina Children's Hospital of the University Medical Centre Utrecht.

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Chapter Seven

Summary

The focus of this thesis was to investigate various aspects of pneumococcal – host interactions in the respiratory tract of the elderly. Furthermore, we aimed to address the paucity of information regarding the underlying mechanisms of disease in this high risk group. Since *Streptococcus pneumoniae* is a frequent asymptomatic colonizer of the human upper respiratory tract, which is a prerequisite to disease, we approached this common medical problem through studies of colonization of the URT. In this thesis we present data from different angels, i.e. using *in vitro* experiments, mouse models of pneumococcal colonization in elderly mice, and human observational studies of colonization in the elderly.

In **chapter 2** we reviewed what is currently known about mucosal respiratory immunosenescence and how this impacts host-pathogen interactions with *S. pneumoniae.* We describe a general lack of research focused on innate mucosal immunosenescence and recommend future research to focus in this area. Then we address the discrepancy between the observed low carriage prevalence yet high invasive disease rates in elderly. We provide evidence from early literature for the hypothesis that pneumococcal carriage in the elderly is under-estimated due to sampling bias and low sensitivity of conventional culture of swabs in adults and elderly. We conclude with updated recommendations for sampling in future elderly carriage surveillance studies; molecular, DNA-based detection methods of pneumococci using saliva samples should be strongly considered.

In **chapter 3** we use an elderly mouse model of pneumococcal colonization to address our hypothesis that immunosenescence leads to aberrant innate mucosal immune responses, especially cellular responses, in the upper respiratory tract of elderly mice, with a direct impact on clearance of pneumococcal colonization. We show that already at baseline, elderly mice have increased expression of pro-inflammatory mediators in the local lymph nodes, with increased expression of tolerance mediators in the respiratory epithelium. We also show that pneumococcal colonization is prolonged in elderly mice and that this is correlated with absent and delayed response by the innate immune system. The inflammation and dysregulated homeostasis we found in the elderly mice also correlated with an altered upper respiratory tract microbiota profile in elderly as compared to young-adult mice.

In **chapter 4** we describe the overall bacterial microbiota composition in relation to acquisition and clearance of *S. pneumoniae* in young-adult and elderly mice. Introduction of *S. pneumoniae* into the niche caused a temporary dysbiosis, in particular a significant loss of bacterial diversity, richness, and evenness in both age groups. An important group of anaerobes, i.e. Bacteroidetes, specifically the Porphyromonadaceae family, was present in higher abundance in young compared to elderly mice, and was the first commensal to re-establish the niche after pneumococcal clearance. Several genera of bacteria were enriched in the altered niche after pneumococcal clearance in both age groups, including *Staphylococcus*.

In **chapter 5**, we further study our hypotheses that 1. pneumococcal colonization rates in elderly are higher than assumed, and 2. the low sensitivity of carriage detection by conventional culture can be addressed by testing saliva samples from elderly adults with molecular methods. To study these, we performed a cross-sectional observational study in the elderly human population. We found a significantly higher prevalence of pneumococcal carriage in elderly when molecular detection is used instead of conventional culture. Furthermore, we found a significantly higher prevalence of pneumococcal carriage in saliva samples compared to nasopharyngeal swabs. The point prevalence of over 35% carriage in the elderly is more than 6 times higher than the general rates reported for conventional culture. The period prevalence of 50% that we report is even in line with historical rates found in saliva.

In **chapter 6**, the interest in the use of saliva as a diagnostic or surveillance tool prompted optimization experiments, since the current recommendation of snap freezing of saliva upon collection is financially and logistically difficult to apply in large surveillance studies in the field. We tested the feasibility of using dried saliva spots (DSS) instead of raw saliva for molecular detection of pneumococcal carriage. We found that detection of pneumococcal DNA is equivalent in raw saliva compared to DSS for both mock spiked samples and clinical samples. Furthermore, pneumococcal DNA in DSS is stable at a variety of temperatures ≤30°C for up to 35 days, and for up to 7 days at 37°C. Furthermore, the stability is not greatly affected by the pneumococcal strain.

The major conclusions of this thesis are that ageing and immunosenescence seem to contribute significantly to an increased and prolonged colonization of the upper respiratory tract in elderly mouse models. Furthermore, in line with these findings, carriage in the elderly population is largely underestimated by the currently applied conventional culture methods of oro- and nasopharyngeal swabs, and is similar to the period prevalence of 40-65% already reported in historical data from early 20th century. This indicates that the pneumococcus is still largely a commensal even in elderly humans. With high rates of pneumococcal carriage in the oral cavity in the elderly, research should focus on reasons why the elderly as a group are highly susceptible to developing pneumococcal pneumonia and what makes an individual at high risk of developing consecutive disease. Microbiota studies of the upper respiratory tract, for example, may reveal important factors for microbial dysbiosis in the elderly, which may be relevant for development of new alternative preventive and therapeutic tools in the future. Better understanding of the process of immunosenescence at the upper respiratory tract and reasons behind disease development is essential for contributing to healthy ageing of a rapidly growing elderly population worldwide.

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Chapter Eight

General Discussion

The focus of this thesis stems from a major public health concern of today: the ongoing burden of community acquired pneumonia in the growing population of elderly (1, 2). Acute respiratory infections (ARIs) are one of the most diverse and deadly infections known to man. Often the exact etiology of ARIs is difficult to assess, but despite this, *Streptococcus pneumoniae* is considered one of the major causes of community acquired pneumonia at all age groups causing the highest burden of ARIs globally (3). There are three major risk groups for developing pneumococcal pneumonia: the very young, those suffering from immunodeficiencies and particular comorbidities, and the elderly. This thesis focuses on the elderly.

We studied in elderly several factors potentially involved in the increased susceptibility to pneumococcal disease: the role of host immunity, the pathogen reservoir in the ageing human host, and the role of commensals or microbiota in the upper respiratory tract.

