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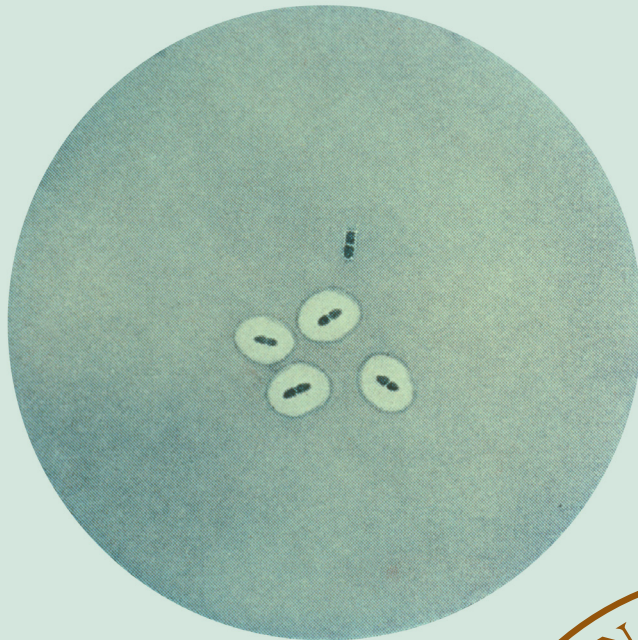
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Streptococcus pneumoniae infections before and after the introduction of a conjugated pneumococcal vaccine

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DEPARTMENT OF TRANSLATIONAL MEDICINE | LUND UNIVERSITY



Streptococcus pneumoniae infections before and after the introduction of a conjugated pneumococcal vaccine

Nils Littorin



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Title and subtitle: <i>Streptococcus pneumoniae</i> infections before and after the introduction of childhood vaccines		
<p>Abstract</p> <p>Pneumococcal infections are among the leading causes of death in children in developing countries. In developed countries the main burden of severe infections, such as invasive pneumococcal disease (IPD) is carried by the immunocompromised and the elderly. Different pneumococcal serotypes vary widely in their ability to colonize the nasopharynx and cause diseases such as Acute Otitis Media (AOM) and IPD. Introduction of pneumococcal conjugated vaccines (PCV) for children marks a new era in the battle against the pathogen.</p> <p>This thesis aims to examine changes in pneumococcal epidemiology in relation to the introduction of PCV in southern Sweden and to explore the immunogenicity and virulence of serotypes included in the vaccine. We monitored the prevalence of clinical nasopharyngeal samples positive for <i>Streptococcus pneumoniae</i> and two related pathogens before and after PCV introduction. IPD incidence is continuously evaluated by the Swedish National Board of Health. But less is known about the ability of individual serotypes to cause severe infections. To determine whether serotypes are associated to clinical outcome we conducted a retrospective investigation on IPD cases in Skåne. In order to protect itself the human host develops a strong antibody response to pneumococcal antigens. However, in some settings vaccination with polysaccharides has led to a suboptimal hyporesponse. Can natural immunization by <i>S. pneumoniae</i> lead to a weakened immune response and is serotype a determining factor? In immunological assays on sera from sepsis survivors we investigated the antibody response after IPD and pneumonia caused by different serotypes.</p> <p>Bacterial cultures from the upper respiratory tract referred to Clinical Microbiology at the University hospital of Skåne were analyzed. 14,473 cultures from the years 2004-2017 were included. Serotyping of <i>S. pneumoniae</i> was performed with <i>Quellung</i> technique. In order to determine disease severeness in cases of IPD, medical history of 513 patient sepsis patients were retrospectively reviewed. Quantification of antibodies in patient sera was performed with ELISA and an Opsonophagocytic assay was used to test their functionality in inducing opsonization of live bacteria.</p> <p>We found serotype-dependent variations in disease severity in patients with IPD. For instance, serotype 3 was significantly associated to septic shock. In parallel, sera from sepsis survivors had serotype dependent differences in their antibody response after disease. According to our data, some serotypes induce a poorer antibody response in the host compared to other serotypes. A poor immune response was found predominantly in IPD patients, compared to patients suffering from pneumonia without sepsis.</p> <p>In two observational studies of PCV effects in the southern county of Skåne, we found that nasopharyngeal cultures of vaccine type <i>S. pneumoniae</i> decreased markedly, while non-vaccine types increased. The net result was a steady decrease in pneumococcal infections during the years investigated (2004-2017). Interestingly, nasopharyngeal cultures of <i>H. influenzae</i> and <i>M. catarrhalis</i> also decreased in prevalence following introduction of PCV. Relative to total cultures taken, however, <i>H. influenzae</i> prevalence appeared unaffected. The reduction of <i>M. catarrhalis</i>, may partly be attributed to a positive association between PCV serotypes and <i>M. catarrhalis</i> discovered by us.</p> <p>In summary, we have found evidence for a change in prevalence of positive bacterial cultures from the nasopharynx of symptomatic children post PCV. There was a relative increase in prevalence of non-vaccine serotypes and other pathogens. Vaccine serotypes were, however, not completely eradicated. Some of them, such as serotype 3, was associated to a worse clinical outcome and to induce a poor antibody response. These findings are important for the evaluation of PCV.</p>		
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Nils Littorin



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Coverphoto by Centers for Disease Control and Prevention (CDC) depicting *Quellung* reaction in *S.pneumoniae* with and without capsule.

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Para Vida que es la vida y Yaiza que es el amor

Gracias a Margarita, Lazarus y el Lobo

Table of Contents

List of Papers.....	8
Abbreviations	9
Summary	10
Förenklad svensk sammanfattning	12
Introduction	13
<i>Streptococcus pneumoniae</i> in the history of man and microbiology	13
The need for more research.....	15
Basic facts about <i>Streptococcus pneumoniae</i>	17
Identifying <i>S. pneumoniae</i>	17
Serotyping	17
Genetic analysis.....	18
Virulence factors	18
Gene transfer	19
Related upper airway bacterial pathogens.....	20
<i>Haemophilus influenzae</i>	20
<i>Moraxella catarrhalis</i>	20
Microbiological interactions	21
The natural habitat.....	21
The commensal flora.....	22
Biofilm formation.....	22
Interactions with bacterial pathogens.....	23
Viral interactions.....	23
The host immune system.....	25
Innate immunity is the first line of defence.....	25
Functions of immune cells.....	25
The Complement System	26
The adaptive immune system.....	27
The antibodies	29
From colonization to disease.....	31
Pneumococcal disease	31
Pneumonia	31
Invasive pneumococcal disease (IPD).....	32

Meningitis.....	32
Acute Otitis Media	32
Aspects of serotype and infection.....	33
Serotype 3	33
Who contracts pneumococcal disease?	34
Carriage and transmission	34
When commensals misbehave	35
Breaking the epithelial barrier	35
Treatment and prevention	37
Antimicrobial resistance.....	37
The pneumococcal vaccines.....	37
Impaired immune response to polysaccharide vaccines.....	38
PCV effects on IPD in Sweden	39
Serotype replacement	39
PCV affects polymicrobial carriage and disease	40
Objectives	41
Material and Methods.....	43
Study design.....	43
General methods and definitions.....	43
Vaccine schedule	43
Sepsis definition	44
Culturing and serotyping	45
Invasiveness of serotypes	45
Statistical methods.....	45
Study specific methods and hypothesis.....	46
Results.....	51
Paper I	51
Paper II.....	53
Paper III.....	53
Paper IV	54
Paper V	56
Discussion	57
Serotype as a risk factor for septic shock in IPD	57
Serotype replacement after PCV introduction.....	57
IPD induces a poor antibody response	58
PCV effects on nasopharyngeal pathogens	60
Acknowledgements	61
References	63

List of Papers

The thesis is based on the following Papers:

- I. High incidence of septic shock caused by *Streptococcus pneumoniae* serotype 3 - a retrospective epidemiological study. Ahl J, **Littorin N**, Forsgren A, Odenholt I, Resman F, Riesbeck K. BMC Infectious Diseases. 2013 Oct 22;13:492.
- II. Reduction of *Streptococcus pneumoniae* in upper respiratory tract cultures and a decreased incidence of related acute otitis media following introduction of childhood pneumococcal conjugate vaccines in a Swedish county. **Littorin N**, Ahl J, Uddén F, Resman F, Riesbeck K. BMC Infectious Diseases. 2016 Aug 11;16(1):407.
- III. Decreased prevalence of *Moraxella catarrhalis* in addition to *Streptococcus pneumoniae* in children with upper respiratory tract infection after introduction of conjugated pneumococcal vaccine – a retrospective cohort study. **Littorin N**, Rünow E, Resman F, Ahl J, Riesbeck K. Clinical Microbiology and Infection. 2020, accepted for publication.
- IV. Serotypes With Low Invasive Potential Are Associated With an Impaired Antibody Response in Invasive Pneumococcal Disease. **Littorin N**, Uddén F, Ahl J, Resman F, Slotved H-C, Athlin S, Riesbeck K. Front Microbiol. 2018 Nov 15;9:2746.
- V. A non-functional opsonic antibody response frequently occurs after pneumococcal pneumonia and is associated with invasive disease. Uddén F, Ahl J, **Littorin N**, Strålin K, Athlin S, Riesbeck K. mSphere. 2020 Feb 5;5(1). *In press*.

Papers not included in the thesis

I: Jalalvand F, **Littorin N**, Su YC, Riesbeck K. Impact of immunization with Protein F on pulmonary clearance of nontypeable *Haemophilus influenzae*. Vaccine. 2014 Apr 25;32(20):2261-4.

2: Thofte O, Su YC, Brant M, **Littorin N**, Duell BL, Alvarado V, Jalalvand F, Riesbeck K. EF-Tu From Non-typeable *Haemophilus influenzae* Is an Immunogenic Surface-Exposed Protein Targeted by Bactericidal Antibodies. Front Immunol. 2018 Dec 18;9:2910.

Abbreviations

AOM	Acute Otitis Media
aPR	Adjusted prevalence ratio
CCI	Charlson comorbidity index
CAP	Community Acquired Pneumonia
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IPD	Invasive pneumococcal Disease
MHC	Major histocompatibility complex
MIC	Minimum inhibitory concentration
OR	Odds ratio
PcV	Penicillin V
PCV	Pneumococcal Conjugated Vaccines
PCR	Polymerase chain reaction
ST	Sequence type
URTI	Upper respiratory tract infection

Summary

This thesis aims to evaluate vaccine effects on upper respiratory tract infections and to explore the virulence and immunogenicity of bacteria causing invasive pneumococcal disease (IPD) and pneumonia. The area of investigation is the county of Skåne, Sweden.

S. pneumoniae is enveloped by a polysaccharide capsule that protects the bacteria from the human immune system and is considered to be its' most important virulence factor. There are numerous variations in the chemical structure of the capsule, giving rise to over 90 known serotypes.

Since 1983 a vaccine against *Streptococcus pneumoniae* intended for the elderly and immunocompromised has been available. Polysaccharide antigens from 23 different capsules (serotypes) were included in the vaccine as target antigens. Due to its pure polysaccharide formula insufficient protective antibodies is elicited in infants. In 2000 a pneumococcal conjugated vaccine (PCV), immunogenic in children, was introduced in the USA and in 2009 in Sweden. It included antigen from seven serotypes conjugated to a diphtheria-protein and proved to be effective in preventing Invasive pneumococcal disease (IPD). Since then, two vaccines of higher valency have been introduced and two others are being researched.

The serotypes included in the vaccine were chosen due to their high incidences of IPD in the USA. However, whether serotypes differ in their capacity to cause severe disease, such as septic shock, is a debated issue. In the first Paper we investigate clinical outcome in 513 cases of IPD. Interestingly, we found that heavily encapsulated serotype 3 was associated to septic shock. Since vaccine effect against serotype 3 is poor this information is valuable in the evaluation of PCV. One reason for a poor vaccine effect could be the inability of certain serotypes to induce a functional antibody response. In some settings, related to repeated immunization with polysaccharides, such a hyporesponse has been reported. In Paper IV and V we explore the antibody response in patients naturally immunized by IPD and pneumonia. Our findings suggest that IPD in a majority of cases elicits a non-functional antibody response and that serotype is an independent factor for this outcome.

Abundant data has been published on the positive effects of PCV on IPD. However, the effect on URTI is less elucidated. Serotypes not included in the vaccine may occupy the vacant niche after eradication of vaccine-types. A phenomenon known as serotype replacement. In the the second and third papers upper respiratory tract isolates before and after the introduction of PCV were analyzed. Vaccine serotypes became less frequent. Non-vaccine serotypes increased in prevalence, a finding consistent with international reports. The finding of serotype replacement gave rise to the question of a potential replacement of vaccine type pneumococci with other

bacterial pathogens, also residing in the nasopharynx. In a 14-year observational study of nasopharyngeal cultures positive for three pathogens we found that all decreased substantially in prevalence. The sharpest decrease was found cultures with *S. pneumoniae* and *M. catarrhalis* co-colonization. In addition, we found positive associations in co-colonization between *M. catarrhalis* and *S. pneumoniae* serotypes included in the vaccine formulas, which may indicate that part of the *M. catarrhalis* reduction was related to the introduction of PCV. A novel finding that may be the impetus for future mechanistic studies.

Förenklad svensk sammanfattning

Streptococcus pneumoniae (pneumokocker) är den vanligaste bakteriella orsaken till lunginflammation och öroninflammation och kan även leda till blodförgiftning (sepsis) och hjärnhinneinflammation (meningit). Pneumokocker har en skyddande kapsel som är uppbyggd av sockerkedjor. Kapselns kemiska struktur skiljer sig åt och ger upphov till över 90 kända kapseltyper. År 2009 infördes ett barnvaccin (PCV) mot pneumokocker i barnvaccinationsprogrammet. Barnvaccinet skyddar bara mot några av de vanligaste kapseltyperna. Denna avhandling har som syfte dels att undersöka hur PCV påverkar övre luftvägsinfektioner och dels undersöka vilka kapseltyper som orsakar allvarlig sepsis och hur patienters immunförsvar reagerar vid sådan sjukdom.

Vi upptäckte att samtidigt som kapseltyper inkluderade i vaccinet minskade efter att vaccinet infördes så ökade kapseltyper som inte ingår. Denna kunskap är viktig för att utvärdera vaccinet. Sedan undersökte vi om PCV kunde ha en effekt även på övre luftvägsinfektioner orsakade av andra bakterier än *S. pneumoniae*. I en observationsstudie över en 14-års period kunde vi påvisa att bakterien *Moraxella Catarrhalis* minskade i förekomst i bakteriekulturer från nashålan efter att vaccinet infördes. En viss minskning sågs också för bakterien *Haemophilus influenzae*, men när vi kontrollerade för totala antalet prov tagna före och efter PCV så var minskningen inte längre statistiskt signifikant.

Sepsis är ett allvarligt tillstånd med hög dödlighet. I en undersökning av sjukdomsfall av pneumokocksepsis före införandet av vaccinet kunde vi visa att kapseltyp 3 orsakade mer allvarlig sepsis än andra kapseltyper. Samtidigt har vi kunnat visa att pneumokocksepsis för det mesta leder till ett svagt antikroppsvar i patienter som överlevt sjukdomen. Särskilt kapseltyp 3, tillsammans med vissa andra kapseltyper, gav oftare upphov till ett svagt antikroppsvar. Detta har inte tidigare visats och ger intressanta uppslag för vidare forskning.

Sammantaget visar resultaten att efter pneumokockvaccinet införts har stora förändringar skett i förekomsten av pneumokocker och andra vanliga luftvägsbakterier. Våra data tyder på att vissa kapseltyper är förenade med allvarligare sjukdom och ett sämre immunsvär.