Colonization in elderly – overlooked contribution to disease

Although colonization is a known prerequisite for disease (4, 5), the role of colonization in pneumococcal disease has been questioned in the elderly due to low carriage rates observed in carriage studies of the last few decades (6). In this thesis we present evidence to support the hypothesis that pneumococcal carriage in the elderly is underestimated and that sampling and technical bias may explain this. After nearly half a century of epidemiological research on pneumococcal carriage during the early 20th century, the consensus of scientists was that at least 40-60% of all adults were carriers of pneumococci at any given moment (7). A great deal of research had established that pneumococci were difficult to detect in human samples due to the polymicrobial nature and therefore it was recommended that multiple samples be taken at once, or over a period of time in order to increase sensitivity of pathogen detection. Furthermore it was known that saliva was the most sensitive sample when compared to either transorally or transnasally obtained nasopharyngeal swabs (7, 8).

The study we present in chapter 5 was designed to address this potential sampling bias in the elderly. Swabs and saliva were taken at two time points from elderly participants. Using a molecular, DNA-based pathogen detection method (qPCR) we determined that in our sample set this method was superior to conventional culture. Furthermore, saliva samples were superior to either swabbing method. The average point prevalence of colonization in this study was 37% and the period prevalence was 50%. The point prevalence was over six times larger than what is generally reported for carriage prevalence in the elderly when using conventional culture and swabs (9-12); whereas the period prevalence was similar to rates reported in the early 20th century when saliva injected into mice was used for surveillance (7). Based on recent culture data it is assumed that the elderly are rarely colonized in the URT with potential pathogens. It seems unjustified though to generalize data generated on nasopharyngeal samples alone to the entire URT. Indeed,

as our study demonstrates, the use of swabs greatly underestimates pneumococcal carriage, with the bulk of carriage in the elderly occurring in the oropharynx. Studies on aspiration pneumonia also report that increased oral hygiene aiming to reduce bacterial density lowers the risk of aspiration pneumonia in the elderly (2, 13, 14). In this context, the findings of our study seem to be in line with these reports and in contradiction to most surveillance studies that focus exclusively on pneumococcal nasopharyngeal carriage. The period prevalence of 50% in elderly indicates not only that pneumococcal colonization goes largely undetected in elderly, but also that pneumococcus may still be a common commensal not only in very young children but in human elderly as well.

Duration of pneumococcal colonization in mice and men

In our mouse model of pneumococcal colonization, we found that pneumococcal colonization duration was significantly prolonged in elderly mice compared to young-adult mice (chapter 3). No differences were found between the initial densities of pneumococcal colonization amongst the age groups. Although clearance, even in young mice, is a heterogeneous process, most young-adult mice were significantly less densely colonized by *S. pneumoniae* by 7 days post-inoculation, and fully cleared the carriage of this pathobiont by 21 days. Elderly mice, however, were delayed in this process and were still colonized at 28 days post inoculation. Future studies should also look into inter-strain -serotype variation in these type of studies as well as into duration of carriage in naïve mice, in mice earlier in life exposed to live *S. pneumoniae* in URT or when immunized with pneumococcal antigens.

Carriage duration is thought to be longest and highest in young children and to decline with age due to immune maturation (15). Immunosenescence and carriage duration has not been studied before. Our observational study in human elderly presented in this thesis, provides evidence not only for much higher pneumococcal colonization prevalence in the elderly than recently reported but also prolonged carriage. Having two sampling moments 6-8 weeks apart gave us the opportunity to look in to long-term colonization in the elderly. We observed that a large sub-population (35 persons or 24%) had a pneumococcal specific signal in their samples at both time points. With carriage prevalence at the first sampling moment of 38% and 36% at the second sampling moment, chance alone dictates that the probability of a person being double positive is 13.7%. The observed 24% is therefore significantly higher than expected by chance alone (Fisher's exact p=0.02). Furthermore, preliminary results of sample serotype composition determined with molecular method indicate that when a serotype could be confirmed, samples from both time-points were positive for the same serotype. Although this is a first small study, and these results need to be confirmed, it is potentially very interesting since this subset of elderly match our observations on long carriage duration from the mouse model.

Few contemporary studies have performed longitudinal surveillance in children (15-18), fewer still looked at parents or adults (17), and studies in elderly are scarce. In children, the duration of carriage found using swabs and conventional culture seems highly variable depending on the serotype. Most studies report an average duration of carriage in very young, under 4 years of age, of 50-60 days, with the longest carriage of some serotypes, notably 19A and 6B, lasting for an average of 100 days (16-18). Duration of nasopharyngeal carriage in adults is reported to be much shorter, with an average of 30 days (17). In historical studies sampling more frequently (1-2 times per week) and using oropharyngeal swabs, duration of carriage is already reported to be higher in adults, with averages of 10-12 weeks (19, 20); a subset of adults (21%) were identified as chronic carriers carrying the same serotype for 3 months to 3 years. In studies using saliva, carriage duration in adults was reported to vary from 1-2 months for some carriers to 3-6 months for others (21, 22). It is uncertain to what extent the elderly are comparable to younger adults, therefore longitudinal sampling of saliva of adults of different ages should be undertaken to determine if the duration of carriage is indeed longer in the elderly.

Host-pathogen interactions in elderly

It is widely debated if the true origin of immunosenescence is genetic decline, physiological decline, a consequence of infections with pathogens throughout life, a result of microbiota imbalance, or a some combination therein (23-25). In order to better understand the interaction between immunosenescence and *S. pneumoniae*, we again used the elderly mouse model of pneumococcal colonization described before. At the mucosal surfaces there is presumed to be a fine balance between tolerance to commensals (including dampening of inflammation to decrease epithelial damage) and a swift pro-inflammatory response to pathogens (26, 27). In our elderly mice we observed an imbalance in this homeostasis in the URT of elderly mice as compared to younger adult mice. We found evidence of baseline inflammation in the nasal-associated lymphoid tissue (NALT) of mice, with significantly higher expression of TLR1, NLRp3, and IL-1β in elderly mice than in young-adult mice. We also saw a strong trend of higher expression of CCL2 in elderly mice. Furthermore, various key innate genes failed to be upregulated upon pneumococcal colonization in elderly mice, whereas young-adult mice significantly upregulated TLR1, TLR2, NOD2, NLRp3, and IL-1β very early in colonization (3-7 days post-inoculation). Elderly mice however, only upregulated NOD2 and that was at 14 days post-inoculation. This suggests that the pre-existing inflammation in elderly mice was masking some of the normal danger signals that would have been perceived by younger mice. Furthermore, when we focused on the URT epithelium of the elderly mice, we found a significant over-expression of a negative regulator, PPARγ, at baseline and throughout the course of colonization. A downstream product that would be affected by this negative regulation is the mouse cathelicidin CRAMP, which was indeed significantly reduced in elderly compared to young mice. Furthermore, elderly

macrophages exhibited defects in killing pneumococci and in producing CRAMP in response to stimulation with pneumococcal antigens. This lack in response in the elderly correlated with the significantly longer colonization.