Introduction

Streptococcus pneumoniae in the history of man and microbiology

In 1927 the proletarian author Ivar Lo-Johansson went to Northumberland in north-east England to live among the English coal-miners. His purpose was to write about their lives. He spent many hours, day and night, in the dark shafts of the mines. His health deteriorated, his hair fell out, giving him the appearance of a rutabaga. Finally, he lodged himself in the Sun's hotel in Newcastle where he felt that he was dying. He was sweating, shaking in ague and had feverish dreams. At the same time he wrote about the interiors of the mine. He had to complete his work before dying. He prayed to God to recover. The hotel management had without his knowing sent word to a doctor, who diagnosed him with pneumonia and gave him a slim 5% chance of survival. After the doctor's visit he felt at ease, praying again for comfort. He later managed to get dressed and embark on a freighter to Gothenburg. He was unconscious almost the whole voyage. Once in Stockholm, in fear of going to the hospital he rented a room but was kicked out because of the risk of contagion. Now he was forced to visit the Sabbatsberg hospital where he was urgently admitted. An x-ray was performed and he was told that he had contracted pneumonia that had progressed to an empyema. The x-ray also revealed two caverns, rests of tuberculosis he had contracted in his youth. Ivar Lo then spent time at a convalescence home in rural Dalarna, where he recovered. His book about coalminers was published a year later[1].

This dramatic episode in the life of one of Sweden's foremost authors was likely caused by *S. pneumoniae*, the most common cause of community-acquired pneumonia, hence the name. Several aspects of pneumococcal disease are captured in the short story. The sudden onset of high fever, the severity of disease almost killing him and the empyema developed as a result of prolonged disease with no antibiotic treatment available at the time. Doctors then had little choice but to be fatalistic about deaths from pneumonia. Sir William Osler, sometimes called the father of modern medicine, a century ago famously called it a "friend of the aged" because it was seen as a swift, relatively painless way to die[2]. Ivar-Lo was not old at the time but the polluted air of the mines could give rise to a chronic inflammation in the lung-epithelial, making him more susceptible to bacterial infection. Poor people, living in crowded areas with unhealthy sanitary conditions and low access to health-care still today carry the main burden of disease from "the captain of the

men of death”, yet another expression used by the witty Osler. The disease ultimately took Osler's own life. His final illness began with a cold that progressed to pneumonia caused by, in his words, "No. 3 pneumococcus & *M. catarrhalis* the organisms"[3].

S. pneumoniae has played an important role in the history of microbiology, medicine and science. Isolated simultaneously in 1821 by Louis Pasteur in France and in the United States by Sternberg it was named *Diplococcus pneumoniae*, because of its appearance in Gram-stained sputum. In 1974 it was renamed *Streptococcus pneumoniae* due to its growth in chains in liquid medium. In the 1880s the Klemperers, German scientists, showed that immunization with killed pneumococci protected rabbits against subsequent pneumococcal challenge and that protection could be transferred by infusing serum (“humoral” substance) from immunized rabbits into naïve recipients. This was the first unearthing of the humoral immune system and before the discovery of penicillin, serotherapy was used to treat pneumonia and sometimes even acute otitis media[4].

Fred Neufeld revealed that the effect of the immunized serum from animals was on the pneumococci, facilitating phagocytosis (derived from Greek “to engulf”) by white blood cells. Neufeld made several important discoveries about the pneumococcus, such as the *Quellung* reaction in 1902[5]. The technique of using capsule specific antisera to cause the bacteria to swell, agglutinate and immobilize, is still today the Gold Standard for serotyping pneumococci[5]. Using this method, he was the first to describe the differentiation of pneumococci into serotypes. Neufeld's findings were essential for the development of effective multivalent pneumococcal polysaccharide vaccines. However, the first attempt to prevent pneumococcal pneumonia was made through immunization with killed whole cell bacteria. In 1911, Wright and colleagues vaccinated South African coal miners which lowered their high incidences of pneumonia[4].

Avery and Heidelberger in 1923 published their findings on the “Soluble specific substance of Pneumococcus”, which they suggested consisted of carbohydrate which appeared to be a polysaccharide[6]. The type-specific pneumococcal antigens had thus been revealed, and following their report there were many efforts to develop a polysaccharide pneumococcal vaccine, all ending in failure. Success was not achieved until a clinical trial at a US Army military base in 1944[7]. But for many technical and practical reasons, the current pneumococcal vaccine for adults was not developed until the 1970s[8].

In their search to control pneumococcal pneumonia the scientists made one outstanding discovery: the function of DNA. In experiments in mice, Griffith had found that avirulent pneumococci lacking a capsule, could be made virulent and kill the mouse when coinoculated with a virulent strain killed by heat. Griffith concluded that the live avirulent cocci had engulfed material from the heat-killed virulent bacteria, a process that enabled expression of a novel carbohydrate

capsule. This observation, which he called transformation, intrigued Avery and collaborators and they set out to identify the chemical nature of the material. Their historic discovery of DNA as the transforming principle in 1943 was made in experiments on *S. pneumoniae*, combining the fields of microbiology, genetics and chemistry.

Adding to the history of the pneumococcus, it was among the first bacteria to be treated with an antimicrobial agent, Optochin. The microbe was also among the first to develop antimicrobial resistance against such therapy. Early experiments that demonstrated antibiotic resistance was, as current pneumococcologists put it, "...premonitory for the remarkable resilience of bacteria, and *S. pneumoniae* in particular, that have evolved and adapted over many millions of years to subvert our efforts to control and eradicate them with antimicrobials and vaccines developed in the last 100 years." [9]

The need for more research

Even though vaccines and antibiotics are effective in preventing and treating *S. pneumoanie* the microbe is still responsible for significant mortality and morbidity worldwide. One important question that remains unanswered is the effect of pneumococcal vaccines on other pathogens residing in the nasopharynx. Long term prevalence studies covering the period before and after vaccine of such bacteria as *Moraxella catarrhalis* and *Haemophilus influenzae* are scarce. Such surveillance is important to detect surges or decreases in infections related to PCV. Another essential field of research is serotype replacement. The increase in non-vaccine type pneumococci after the introduction of PCV is a well-known phenomenon. However, since the changes in serotypes differ widely over time and geographically, local studies are needed. The center of attention for the epidemiological work in this thesis is southeastern Skåne, the southernmost district in Sweden. It is known for its proximity to the continent and the residential center of Malmö for its multi-ethnic population. It has a fast growing population with a high proportion of pre-school children that constitute the main reservoir for pneumococcal colonization. This international, young and developing area provides an interesting challenge for epidemiological research.

Very little research has been published on the effects of natural immunization through IPD or pneumonia on the immune response in humans. Is it possible that instead of triggering a strong antibody response, an episode of pneumococcal disease does the precise opposite? This vital question is the focus of the immunological research in this thesis. Finally, there is debate on whether serotypes are a risk factor for serious infections or not. Some researchers attest that host factors such as age and comorbidity are more important, whereas some studies on case

fatality suggest otherwise. It is imperative to know more about the virulence of serotypes responsible for IPD so that the most lethal ones can be included in future vaccine formulas.

Basic facts about *Streptococcus pneumoniae*

Identifying *S. pneumoniae*

Species of the *Streptococcaceae* family vary in color and shape and several members of the family may cause disease in humans, whereas others are commensal inhabitants of different body tissues. The gram-positive coccus *Streptococcus pneumoniae* is lancet-shaped and often appears in pairs (diplococcus) or chains (streptococcus). On blood agar the colonies are grey and glistening, surrounded by a subtle greenish α -hemolysis. The *S. pneumoniae* typically have depressions in their centres after overnight culturing and some serotypes may be mucoid due to abundant polysaccharide capsule production[10]. It is a fastidious facultative anaerobe that requires enriched growth media and elevated levels (5%) of CO₂ for optimal growth[11]. The classical diagnostic identification of pneumococci is based on colony morphology (α -haemolysis and characteristic colonies), optochin sensitivity, and some biochemical activities, such as the lack of catalase production and bile solubility[11].

Serotyping

The chemical structure of the capsule antigen enveloping the bacteria provides the basis for differentiation into 94 serotypes[12]. Polyclonal antibodies from sera derived from rabbits immunized with *S. pneumoniae* is used in the standard method of serotyping. The classical method is observing the *Quellung* reaction in phase microscope but newer techniques include latex particles covered in sera for rapid and simple typing by agglutination visible for the eye[13]. Cross-reactive serotypes are categorized into a serogroup and given a letter (e.g. serotypes 6A, 6B, 6C within serogroup 6).

A PCR method of serotyping was developed in the early 2000s with designed primers based on the sequences available for some of the most clinically relevant capsular types[14]. The PCR method is faster, cheaper and equally accurate compared to conventional serotyping, but only identifies a minority of serotypes. Non-encapsulated strains, often found in conjunctivitis, are not typeable by conventional methods and are sometimes referred to as non-typeable[15]

Genetic analysis

Knowledge of the genetic relatedness of clinical isolates has gained in importance after the increase of non-vaccine serotypes following PCV and the emergence of antibiotic resistant clones spreading globally. Tracking the emergence of multidrug resistant isolates and investigating the origins of newly defined serotypes can be performed by different methods such as Multi locus sequence typing (MLST) and Whole genome sequence analysis (WGS)[16,17].

Virulence factors

The pneumococcus produce a wide range of colonization and virulence factors important for survival and disease, several of which have been identified (Table 1). The capsule is considered the major virulence factor, as illustrated by the fact that virtually all clinical isolates are encapsulated and non-encapsulated strains are generally avirulent[15]. Protection from phagocytosis and facilitating colonization of the nasopharynx are features of the capsule that promotes survival and infection[18]. The capsule is made of oligosaccharide repeat units linked to the peptidoglycan of the cell wall. Some serotypes have a tendency to more often asymptotically colonize the nasopharynx (e.g. 19F, 23F) while others are found to be carried less frequently (e.g. 1, 5) but are more often associated to invasive and mucosal disease[19]. The reason for this difference might be that strains prevalent in carriage express higher levels of the capsule and are more resistant to neutrophil-mediated killing. These capsule structures have been suggested to be less metabolic demanding, enabling the bacteria to produce more and reside in the nasopharynx for longer periods of time [20]. The capsule interferes with the classical pathway by inhibiting complement opsonization as well as antibody to Fc-receptor interaction. In parallel, it prevents CRP deposition to the cell surface. In order to escape the mucociliary clearance in the airways, the pneumococcus upregulates the capsule production, which repels the negatively charged mucus[21]. When subsequently adhering to the epithelial cells the capsule expression is down-regulated and the expression of adherence molecules are up-regulated. The reduced amount of capsule facilitates epithelial contact and uptake of the bacteria[22].

Another major virulence factor is pneumolysin, a cytolytic protein released during the log phase of bacterial growth. It has two major functions; lytic activity as it interacts with cholesterol in cell membranes to form large pores and complement activation. Pneumolysin triggers inflammation and is a major cause of lung tissue damage in pneumonia[23].

Table 1. A selection of virulence factors of *S. pneumoniae* important for adhesion, invasion and escaping host immunity.

Virulence factor	Molecule	Proposed function	Reference
IgA protease	Enzyme	Cleavage of protective IgA antibodies	[24,25]
Hyaluronidase	Enzyme	Degrades connective tissue, aids bacterial spread and colonization. Promotes inflammation.	[26]
Neuraminidases	Enzyme	Cleave terminal sialic acids from glycoconjugates. Facilitates adhesion, colonization.	[27]
Pilus	Protein	Mediates binding to cells.	[28]
PspA	Lipoprotein	Choline binding protein. Inhibits complement deposition, blocks bactericidal activity of lactoferrin	[29]
PspC	Lipoprotein	Choline binding protein. Binds to Vitronectin and Factor H, inhibiting complement and promoting adhesion.	[30]
Hydrogen peroxide	Chemical compound	Celltoxic, pro invasive. Also used to kill other bacteria.	[31,32]
PsaA	Lipoprotein	ABC-transporter. Helps evade oxidative stress from host.	[33]

Gene transfer

S. pneumoniae is a naturally competent organism. It has the ability to take up genetic material from its surroundings, from other pneumococci and even other bacterial species. This can be used to acquire virulence, and uptake of DNA is often induced in the exponential growth phase or under stress (antibiotic treatment, DNA damage)[34].

Horizontal transfer of genetic material can substantially increase the heterogeneity of the pneumococcal gene pool. High levels of recombination, as has been described for clones that have undergone decades of evolution[35], ensure the success of a clone over the long term, as it is faced with environmental challenges such as antibiotics or vaccines. Even during a single episode of infection, horizontal gene transfer between pneumococcal strains has been observed [36]. An illustrative example of the importance of genetic variation is capsular switching. The pneumococcus has the ability to generate novel combinations of serotype and genomic backbone. The first recognized multidrug resistant clone carried the capsule 23F, included in PCV formulas. Following the introduction of PCV7 in the USA this sequence type escaped impediment by switching capsule to non-vaccine serotype 19A, which then became a major cause of disease[35]. Recently, the recombinant clone CC156, which before PCV7 carried a serotype 14 or 9V capsule, has emerged as serotype 11A[37].

Related upper airway bacterial pathogens

Haemophilus influenzae

In the 20th century *Haemophilus influenzae* type b (Hib), an encapsulated bacteria, was known to cause severe infections such as meningitis and epiglottitis in children. Since the introduction of a childhood vaccine against Hib, other types of *H. influenzae*, predominantly the unencapsulated and hence nontypeable *H. influenzae* (NTHi), have become increasingly common as opportunistic pathogens in the upper respiratory tract. The rod-shaped, gram negative bacteria share the propensity of *S. pneumoniae* to colonize the airway epithelia in children and resides most of the time in the nasopharynx without causing disease. However, in situations where the host immune system is weakened and the airway epithelia is disrupted *H. influenzae* can cause acute otitis media (AOM) in children and chronic obstructive pulmonary disease (COPD) exacerbations and pneumonia in adults[38]. Its' a resilient colonizer of the airways and readily forms biofilms which is reflected in the chronicity of respiratory infections[39]. In children with recurrent and chronic otitis media *H. influenzae* the importance of biofilm formation has been highlighted in several reports [40,41].

Since the introduction of PCV, *H. influenzae* has gained in relative importance to *S. pneumoniae* as cause of AOM[42]. *H. influenzae* frequently produces betalactamase and is resistant to Phenoxymethylpenicillin (PcV), an oral narrow spectrum penicillin used to treat URTI caused by *S. pneumoniae*. The incidence of betalactam resistant strains has increased in Sweden[43].

Moraxella catarrhalis

Moraxella catarrhalis is Gram-negative, unencapsulated and expresses a type IV pili structure. It was previously discounted as a simple commensal organism with limited potential for pathogenesis. Now it is recognized as a respiratory pathogen causing AOM and sinusitis in children as well as lower respiratory tract infections in adults with underlying diseases. The bacterium rapidly colonizes the nasopharyngeal cavity soon after birth and is part of the nasopharyngeal microbiome described below[44].

After *S. pneumoniae* and *H. influenzae* it is the the third most common cause of AOM and is the second most common cause of exacerbations in COPD[45]. The vast majority of all *M. catarrhalis* isolates are betalactam resistant but are susceptible to amoxicillin-clavulanic acid and broad-spectrum antibiotics.

Microbiological interactions

The natural habitat

The upper respiratory tract consists of the nasal cavity, the nasopharynx, oropharynx, the larynx and the upper part of trachea as well as paranasal sinuses. The nasopharynx is located dorsally of the nasal cavity and just cranially of the uvula[46]. Lymphoid tissue constitutes the posterior and upper walls and in the middle is the opening of the auditory tube, which connects the middle ear to the upper airways. Intraepithelial leukocytes, including IgA secreting B-cells, and macrophages are part of the lymphoid tissue. The surface of the tube is made up of ciliated respiratory epithelial cells and goblet cells near the nasal cavity and squamous cells closer to the pharyngeal isthmus. In this connection site between the respiratory pathway of the nose, the tuba auditiva and the lower respiratory airways, an intricate milieu for bacterial colonization has been created. It is the site where inhaled antigens first come in contact with human tissue. The anatomical connections with the middle ear and the lungs, as well as proximity to nasal sinuses facilitate bacterial spread to these sites, with unwanted consequences such as pneumonia, AOM and sinusitis. But more than being a place of departure for pathogenic bacteria, the nasopharynx is a place where commensals live in symbiosis with the host[47].