Dysregulation of TLRs has been found in both elderly mice as well as elderly humans (28) at various sites and in response to a range of stimuli. Furthermore, dysfunction of TLRs 1 and 2 was related to increased pneumococcal pneumonia severity in elderly mice (29). However, very little is known regarding the functioning of intracellular PRRs such as NOD and NOD-like receptors in the elderly with respect to recognition of Gram-positive bacteria (30). Baseline inflammation has also been found in various sites of both elderly humans and elderly mice (30), and it has been hypothesized that this inflammation in part hampers responses to PAMPs and danger signals (31). Our data is in line with these observations, showing that these deficits are also occurring in the URT. Furthermore, we show an imbalance in URT homeostasis with increased expression of genes involved in mucosal tolerance. Future work could focus on this imbalance in URT homeostasis to determine to what extent this is increasing pneumococcal colonization duration in the elderly.

The combined observation of higher inflammation in the local URT lymph nodes with higher tolerance in the URT epithelium present in elderly mice caused us to question if the microbiota might be altered as a result or as a cause for this imbalance in immunosenescence.

Microbiota of the URT

In this thesis we present data from the first culture-independent survey of microbiota in the URT of mice. Since this is the first report on what is present in the niche, we cannot be certain that what we see is the core mouse URT microbiota; additional work with different strains of mice from different breeders will be necessary to determine this. However, the bacterial communities we observed in this set of mice are interesting, and may relate to immunological observations from these mice, as discussed above.

Naïve young-adult mice had higher abundance of normal commensals (i.e. Bacteroidetes and Firmicutes), whereas elderly mice in comparison had more aerobic bacteria and potentially pro-inflammatory bacteria. In addition to significantly higher Porphyromonadaceae presence, we also found in URT samples from young mice a trend towards more *Lactobacillus* reported to improve mucosal epithelial barrier function against pathogen entry in mice (32) and to stimulate beneficial immune responses reducing damage from pneumococcal pneumonia in mice (33). Also in higher abundance in young-adult mice than elderly mice were Clostridiales which were described to induce a regulatory T-cell response protective against inflammation in mice (34) and streptococci which have been shown to promote URT homeostasis (35). In comparison, elderly

mice had higher abundance of Betaproteobacteria, *Corynebacterium*, and *Staphylococcus*. Higher abundance of Proteobacteria in relation to Bacteroidetes is associated with the inflammation seen in Crohn's disease (26). Many species of the Gram-positive *Staphylococcus* are potential pathogens (36) that can either have immuno-stimulatory or have a pro-inflammatory effect via their lipoteichoic acid (37). This may result in the high expression of PRR and genes related to responses to Gram-positives already at baseline that we observed. In addition, certain species of *Corynebacterium* (38, 39) and *Staphylococcus* (39) have been indicated to contribute to dysbiosis of the URT leading to chronic rhinosinusitis. Also, a shift from Lactobacillus to Corynebacterium in mouse URT has been associated with decreased caspase signaling in response to respiratory viruses (40). However, significance of these interactions for dynamics within URT mucosa of the imunosenescencent host still needs to be determined.

Furthermore, there was still evidence of dysbiosis in the microbiota profiles of the elderly mice after pneumococcal carriage clearance indicating that there are certain pathobionts, in particular staphylococci, able to grow out and dominate the niche. This is in line with previous epidemiological findings suggesting interspecies interctions between *Streptococcus* (*S. pneumoniae*) and *Staphylococcus* (*S. aureus*) (41, 42). This was also observed in children, where a temporary increase in *S. aureus* colonization and disease was observed in children vaccinated with a pneumococcal conjugate vaccine versus non-immunized children, both in randomized controlled trial settings as well as in surveillance studies in the population (43, 44). If our observations hold that the URT of elderly contains less commensal anaerobes and more inflammatory pathobionts, using pre- or pro-biotics to selectively promote commensals could be considered as preventive or therapeutic option in lowering burden of pneumococcal disease in the elderly.

Distinct niches of URT and dysbiosis

Two key observations gathered in the course of this thesis may seem to be at odds, but they are not mutually exclusive. First, is the observation that introduction of *S. pneumoniae* into the nasopharynx of mice leads to dysbiosis. Second, is the observation of high pneumococcal carriage prevalence in the saliva of elderly, and our hypothesis that the pneumococcus is more of a commensal existing asymptomatically in a large proportion of the population. There are several key points to consider however. The mice we inoculated were naïve, and had never encountered pneumococci before. And although they have a commensal microbiota, they were kept pathogen free and thus their immune system may not be representative of that of humans. Also, these mice never appeared sick in any way during the study. It is possible that an asymptomatic temporary dysbiosis occurs, and that the niche returns to baseline in time, but our study did not continue long enough to observe this. Another point is that for the mouse microbiota study we used a technique to sample the nasopharynx (45), and the nasopharynx is different in physiology and composition than the oropharynx and oral cavity and this may even

change with age and altering anatomy. Saliva contains many antibacterial proteins and therefore the pneumococcus and the oral cavity may come to a different homeostasis than the upper regions of the URT. Since other streptococcal species that are closely related to pneumococci have evolved to live in the oral cavity of humans, it is possible that so have pneumococci and that the oral cavity is the main niche and pneumococci spread into the nasopharynx from there. It may be that initial introduction of *S. pneumoniae* into the nasopharynx causes a dysbiosis there, but not in the entire URT or oral cavity. Future studies on the complex interactions between the URT niches and the microbiota in time could shed more light, especially on dynamics of the reservoir of pathobionts in the elderly.