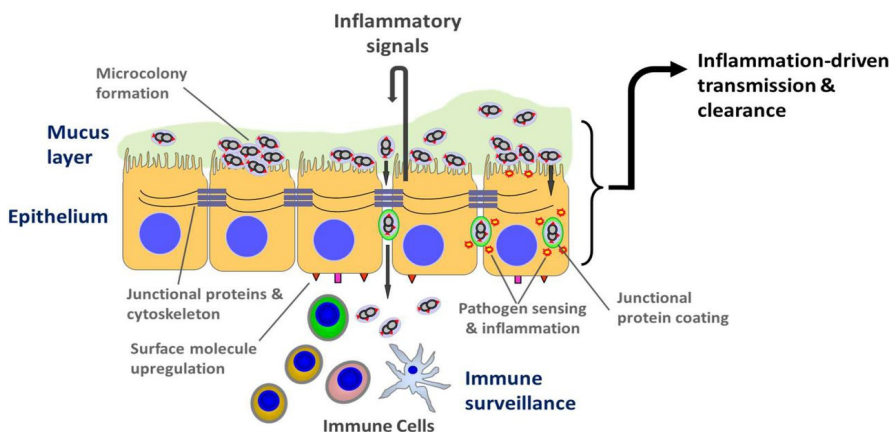


Figure 1. The airway epithelium of the nasopharynx controls colonization of *S. pneumoniae* at the mucosal surface. Image used with permission from the publisher and creator [48].

The commensal flora

The healthy nasopharyngeal microbiome constitutes of different genera such as *Moraxella*, *Streptococcus*, *Corynebacterium*, *Staphylococcus*, *Haemophilus* and *Alloiococcus*. In young children the microbiota varies between seasons independent of antibiotic use or viral co-infection[49]. The pneumococcus shares the nasopharyngeal niche with an array of bacteria and viruses that interact with the immune defence of the host and each other. This commensal flora is considered to be beneficial since it stimulates the immune system and functions as a protective barrier against invading pathogens. Nasopharyngeal colonization by bacterial species can be immunizing events, stimulating both humoral and cellular adaptive immune responses that protect against either re-colonization or subsequent invasive disease[50]. When the microbial balance is disturbed, for example during the influenza season, the risk of infection increases [49]. During symptomatic viral infection pathogenic bacteria increase their colonization, and the relative abundance of commensals is reduced, but specific commensals are affected differently[51]. Antibiotic treatment might also alter the protective balance in the commensal flora, favouring selection of *Streptococcus*, *Haemophilus* and *Moraxella* genera[52].

Biofilm formation

Low temperature, deprivation of nutrients and constant danger of attacks from the immune system of the host as well as vulnerability to antimicrobial therapy hardly makes the nasopharynx an ideal site for bacterial growth. *S. pneumoniae* therefore frequently appears in biofilm formations where growth rate and virulence factors are downregulated and adherence proteins are upregulated. Metabolism, gene expression and protein production are different to those of planktonic cultures[53]. The biofilm produces extracellular matrix composed of DNA, proteins and noncapsular polysaccharides which provide mechanical stability as well as protection. Horizontal gene transfer occur in biofilms, and is promoted by the simultaneous colonization of different serotypes[53]. Interestingly, it was suggested by Hammerschmidt and colleagues that in transition from biofilm to IPD the pneumococcus upregulates its capsule expression[22]. One of the most important and persistent problems posed by biofilms is the inherent tolerance of their associated communities to antibiotic therapy and host defence mechanisms. Therefore, therapies targeting biofilms is an active field of research.

Interactions with bacterial pathogens

Moraxella catarrhalis and *H. influenzae* share the propensity of the pneumococci to colonize the upper airways. They interact with each other and other commensal bacteria in a not yet fully elucidated interspecies interaction. In certain age groups, particularly infants, the nasopharyngeal colonization of both *S. pneumoniae* and *H. influenzae* can exceed 50%. In such asymptomatic carriage *S. pneumoniae* is frequently detected together with *M. catarrhalis* and *H. influenzae* in the nasopharynx, forming polymicrobial biofilms [54,55]. Bacteria co-colonizing the nasopharynx may be positively or negatively associated in respiratory tract infections based upon synergistic or antagonistic interactions between the various species. In polymicrobial colonization of the nasopharynx, *H. influenzae* seems to predominate over *S. pneumoniae* and *M. catarrhalis* in causing AOM [56]. In parallel, *S. pneumoniae* and *H. influenzae* are negatively associated in URTI [57].

Interestingly, Lysenko *et al.* reported that co-colonization with *H. influenzae* and *S. pneumoniae* in a murine mouse model resulted in a rapid clearance of the latter species after *H. influenzae*-dependent activation of phagocytosing neutrophils [58]. In contrast, *S. pneumoniae* has developed different strategies to clear *H. influenzae*, including the release of bactericidal hydrogen peroxide [32].

Only a few papers have been published on the microbiological interplay between *M. catarrhalis* and *S. pneumoniae*, but biofilms consisting of the two bacterial species may promote antibiotic resistance of pneumococci [59] as well as bacterial persistence and growth of *M. catarrhalis* in a mouse infection model [60]. It thus remains to fully prove whether the relationship between *H. influenzae*, *S. pneumoniae* and *M. catarrhalis* is competitive or cooperative or depends on the context.

Viral interactions

In a thorough re-investigation of the 1918/1919 influenza pandemic, researchers concluded that most deaths were caused by bacterial superinfections. The pandemics of 1957 and 1968 were also analysed and were consistent with these findings, albeit based on less substantial data[61]. Coinfections occurred with above all *S. pneumoniae* but also involves *Staphylococcus aureus*, *Haemophilus influenzae* and other *Streptococcus spp.* During the 20th century bacterial pathogens occurred more frequently in pandemic compared with seasonal influenza periods[62]. In a literature review of the most recent influenza pandemic, 2009 H1N1, coinfection was found in 23% of fatalities with *S. pneumoniae* the most common bacteria identified[63]. The involvement of bacteria might have been even greater, suggested by autopsies from 34 cases who died from the 2009 pandemic. Over half displayed

signs of secondary bacterial infections in postmortem lung cultures and histological evaluation[64].

Aside from seasonal Influenza A and B, other viruses such as RSV and Rhinovirus have been implicated in promoting pneumococcal transmission and predisposing to infection[65,66]. How Influenza viruses enhance pneumococcal adherence, invasion and disease is not yet fully elucidated. One important factor is viral damaging of the normally protective epithelial layer of the upper airways and lung which prepares access to extracellular matrix molecules and basement membrane elements to which bacteria can adhere[67]. There is limited data on superinfections associated with the current pandemic disease caused by the novel coronavirus SAR-CoV-2.

The host immune system

Innate immunity is the first line of defence

The first line of defence against antigen and pathogens is the nasal fimbriae which filters away larger particles. Smaller organisms that pass on to the airway may encounter the rhythmic beating of cilia on the epithelial cells lining the mucosa. These movements capture microorganisms in the terminal bronchioles and transport them to the trachea and the oral cavity where they are swallowed. Secretion of mucus from mucosal cells helps in this process called mucociliary transport. The mucus, built up by mucins and proteoglycans function as a barrier to bacteria[68]. Mechanically, the epiglottis and cough reflexes are important to clear mucus from the lower airways and keep the environment in the lung antiseptic. The pseudostratified columnar epithelium is joined by tight junctions between the cells that constitute a mechanical barrier against microorganisms (see Figure 1). Particles or microorganisms that avoid the mechanical barriers confront a range of soluble mediators with antibacterial activity, such as lactoferrin and defensins, produced by cells of the respiratory tract. These molecules have the ability to directly lyse pathogens or destroy them by opsonization or recruitment of inflammatory cells[69]. In addition, macrophages and dendritic cells are phagocytes located in close proximity to the epithelial surface of the airway system which sample and examine the air-borne and blood-borne material. They are gate-keepers that help to keep the airways free of any invading pathogens[70].