Pneumococcal detection in polymicrobial niches

It has been touched on several times in this thesis that the upper respiratory tract is a niche containing a dynamic microbiota. While our microbiota are crucial in training our immune system in defense against invasions by pathogens, they are also the source of problems in detecting *S. pneumoniae* carriage. One of the reasons that conventional culture is not a sensitive method in *S. pneumoniae* detection URT swabs or saliva samples is overgrowth of co-colonizing microorganisms. This overgrowth is not inhibited even when media designed to be selective for *S. pneumoniae* are used (gentamicin supplemented plates are the standard selective medium used to detect *S. pneumoniae* culture). Competition among and between bacteria in the niche and during culturing can inhibit growth or cause death of some bacteria, thus skewing culture results. Furthermore, many species of streptococci are human commensals and can be difficult to distinguish from *S. pneumoniae* on an agar plate (46). The density of bacteria in swabs is lower than in saliva, which make live *S. pneumoniae* isolation from swabs easier compared to saliva cultures. Using molecular, DNA-based methods can greatly improve the sensitivity of pneumococcal detection. However, several factors can affect the outcome of molecular detection as well. We observed a significant variation in quantity of pneumococcal DNA detected depending on the method used for DNA extraction with up to 6 C_T difference in quantity of *S. pneumoniae*-specific sequences in samples processed in two different protocols described in this thesis. The method that performed the best in the end was a method used by our group to extract DNA for URT microbiota studies (47) which involved bead-beating the sample in a phenol-based extraction buffer coupled with magnetic bead separation of the DNA (used in chapters 4 and 5). This is the method we recommend for all future molecular based pneumococcal detection studies.

Additionally, we found a difference between culture-enriched (CE) samples and samples minimally processed prior to DNA extraction. The process of culture-enrichment does inhibit some undesirable bacteria from growing while also increasing the growth of *S. pneumoniae* resulting in higher quantities of sequences targeted in the *S. pneumoniae*-specific qPCR. However, we found some samples of saliva qPCR-positive for

S. pneumoniae only in raw and not in culture-enriched samples. Thus, it would be prudent to test both culture-enriched and raw samples when using saliva for pneumococcal carriage detection with molecular methods. Reasons for the discordance between raw and culture-enriched samples were discussed in chapter 5. Another possible explanation might be presence of a residual pneumococcal DNA and not live *S. pneumoniae* cells. Although this is a possibility in raw whole saliva, we have found no evidence to support this. Indeed for the DSS study, over the course of 8 months, we repeatedly sampled the saliva of the members of the group, who have daily contact with both live pneumococcus and pneumococcal DNA, and rarely did we detect any pneumococcus specific signal in these samples before they were spiked. This may be evidence to support our presumption that the pneumococcal DNA we measure in saliva samples is not residual DNA or any kind of DNA contamination from the lab. Despite the minor discordance with raw samples, our group (48) and others have shown that there is a clear benefit in using culture enrichment (49). Culture enrichment and molecular methods are not perfect, but they seem superior to conventional culture for detection of pneumococcal DNA in URT samples.

Alternative sampling methods

The difficulties of using a highly polymicrobial sample such as saliva were discussed above, however, it should be noted that there are many advantages of replacing swabs with saliva in surveillance studies on pneumococcal carriage. First and foremost is the ease in collection. Saliva collection is non-invasive and causes little, if any, discomfort for the sampled person. The second benefit of using saliva is that saliva can also be used for detection of other clinical parameters, in addition to bacteriological diagnostics. It can be used to measure both antibodies and biomarkers (50, 51). However, currently the collection and use of raw saliva requires snap freezing on dry ice at the point of collection (52). This makes saliva collection expensive and impractical for many studies, and altogether impossible for remote locations. We sought to simplify collection and transport of saliva samples to overcome the cold-chain boundary.

We chose to investigate the utility of dried saliva spots (DSS) for several reasons. First, DNA itself has long-term stability dried on filter paper and can be sent in the mail. Second, dried blood spots are a validated diagnostic technique for molecular detection of many diseases (53, 54), and the cards themselves are standardized and easy to use. Third, there are several historical methods of successful drying of pneumococci, borne out of necessity for scientists wanting to keep stocks of pneumococci for indefinite periods of time. The first account came from 1891 where pneumococcal cultures were dried on silk threads (55). Later reports involved dried sputum, dried animal organs, dried blood and the consensus by 1930 was that almost any type of culture of pneumococci could be dehydrated, kept at a variety of temperatures, and rehydrated for use later without much detriment or change in the virulence of the strain as long as the drying

happened rapidly and the sample was kept completely desiccated for the entire duration of storage (56).

Indeed, we found that using dried saliva spots was an easy and robust method for storing and transporting saliva and detecting pneumococcal DNA. Pneumococcal DNA was stable for up to 35 days at a variety of temperatures ≤30°C, and is stable for up to 7 days at 37°C, and the stability does not vary greatly between pneumococcal strains. Furthermore, there was no difference between pneumococcal DNA detection of DSS compared to raw saliva. We conclude that the DSS method is a promising new method for surveillance studies on pneumococcal carriage, however several optimization steps would still be needed before it can be implemented in clinical studies. First, we used the entire circle, or spot, for DNA isolation and preliminary tests using half a circle and accordingly scaling down reagents yielded even higher DNA recovery than our original protocol. Changing the DNA extraction method from the column-based method detailed in chapter 6 to the more efficient heavy bead-beating method detailed in chapters 4 and 5 also increased the yield of DNA by approximately 5 C_T. Future optimization may develop a protocol where only a fraction of the sample is needed, leaving the remainder for other tests.

Outer limits of the DSS method

Initially we chose the Whatman 903 filter paper cards because they were already in use in many diagnostic labs, but also because they are composed of pure cotton fibers with no additives. Our aim was to also be able to isolate live pneumococci from the filter paper specimens. Unfortunately, the filter paper itself appears to be bactericidal, as live cultures were obtained after desiccation of the same material on plastic Petri dishes. For DNA-based diagnostics this poses no problem and actually makes the DSS quite safe to send in the mail. Another advantage of the DSS method is that preliminary testing shows that it is possible to recover pneumococcal specific antibodies from the DSS and measure them in a Luminex based assay. Again, optimization of the method is necessary, but these tests were encouraging. In the future, it may be possible to perform long term longitudinal pneumococcal carriage surveillance anywhere in the world without trained personnel, and also measure antibodies and biomarkers from one single DSS.