Functions of immune cells

The alveolar macrophage is the first type of phagocytic cell to meet an invader at the alveolar level. Macrophages are also present in the interstitial tissue and the pulmonary capillaries prepared to remove invading microorganisms and to present antigen to lymphocytes using major histocompatibility complex II (MHC class II)[71]. Antigen-presenting cells (APC) also includes dendritic cells that reside in proximity to the basement membrane and extend their dendrites between the epithelial cells of the airway epithelium in order to sample inhaled antigens[72]. After antigen uptake, airway dendritic cells migrate to the paracortical T-cell zone of the draining lymph nodes of the neck or lung, where they interact with naive T-cells. Neutrophils are key immune effector cells that are rapidly recruited to a site

of infection. They are highly motile and are attracted by cytokines expressed by endothelium or immune cells activated in inflammation. Neutrophils use degranulation (release of soluble anti-microbials), phagocytosis and neutrophil extracellular traps to kill bacteria[73].

Immune cells utilize Toll like receptors (TLR) and other pattern recognition receptors (PRRs) to recognize Pathogen associated molecular patterns (PAMPs) in bacteria and virus. PAMPs are molecules such as lipotechoic acids, lipopolysaccharides, lipoprotease or DNA, some of which constitute parts of structures unique to pathogens such as bacterial cell wall or flagella. PRR essentially functions by activating the innate immune system. Opsonization of the pathogen, activation of complement proteins, phagocytosis of the pathogen, activating inflammatory mediators, secretion of cytokines and induction of apoptosis in infected cells are all consequences of PRR-PAMP interactions[71].

The Complement System

An important part of the innate immune system are soluble proteins that can bind to pathogens, coat their surfaces and tag them for opsonization by macrophages containing complement receptors. They are called complement proteins and enhance inflammation and attack the cell membranes of bacteria (Figure 2). The classical pathway of complement activation is usually initiated as a response to antibody-antigen complex formation but can also be activated by C-reactive protein (CRP) and other substrates. When complement protein C1q binds antigen-antibody complexes, the C1 complex becomes activated causing it to undergo conformational changes, activating proteases and initiating a cascade of reactions where complement proteins activate other complement proteins. The reactions result in the cleavage of C3 into two fragments, the anaphylactic C3a, that recruit leukocytes and promote inflammation, and C3b, responsible for further downstream complement activation. Ultimately the chain of reactions leads to formation of the cylindrical membrane attack complex (MAC) deployed at the cell wall membranes where it creates pores causing lysis of bacteria like *S. pneumoniae*[74]. In the alternate pathway complement proteins are spontaneously activated at a low level in the blood with regulatory proteins preventing them from causing damage to the host. Pathogens lacking such regulatory proteins bind to activated complement proteins which leads to the reactions ending with formation of MAC. Finally, the lectin pathway is initiated by Mannose binding lectine, a PRR that recognize carbohydrate structures. Unlike other PRRs it is able to activate the complement system in an antibody and C1-independent manner[75].

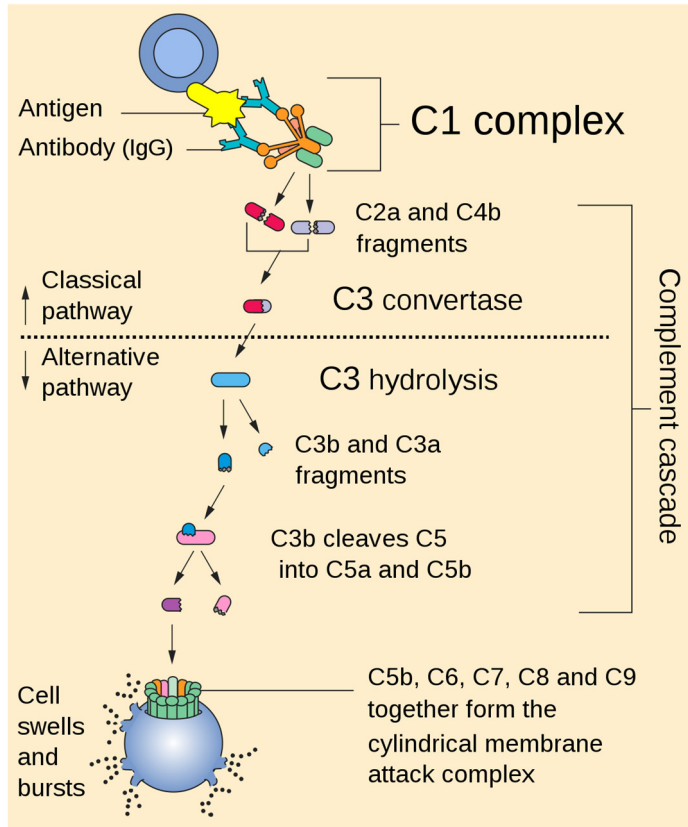


Figure 2. The classical and alternative pathways of the complement system. Both ends with the membrane attack complex and cell lysis. Illustration: US Federal government - Public domain.

The adaptive immune system

The adaptive immune system involves two main activities, the antibody response and the cell mediated immune response, performed by B and T cells. The B cells are produced in the bone marrow and carry receptors designed to identify specific foreign antigens (such as bacterial proteins or carbohydrates). If they encounter a matching protein antigen they consume it, receive signals from a T-helper cell and further differentiates into antibody secreting plasma cells and memory cells (Figure 3). In this process the B-cells undergo affinity maturation in which B-cells expressing high affinity receptors are selected for clonal expansion. However, B-cells can also be activated in a T-cell independent way, as in the case of bacterial capsular polysaccharides. The B-cell then rapidly mature into short lived plasma cells and do not contribute to memory B-cell pools. Polysaccharide vaccines are not

recommended to infants since they are not able to mount a T-cell independent antibody response[76]. Results from clinical trials demonstrate no induction of memory B-cells from pure polysaccharide vaccine PPV23 in adults. On the contrary, a lack of memory B-cells in blood after a primary dose and an attenuated antibody response upon revaccination has been reported [77].

Naïve CD4 or CD8 T-cells become activated when antigen specific to them are presented by APCs. T-cell receptors (TCR) interact with the MHC class I or II protein complexes on the APC that have antigen bound, causing the T-cell to develop from naïve CD8 to cytotoxic T-cells or from naïve CD4 cells into activated T-helper cells. The T-helper cells enhance the immune response by activating B-cells, natural killer cells and macrophages.

The adaptive immune response creates immunologic memory in both T and B-cells. After the primary encounter with an antigen and the subsequent immune response, a small number of lymphocytes remain that make up the cellular part of immunological memory. In addition, antibodies specific for the antigen remain in the body and make up the humoral component of memory. Subsequent encounters with the same antigen results in a fast and more effective response as specific memory B-cells already exist and have undergone Ig class-switching to higher affinity antibodies (e.g. IgG and IgA). By one month after immunization (natural exposure or vaccine), memory B cells are present at their maximal levels[78].

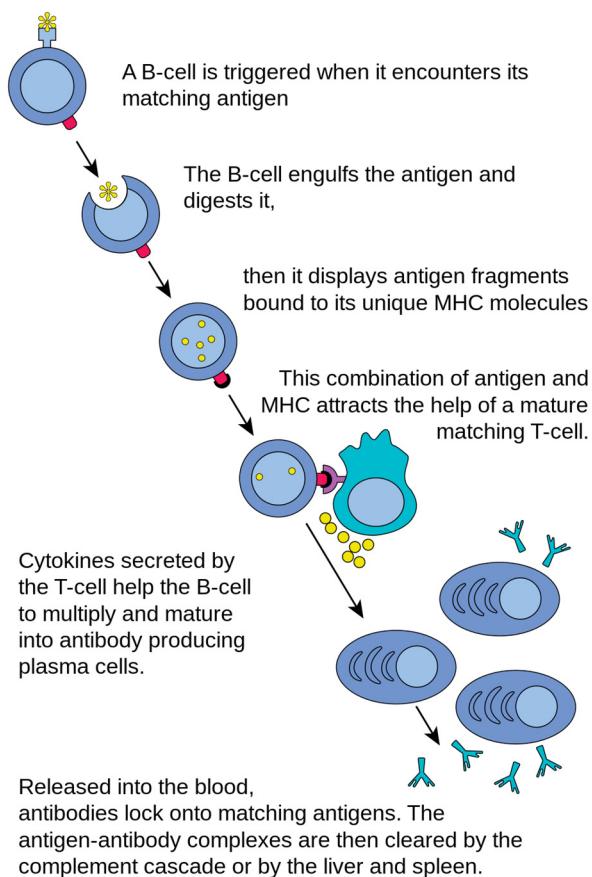


Figure 3. The adaptive immune system and B-cell development to plasma effector cells. Illustration: Fred the Oyster, Creative commons, Public domain.