Final remarks: Ultimate role of *S. pneumoniae* in ecology of URT

Throughout the course of this thesis the pneumococcus has been referred to as a commensal, potential pathogen, pathobiont, or frequent inhabitant of the URT. In any of these cases, the pneumococcus needs the human host to survive. It has been discussed by others that the virulence factors that aid pathogenesis of pneumococcal disease evolved from a requirement to efficiently colonize humans (57), and as such, pneumococcus is an accidental pathogen. An argument can be made to classify pneumococcus as a pathogen in the elderly assuming that this bacterium is no longer carried. But this appears to not be the case. The more we look for the pneumococcus in the entire URT, and not just focusing on the nasopharynx but also oropharynx and saliva, the higher the carriage prevalence becomes, in all ages. This leads us to the ultimate question, if *S. pneumoniae* is asymptomatically carried by a large majority of people at any given time, is it simply a commensal that has co-evolved with us, or is it instead more of a symbiont? Do humans benefit from pneumococcal carriage? It is too soon for a definitive answer, but deeper knowledge of the host-mirobiota-pathogen interactions so far has revealed several host-benefits from pathobionts (26, 34). For example, helminths, previously considered pathogens are now being investigated in clinical trials for treatment of a variety of inflammatory conditions (58). It has also been observed that with pneumococcal vaccine decreasing vaccine-type colonization in children that *S. aureus* and *H. influenzae* are able to establish in the niche (41, 44) and may even be responsible for increased infection as was seen in older children with recurrent otitis media (43). It is entirely plausible that humans benefit from colonization by pneumococcus and that the interaction is, on the whole, benefitting humans more than harming. Therefore, we should carefully design any new preventive strategies aiming at eradication of disease casued by *S. pneumoniae* and carefully monitor the impact on all upper respiratory microbiota, and disease. Surveillance after pneumococcal vaccine implementation should not be limited to the vaccine target alone, but to the overall benefit in disease including disease caused by other pathobionts.

Finally, immunosenescence studies of the upper respiratory tract and microbiota studies appear relevant for development of new alternative preventive and therapeutic tools in the future. Better understanding of the process of immunosenescence at the upper respiratory tract and reasons behind disease development is essential for contributing to healthy ageing of a rapidly growing elderly population worldwide.

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Nederlandse samenvatting

Dit proefschrift beschrijft verschillende aspecten van de interactie tussen de bacterie '*Streptococcus pneumoniae*' (de pneumokok), een bacterie die veel voorkomt in de neus-keelholte, en meestal zonder ziekte te veroorzaken. Jonge kinderen onder de 5 jaar en ouderen boven de 65 jaar worden wel vaker ziek door pneumokokken met longontsteking en bloedvergiftiging en soms hersenvliesontsteking. Kinderen kunnen ook vaak een oorontsteking krijgen door pneumokokken. Bij kinderen is de afweer tegen pneumokokken nog onrijp maar bij ouderen is minder duidelijk waardoor zij weer zo kwetsbaar worden voor pneumokokken. We hebben ons voor dit onderzoek gericht op de leemte in kennis ten aanzien van mogelijk onderliggende mechanismen van ziekte bij deze risicopopulatie. Aangezien pneumokokken de menselijke luchtwegen frequent koloniseren, hebben we ons primair gericht op het bestuderen van de dynamiek van pneumokokken kolonisatie van de bovenste luchtwegen bij ouderen. We hebben dit bestudeerd middels in vitro experimenten, muismodellen en humane studies.

In **hoofdstuk 2** beschrijven we wat momenteel bekend is over het proces van veroudering van het afweersysteem, ook wel 'immunosenescence' genoemd, in het bijzonder de gevolgen hiervan voor de lokale afweer tegen pneumokokken op de slijmvliezen. We concluderen dat er nog weinig onderzoek is gedaan naar de rol van immunosenescence in pneumokokken dragerschap en ziekte, en dat er duidelijke hiaten zijn in onze kennis hierover. We bevelen daarom aan om aanvullend onderzoek te verrichten naar specifieke aspecten van dit probleem. Tevens bespreken we de discrepantie tussen de zeer lage prevalentie van pneumokokken dragerschap bij ouderen zoals die nu in de literatuur staat, en de hoge incidentie van pneumokokken ziekten zoals longontsteking bij ouderen. Onze hypothese is dat er eigenlijk geen sprake is van een lage prevalentie in dragerschap bij ouderen, maar dat dit dragerschap in onderzoek frequent wordt gemist met de gangbare detectie methoden. We onderbouwen dit onder andere door gegevens uit vooroorlogse literatuur. Met de huidige methoden van kweken van neus- en keeluitstrijkjes wordt de prevalentie in dragerschap bij volwassenen en ouderen waarschijnlijk ernstig wordt onderschat. In de eerste helft van de twintigste eeuw vond men een hoog dragerschap bij volwassenen als met muizen serieel inspoot met speeksel. We besluiten dit hoofdstuk dan ook met een aantal aanbevelingen ten aanzien van toekomstige surveillance studies bij volwassenen en ouderen, en adviseren om moleculaire, op DNA gebaseerde detectiemethoden te gebruiken voor het aantonen van pneumokokken in oropharyngeale of speeksel monsters.

In **hoofdstuk 3** gebruiken we een muismodel om de dynamiek van pneumokokken kolonisatie te bestuderen bij jong- volwassen en oudere muizen. Onze hypothese is dat immunosenescence aanleiding geeft tot verstoorde mucosale immuunreacties, in het bijzonder cellulaire reacties, in de bovenste luchtwegen van oudere muizen, en dat dit resulteert in een verminderde klaring van pneumokokken in deze niche bij ouderen. We zien inderdaad in het algemeen een verhoogde expressie van pro-inflammatoire markers in lymfeklierweefsel in de luchtwegen van oudere muizen, en een verhoogde expressie

van markers betrokken bij tolerantie in het luchtwegepitheel zelf. Tevens tonen we aan dat pneumokokken kolonisatie minder snel wordt geklaard bij oudere muizen en dat dit is geassocieerd met een afwezige of vertraagde reactie van het aangeboren immuunsysteem bij oudere muizen. Deze bij immunosenescence passende bevindingen zijn tevens geassocieerd met een veranderde bacteriële samenstelling (microbioom) van de bovenste luchtwegen bij oudere muizen in vergelijking met jong-volwassen muizen.

In **hoofdstuk 4** beschrijven we de relatie tussen de samenstelling van luchtweg microbiota en de klaring van *S. pneumoniae* in jong volwassen en oudere muizen. Introductie van *S. pneumoniae* in de luchtwegen veroorzaakt een tijdelijke disbalans in microbiota, in het bijzonder een aanzienlijk verlies van bacteriële diversiteit en een verlies van de normale verhoudingen tussen bacteriën bij zowel jong-volwassen als oudere muizen. Ook het herstel loopt anders bij oudere muizen. Een belangrijke groep van anaerobe bacteriën, de Bacteroidetes met in het bijzonder de Porphyromonadaceae familie, was al bij aanvang meer abundant aanwezig bij jonge dan bij oudere muizen, en was de ook eerste commensaal die zich in aantal en abundantie herstelde nadat pneumokokken werden geklaard en sneller bij jonge dan bij ouder muizen. Verschillende bacteriële genera waaronder *Staphylococcus* soorten groeiden bovendien uit na klaring van de pneumokokken.

In **hoofdstuk 5** bestuderen we pneumokokken kolonisatie bij oudere mensen in een cross-sectionele observationele setting, waarbij de gangbare afname en detectie technieken (het kweken van een uitstrijkje van de neus en keel) afzetten tegen moleculaire detectie van pneumokokken in speeksel monsters. We bevestigen de eerdere bevinding dat moleculaire detectie een hogere sensitiviteit heeft vergeleken met die van conventionele kweken. We vonden bovendien een significant hogere prevalentie van pneumokokken in speeksel vergeleken met nasopharyngeale monsters. De door ons waargenomen prevalentie van pneumokokken dragerschap bij ouderen van ruim 35% is meer dan 6 keer hoger dan tot nu toe in de literatuur beschreven prevalenties met conventionele kweekmethoden. De prevalentie aan pneumokokken dragerschap door ons waargenomen is in lijn met de historische data die ook gebaseerd waren op het aantonen van pneumokokken in speeksel van volwassenen.

In **hoofdstuk 6** gaan we verder in op het mogelijk gebruik van speeksel voor het aantonen van pneumokokken dragerschap in grotere studiecohorten. Aangezien de gouden standaard voor het gebruik van speeksel voor diagnostische methoden vereist dat het direct wordt ingevroren, is dit een belangrijke praktische probleem bij het uivoeren van grote epidemiologische veldstudies. We hebben daarom de methode verder uitgewerkt en geanalyseerd of met onze methoden het aantonen van pneumokokken in gedroogd speeksel net zo goed werkt als het aantonen van pneumokokken in ingevroren speeksel. We vonden geen verschil tussen detectie in gedroogde versus ingevroren speeksel. Ook was er geen invloed van het droogproces op het aantonen van pneumokokken in

speeksel dat werd bewaard gedurende 35 dagen bij temperaturen tot 30 °C, en in speeksel bewaard gedurende 7 dagen bij een temperatuur tot 37 °C. Ook had de genetische achtergrond van de gebruikte stammen weinig effect op de stabiliteit van pneumokokken DNA in de gedroogde speeksel monsters .

De belangrijkste conclusies van dit proefschrift zijn dat immunosenescence een mogelijke rol speelt bij vertraagde klaring van kolonisatie van de bovenste luchtwegen met pneumokokken bij ouderen. Bovendien lijkt, in lijn met deze bevindingen, de prevalentie van pneumokokken kolonisatie bij oudere mensen hoger te zijn dan tot op heden aangenomen. Dit betekent dat de pneumokok een belangrijk onderdeel is van de commensale flora bij oudere mensen. Dit is een belangrijk bevinding die als uitgangspunt kan dienen voor nieuw onderzoek naar de exacte oorzaak van de verhoogde gevoeligheid voor longontstekingen bij ouderen in het algemeen, maar ook op individueel nivo. Studies naar de samenstelling van microbiota van de luchtwegen zouden bijvoorbeeld bepaalde bacteriën kunnen identificeren die verhoogde of verlaagde barrière functie van het microbioom kunnen geven. Het identificeren van deze bacteriën kan uiteindelijk leiden tot het ontwikkelen van nieuwe strategieën om longontstekingen te voorkomen of te behandelen. Meer inzicht in het proces van immunosenescence in de bovenste luchtwegen in relatie tot het ontstaan van infecties kan een belangrijke bijdrage leveren aan het proces van gezond ouder worden, wat mogelijk bijdraagt aan een betere kwaliteit van leven voor deze snel groeiende bevolkingsgroep.

10

Acknowledgements

"I get by with a little help from my friends" ☙ The Beatles

Nothing is ever truly accomplished alone, and this book is no exception. To say that this has been a long, strange trip is an understatement, because in reality this trip started long before I arrived in the Netherlands. Many people have helped me along my journey, either directly or indirectly, and now it is my turn to say thanks. If this section of the book sounds less friendly and more stiff than you imagined it would be, it's probably because I find writing so difficult (many of you already know this), but it's certainly not because my gratitude is not from the heart.

First, of course, my supervisors / co-promotors. Debby and Krzysztof, from HSPH to UMCU, through good times and difficult times, you guided me through this PhD-training. I have learned a lot from the both of you, and many things I probably don't even realize I've learned will become clear in the years to come. Debby thank you for giving me the opportunity to do this thesis project. And Debby and Tonny thanks for all the help in the beginning, I was so naïve, I didn't even realize so many little things (like the symbols and words on washing machines) could be so different here in NL. KT thanks for all of the sweet treats from Poland and for taking time in the beginning in the mouse facility. I never knew making mice sneeze could be so fun and rewarding. Debby and KT both of you bring different opinions and insights to the work we did and I hope that I have absorbed some of the creativity and good ideas you've had in the process.

Lieke, my promotor, your encouragement and support in these four years has gone a long way to get me to this place, the finish line. Believing that I could finish in time, despite my slow writing progress, and spending extra time on the manuscripts with me has been a great help. Reminding me to worry less and cultivate outside activities like my art has also had its part in ensuring my success. I enjoyed our meetings not only for the scientific discussions but also because I always left them in a relaxed state - you have a way to calm even the most stressed and worried of PhD students.