The antibodies

In humans, immunoglobulin isotypes (Ig) A, D, E, G and M are secreted by B-cells to protect from pathogens. IgM or IgD are utilized as a first line of defence whereas IgA, IgG or IgE are more specific with special roles in the immune system [79]. IgA is the primary Ig isotype induced at mucosal sites where it inhibits absorption of antigens from mucosal surfaces by forming large immune complexes. In addition, IgA coats bacteria preventing their adherence to epithelial cell receptors[80,81]. It has also been demonstrated that IgA recognizing the capsular polysaccharide mediates pneumococcal killing by phagocytes[81]. IgG is the main antibody in the lymph, blood, cerebrospinal fluid and peritoneal fluid and forms 15% of total serum protein[82]. It is separated into four subclasses of which IgG2 has been suggested

as the most important against encapsulated bacteria such as *S. pneumoniae* [83,84]. Deficiencies in total IgG or subclass IgG2 is related to an increased risk for airway infections by encapsulated bacteria and damage such as bronchiectasis. The immunologic functions of IgG are diversified and includes opsonization of pathogens by tagging them to promote phagocytosis. Agglutination by IgG has been suggested as an important defence against pneumococcal colonization[85]. Furthermore, IgG is one of the isotypes that can induce the classical pathway of the complement system. The adaptive response to pneumococcal colonization includes acquisition of anti-capsular and anti-protein antibodies as well as T-helper cellular response targeting proteins[86]. In response to pneumococcal carriage, animals[50] and humans have been shown to develop increased serotype specific IgG antibody titres. Adults can develop protection from subsequent colonization, but evidence of a reduced risk for children is less clear[87,88]. Pneumococcal infections and carriage, so relatively common in childhood, decreases with age independent of anti-capsular antibody levels, which indicates that antibodies against proteins, cellular and matured innate immunity are involved in the immunity[86]. Animal models have demonstrated that colonization induces anti-protein antibodies and cellular responses that protects against mucosal colonization, pneumonia and sepsis. Several experimental studies have suggested similar protection in humans[89,90].

From colonization to disease

Pneumococcal disease

Pneumonia

Pneumonia accounts for 15% of all deaths of children under 5 years old, killing 808,694 children in 2017. *S. pneumoniae* is the leading cause of childhood pneumoniae and the vast majority of annual deaths are in developing countries[91]. In the USA and Europe the main burden of disease is in the elderly and immunocomprised [92]. The yearly incidence for communitiy acqiered pneumonia (CAP) in developed countries is about 1 percent in the population and about 20-40% require admission to hospital. Average mortality for pneumonia in Sweden was 6.9 per 100.000 individuals for women and 12.4 for men in the years 2012-2014. Since 2001-2003 mortality had decreased by almost 40%[93]. Aetiology before PCV was dominated by *S. pneumoniae* followed by *H. influenzae* and *Mycoplasma pneumoniae* and some viral agents. Acute onset of fever, chestpain and WBC $>15 \times 10^9$ and x-ray demonstrating a characteristic lobar pneumonia supports the diagnosis of pneumococcal pneumonia. Most reports on causative agents after introduction of PCV indicate that *S. pneumoniae* is still the most common cause of CAP[94,95].

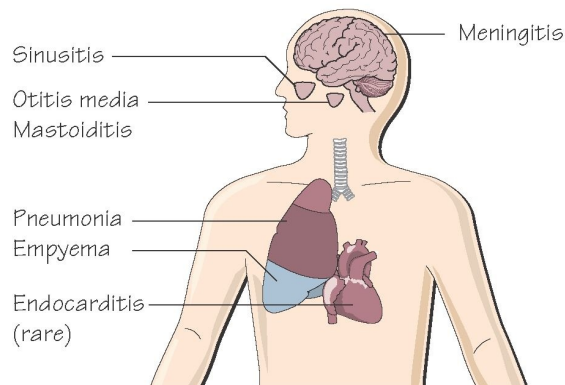


Figure 4. Anatomical sites of infection of *S. pneumoniae*. Image adapted and used with permission from the publisher[96].

Invasive pneumococcal disease (IPD)

IPD is defined as an infection confirmed by the isolation of *S. pneumoniae* from a normally sterile site, such as blood or cerebrospinal fluid. The IPD burden is mainly determined by pneumococcal pneumonia, meningitis and pneumococcal bacteraemia without a primary focus. IPD is a common cause of sepsis, a life threatening condition that according to symptoms and laboratory findings can be classified as severe or septic shock[97–99]. Annual incidence in a wedish study was estimated to 15/100,000 for any IPD and 1.1/100,000 for meningitis and was the highest among elderly followed by children < 2 years. Case-fatality rate (CFR) had dropped from 20 to 10% during a 45 year follow up period (Epidemiology of invasive pneumococcal infections: manifestations, incidence and case fatality rate correlated to age, gender and risk factors), and in our investigation from southern Sweden, included in this thesis, 28-day mortality was 12%[100].

Meningitis

Bacterial meningitis is a severe infectious disease characterized by infection and inflammation of the meninges. On a global scale, as well as in Sweden, *S. pneumoniae* is the most common cause, followed by *Neisseria meningitides*[101]. Aetiology can vary depending on region and time period. Mortality untreated is up to 50% but even with swift detection and antibiotic treatment 8-15% of the patients die. Sequele after meningitis includes hearing loss, brain damage and learning disabilities[102]. Advancements in prevention strategies such as vaccination against Hib, *S. pneumoniae* and *N. meningitidis* have substantially reduced the burden of disease in both vaccinated and unvaccinated populations[101].

Acute Otitis Media

URTI represents the most common acute illness evaluated in the outpatient setting. A common bacterial URTI is AOM, affecting up to 75% of children before the age of 5 years[103]. In Sweden there are 200,000 new cases of AOM every year, and *S. pneumoniae* was until recently the most common causative pathogen, but has since the introduction of PCV been dethroned by *H. influenzae* [104–106]. Together they are responsible for up to 80% of cases and *M. catarrhalis* ranks as the third most important otopathogen[107]. Although more restrictive prescription guidelines for URI and AOM have been adopted, these illnesses are still one of the main reasons for antibiotic use in Sweden [108]. Penicillin V is the first-hand choice based on the high susceptiblity rate of *S. pneumoniae*. Changes in causative bacteria of URI and AOM can have implications for future treatment guidelines since *H. influenzae* is often resistant to penicillin.

Aspects of serotype and infection

About 90 serotypes have been identified all over the world, but only a minority of them are clinically relevant. Global surveillance demonstrates that a limited number of capsular serotypes cause more than 70%-80% of IPD. The distribution of serotypes in the population varies greatly, related to geography, time, use of antibiotics, vaccine status and socioeconomic factors[109]. They also differ in their ability to cause invasive disease. Brueggeman *et al.* examined the relation between asymptomatic nasopharyngeal carriage in children and IPD[19]. The invasive disease potential of a particular serotype is related to the tendency to cause IPD while colonizing the nasopharynx. A significant inverse correlation between invasive disease and carriage rate was observed. Sont *et al.* found some serotypes (i.e: 1, 4, 5, 7F, 8, 12F, 14, 18C and 19A) to have high invasive potential[110]. Sjöström *et al.* postulated that some of these (i.e: 1 and 7F) behave as primary pathogens, infecting mostly younger and previously healthy individuals, but causing relative mild disease with few fatalities. Serotypes/serogroups with lower invasive potential include 19F, 23F, 3, 6A, 6B, 15, 8 and 33. Some of these (i.e.; 19F, 23F), were described as opportunistic pathogens, mainly causing disease in immunocompromised and elderly individuals with higher case fatality rates[111]. Serotype 1 is repeatedly associated to outbreaks of disease in impoverished and crowded areas[112].