Department of Medical Microbiology, the only "home" I ever knew at UMCU (despite being technically part of an entirely different department). Being part of the MMB and yet, not quite, was sometimes confusing at the beginning, but if I had to choose my own department, I would still choose the MMB. So many people from the MMB to thank, so many good times to remember. Koepels, borrells, PhD retreats, ICEA events, Christmas parties, and somewhere in the middle we did some science. Hooray Science! Special thanks to Marc and Jos, not only members of my thesis committee, but also professors of the MMB that encourage the balance of

critical thinking and hard work with borrell-brainstorming and good times. Also thanks to both of you for collaborating with our group by providing lab space and expertise.

I would also like to thank my thesis reading committee, Professors van Dissel, Hoepelman, and Verheij, for taking time out of their summer to assess my thesis.

My "pair of nymphs," Fernanda and Mei Ling: you ladies rock. Fe, we shared so much these past few years, I really am going to miss you and Mauricio. Our woes as North/South American foreigners in a strange land brought us together and our shared interests in beer, good food, rock music, and nerdy hardcore biology cemented our friendship. In our future lives (post-PhD) I am sure all clonings will work, RNA yeilds will be sufficient, and all bacteria will grow to the correct OD; ha, we can dream right? Mei Ling, your addition to our pneumo group brought a much-needed Dutch speaker, but you have been so much more. Thanks for patiently teaching me all about qPCR and for all of the help in the lab in the final stretch. Also thanks for the free ceramic table set, and driving me to IKEA to get a bed. I will miss your humor in the lab and lessons on Dutch sayings – and now the monkey comes out of the sleeve! It is bittersweet that both of you will stand with me at my defense, happy that you are there to share it, sad that I will leave soon after.

Fellow members of the pneumo group, current and past – it has been a pleasure. In the beginning, there were two of us and how our little group has grown! Tomasz, I miss your dark humor, you made the hours in the GDL bearable. Giske, it was so great to share PhD activities with you, and to collaborate on the microbiota work. Many thanks for all your hard work and expertise on the mouse microbiota work. The only thing I wished we had not shared is our 'major publication' sorrows. But at least we can say we learned a lot together these past few years and I cannot wait to see your book when you are done! Wouter and Xinhui thank you both for helpful discussions and advice. To Anne, best of luck on the PhD journey and *"Welcome to the jungle, we've got fun and games."* And to the students Pepijn, Wouter, Lisette, Jody, Lidewij: your enthusiasm for research is inspiring and you brighten the lab with your merriment.

Kirsten, Mireine, and Anna: you were the best students a gal could ask for. Dedicated, willing to learn, and pretty decent pipettors to boot. Kirsten, I enjoyed supervising you for 2 projects, and I'm really glad you took a chance on my 'wacky side-project.' I was honored to participate in the graduations of Mireine and Kirsten, and wish you both all the best. Anna, soon you will finish too and whatever you decide to do after your masters is complete, I know you will be successful. I do have some advice

though: the fastest way to make a sheep costume does not always result in a durable costume.

Colleagues at the Spaarne Hospital, Reinier, Elske, Gerwin, Annemarie, Menno, Judith, Sabine, Astrid, Jacob – our "extended" group is so much richer for having so many talented members. I really enjoyed the meetings and social events in Haarlem, special thanks to Reinier for organizing those. It was also a lot of fun at ISPPD and I was glad to share time in Tel Aviv and Iguaçu with those of you that joined.

Additional Friday morning meeting members Belinda, Susanne, Ewoud, Sander, Patricia, thanks for some great conversations, science and otherwise.

517-AIO-kamer members, Cindy, Axel, Evelien, Fernanda, Ana Maria, Michiel, and eventually Rutger too – *"From there to here, and here to there, funny things are everywhere"* ~ *Dr. Seuss.* You kids make me laugh and cry and laugh again. Science is hard, and a PhD is harder, and shoving 5 people into a tiny room can sometimes lead to grumpiness and toes stepped on. But we managed to not kill each other, and we managed a Lot of hard work in between the fun and games (I know because we ate a Lot of paper-acceptance-appelbollen). Cindy thank you for understanding the pain and suffering of long hours of mouse experiments that fail, and for helping me troubleshoot. And for being a good friend in my early dark years. Axel, Cindy, and Eve thanks for helping me move, twice! As sad as me and Fe were when former office members left, we couldn't have asked for better "replacements" than Ana, Michiel, and Rudy. Somehow the office survived the stress of 2 of us writing and finishing our thesis at the same time, and now Eve, we can sit back and enjoy the rewards for all that work. I will miss our office dinners, field trips, BBQ's, fashion advice, ice cream breaks, and everything in between.

Monday morning / Enterococcal group members: Rob, Willem, Janetta, Maja, Mark, Xinglin, Elena, Miranda, Malbert, Els, Iris, Jan, Marieke, Neeltje and Nina (who joins on Mondays but is more of a *Streptococcus* researcher): thanks for welcoming me to your group and lab space and including me in the journal club and social lunches. The scientific discussions have helped my work to continually improve and the social lunches have helped me to learn about and adapt to Dutch culture.

Other MMB AIOs: Ilse, Dodi, Marieke, Bart, Alex, Bas, Andras, Marjolein, Jori, Guido, Sue, Maaike, Lydia, Daphne, Steven, Evelein B, Claudia, Anna G, Arjan, Manouk, Kirsten, Nienke, Mike, Annemarie – I've enjoyed working with you and getting to know you all, best of luck in all your future endeavors!

Colleagues at RIVM, Josine and Nynke, I enjoyed sharing the exciting data for the ILI project and I look forward to the publication of the results of the study.

I would also like to gratefully acknowledge the companies Yakult, Astellas, GSK, and Pfizer for the financial support for printing this book.

Some important people from my scientific past that have mentored me along the way also need to be mentioned here. Professor John Finnerty – I would not be as good of a scientific public speaker if it was not for my time in your lab. I learned many useful things as an undergrad working with you and Pat Burton, and besides speaking and presentation skills, I think the most important one was that sometimes, science is like watching grass grow. Jyothi Rengarajan and Samantha Sampson – thanks for taking a chance on a freshly graduated bachelor and hiring me for a position that probably required a lot more experience than I had. You both taught me so much about host-pathogen interactions and research in general and I really value your mentorship of me. And of course Professor Barry Bloom, the head of our group who continued to supervise me after Sam and Jyothi left and encouraged me to leave the group and start my PhD.

Andreas Sandgren thanks for the random bits of help – conversations on troubleshooting mouse infections, managing culture shock, attempting to find obscure journal articles, advice on shoes, etc.

Claudette Thompson many, many thanks for urgent library searches from HSPH and sending PDFs of ancient journal articles retrieved from the depths of Countway. And of course Rick Malley and Marc Lipsitch, it was great to see you at ISPPD and thanks for the helpful comments and critiques of my work.

Outside of the lab, dearest Utrecht knitters, Arja, Anna, Brent, Fiona, Jantine, etc. -- life in Utrecht would not have been as enjoyable without you. And yes, despite the difficulties I have had as an expat, there were many enjoyable times. You all were there for me with happy squishy yarn whenever I needed it or had the time for it and for that you are awesome.

To the Lawsons, the family of my "better half," I've really enjoyed getting to know you these past years and you have made Christmas away from home feel more like Christmas at home. That means a lot to this expat.

Mom and dad, the loving parents who never pushed for perfection because I was already pushing enough on my own, you deserve a lot of the credit for this book. Always encouraging me to do what I love, no matter what it was. Probably sometimes you think your weird scientist daughter, soon to be Doctor weird scientist daughter, came from outer space (from the planet of bossy children who argue too much). But really, I get a lot of it from you. Hard work and perseverance, a love of books and learning, a love of nature and history, and a desire to know how and why the world works – all of those I get from you. And the weirdness? Well you did introduce me to Stephen King and Star Wars and Star Trek at a very young age; it's all your fault. Gramma and Grampa, I wish you were still here to see the first doctor in our family get her diploma. And I wish I could thank you for encouraging me to see the world and follow my dreams.

And massive thanks to my lovely boyfriend, Jamie. My anchor in stormy seas. My rock and roll. The Wash to my Zoe. The Kinks said it best, *"The only time I feel all right is by your side."* You have worked so hard to keep me sane, happy, fed, and in clean clothes that this book is as much yours as anyone else's. Everything in my life is so much better with you in it and I can't wait for all of our future travels and adventures.

"And to our health we drank a thousand times, it's time, to ramble on"
Led Zeppelin

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Curriculum vitae

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List of publications

Krone CL, Trzcinski K, Zborowski T, Sanders EAM, Bogaert D. Impaired innate mucosal immunity in aged mice permits prolonged *Streptococcus pneumoniae* colonization. Accepted at **Infection and Immunity**.

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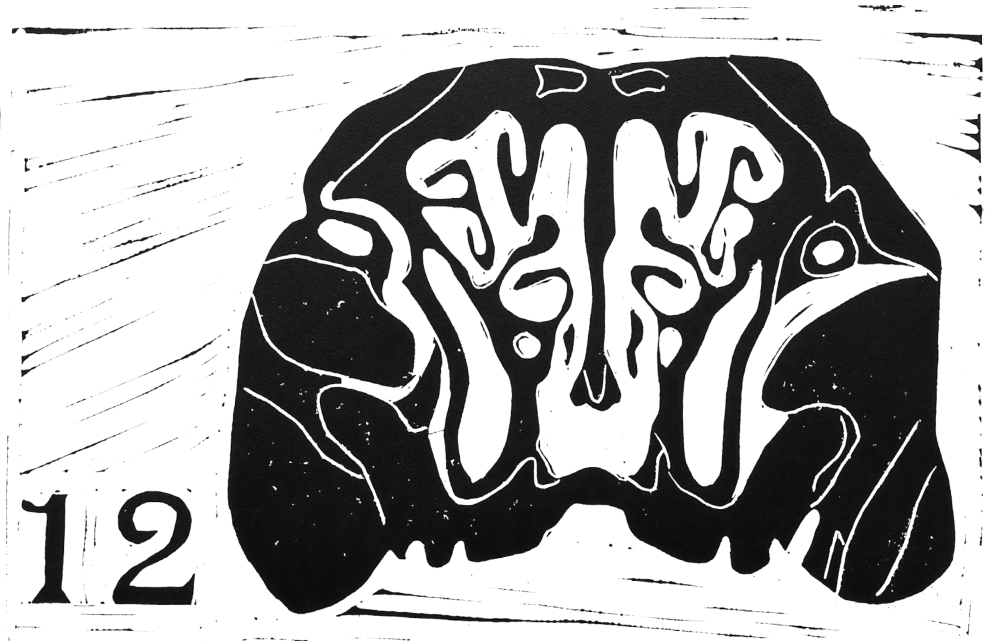
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Biography

Cassandra Lynn Krone was born in LaGrange, Illinois, USA on May 10, 1982. She grew up in Orland Park, IL and graduated from Carl Sandburg High School, with honors, in May 2000. In the same year she moved 1000 miles away to Massachusetts and began her studies in Biology and Visual Arts at Boston University. In 2003 she won two grants from the Undergraduate Research Opportunities Program (UROP) to perform summer research in the laboratory of Prof. Dr. John Finnerty. She continued this work under the supervision of Dr. Patrick Burton for her Bachelor's thesis. She defended her thesis in Biology in May 2004, and obtained her degree, *cum laude*, the same month. In August 2004 she joined the laboratory of Prof. Dr. Barry Bloom at the Harvard School of Public Health as lab manager/research technician. Her work in host-pathogen interactions with *Mycobacterium tuberculosis* was performed under the supervision of Dr. Jyothi Rengarajan and Dr. Samantha Sampson. In 2008 she began her masters study in Molecular Microbiology and Immunology at the John's Hopkins Bloomberg School of Public Health, in Baltimore. Her thesis work was supervised by Prof. Dr. Egbert Hoiczyk and she graduated in 2009.

In June 2009 she moved accross the Atlantic Ocean to Europe and in July she started her PhD training in Infection and Immunity at the University Medical Center Utrecht, the Netherlands, under the supervision of Prof. Dr. Elisabeth Sanders, Dr. Debby Bogaert, and Dr. Krzysztof Trzcinski; working both at the Department of Medical Microbiology and the Department of Immunology and Infectious Diseases at the UMCU Wilhelmina Children's Hospital. The results of this study are presented in this thesis, and are published in various scientific journals. She currently lives with her partner Jamie Lawson and their cat Islay.

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