Serotype 3

Of special interest for this thesis is serotype 3. It is visually distinguished from other serotypes when cultured on blood agar plates where it presents larger, more mucoid colonies. Together with 19F and 23F, which have a thicker capsule *in vitro* as measured with digital fluorescence microscopy, it is more frequently associated with a fatal outcome in IPD[113–115], and is independently associated with a higher incidence of septic shock[116]. In parallel, experimental animal studies revealed that serotypes with a thicker capsule are more virulent[117]. Recent post-licensure studies have revealed that PCV provides significant protection for the vaccine serotypes with exception for serotype 3, even though it generates the most elevated concentration of anti-capsular antibodies after priming[118–120]. The failure of serotype 3 in the PCV13 formula may be due not only to protection from abundant capsule production but the release of capsular polysaccharide during growth, which interferes with antibody-mediated opsonization *in vivo*[121]. The role of the capsules in poor antibody responses is further discussed below.

Who contracts pneumococcal disease?

Colonization is a prerequisite but not enough to cause disease. Multiple risk factors for pneumonia have been defined. Many are associated to poverty such as lack of housing, clean water, malnutrition, cooking facilities and access to basic health care[122]. Indoor air pollution by solid fuel used for cooking, the most common form of preparing food in India and China, increases the risk for severe pneumonia in children[123]. Household crowding and not being vaccinated are other significant risk factors in poor communities[124]. Occupational hazards include exposure to welding- and metal fumes and inorganic dust or chemicals. Metal- and construction workers are at an elevated risk for pneumonia and IPD[125,126].

Chronic infections that impair the immune system is a large risk group. HIV augment the risk multiple fold of contracting pneumonia in both children and adults[127]. Other important risk factors are chronic illnesses such as asplenia, heart and lung disease, kidney failure, liver disease and diabetes. In addition, immune suppressive medications and life style factors like alcoholism and smoking are associated to pneumococcal pneumonia. Dysphagia in elderly and cochlear implants or cerebrospinal fluid leaks also augment the risk[128,129].

Age related factors include high incidences of pneumococcal disease in children < 2 years of age and in adults > 65 years of age.

Carriage and transmission

Colonization of *S. pneumoniae* can occur anytime in life but is most common in childhood with evidence of colonization as early as the first day of life[130]. The prevalence of pneumococcal carriage increases in the first few years of life, peaking at approximately 50% to >70% in hosts 2–3 years of age. In addition to a high prevalence of *S. pneumoniae* carriage, young children also have a higher pneumococcal density in the nasopharynx than older individuals[131]. This might help to explain why children are more efficient in transmitting *S. pneumoniae*. People with pneumococcal disease, or more commonly healthy individuals who carry the organism in the nasopharynx transmit it through respiratory droplets [132]. Animal experiments suggest that transmission can be contact dependent or airborne. In nutrient rich environment, such as saliva, the bacteria survive for days and can be easily cultured from toys in day-care centers[133]. Crowding in places like day-care centres, military camps and prisons promotes horizontal spread and is a risk factor for transmission of drug resistant clones. It has been suggested that the nasopharynx of children is an important global ecological reservoir of drug resistant pneumococci[134]. With increasing age, prevalence of carriage decreases. In geriatric patients in nursing homes and hospitals colonization is rare and risk of spread and infection is low[135,136]. Instead, children have been found to transmit

S. pneumoniae to adults within the household[137]. Interestingly, multiyear surveillance of seasonal trends of IPD demonstrated that older adults, have a high risk of disease caused by paediatric serotypes during holidays when social gatherings usually take place[138].

When commensals misbehave

Pathogens such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* harbouring in the nasopharynx do not cause disease unless there is a change in the milieu. The Eustachian Tube normally regulate airway pressure, clear the middle ear cavity of fluid and protect it from infecting organisms. Infants are susceptible to AOM not only because of immaturity of systemic immunity but also due to immature development of the Eustachian tube and the inability to clear secretions and bacteria from the middle ear[139]. Dysfunction of the tube is a result of inflammation, craniofacial malformations or environmental insults[140]. Viral URI causes nasopharyngeal and Eustachian tube inflammation, mucociliary changes and increases pneumococcal adhesion and invasive abilities, all favouring the entrance of virus and bacteria to the middle ear[141]. Respiratory viruses can be detected in the nasopharynx of up to 90% of children with AOM and in the middle ear fluid of 20%[142]. In pneumonia 54% of children were found to have coinfections with a viral pathogen, most commonly the Influenza viruses[67]. In adults, especially in the elderly, secondary bacterial pneumonia after influenzae is an important cause of mortality. Even without viral promotion of mucal discharge, aspiration of small amounts of oropharyngeal contents occurs in deep sleep, even in healthy individuals, but if the oropharyngeal material includes pneumococcal serotypes associated with invasive infection and if normal clearance mechanisms fail, the colonizers may become pathogens[143].

Breaking the epithelial barrier

When *S. pneumoniae* enters the nasopharynx it undergoes a process called phase variation, expressing a thick, negatively charged capsule that repels the sialic residues of mucus to avoid being trapped. Once the pneumococcus reaches the epithelial cell layer it shifts phase and down-regulate capsule expression and instead expresses proteins that promote adhesion and biofilm formation[144].

The exact method *S. pneumoniae* deploys to overcome epithelial and endothelial barriers to access underlying extracellular matrix and blood stream remains to be determined. It is estimated that pneumococci exhibit over 500 surface proteins[145]. The host secretes antimicrobial IgA to interfere with adhesion and the bacteria counteracts by releasing IgA protease which cleaves IgA. In septic pneumonia, the

bacteria gain access to the blood through the alveolar space, which requires an aggressive interaction with endothelial cells in the capillaries[146]. Adhesins include Pili, the lipoprotein PsA and PavA protein. They interact with host epithelial cell amino sugars (GalNac) and extracellular matrix. Once attached to the human cells, other proteins such as PspA interact with host cell proteins to achieve translocation of bacteria through the epithelial cell [145,146]. Other bacterial surface proteins capture host protease plasminogen to stimulate migration pericellularly and across ECM[147]. As the bacteria replicate they cause tissue damage and inflammation by releasing pneumolysin, neuramidase and hyaluronidase. These enzymes disrupt the connective tissue and ECM to allow even more access to components between cells and below the basement membrane which facilitates access to capillary endothelium and eventually the blood stream[148]. The pneumococcal antigens such as teichoic acid, cell wall complexes and DNA drive the innate immunity and inflammation. These components bind to TLR2, TLR4 and TLR9 and hence contribute to activation of the innate immunity. A strong cell response that involves cytokine release (e.g. IL8 and IL10) is followed by a characteristic neutrophil infiltration of the infected lung[149]. Rapid replication in the alveoles of the lower respiratory tract establishes lobar pneumonia.

Treatment and prevention

Antimicrobial resistance

Beta-lactam antibiotics inhibit the cell wall synthesis of bacteria and is an effective treatment of infections by *S. pneumoniae*. In a classical study in 1964, it was demonstrated that mortality from IPD decreased from 80% to just 17% in patients who received treatment with penicillin[150]. In CAP and upper respiratory tract infections such as AOM, penicillin is recommended in Sweden, whereas in cases of unknown aetiology and severe disease antibiotics with broader coverage might be selected. The first clinical case where an antibiotic resistant *S. pneumoniae* was isolated was in Australia 1967[151]. Since then, resistance to penicillin and is an increasing concern. A key factor influencing the emergence and spread of resistant pneumococci is unnecessary antibiotic use for viral respiratory illnesses. Misdiagnosis of conditions where both viral and bacterial agents cause similar symptoms is common, and patient expectations to prescribe antibiotics is something most primary care physicians face.

Internationally, *S. pneumoniae* non-susceptible to penicillin is increasing but vary widely between countries. In Europe, it varied between 0.6 – 39% in isolates reported from 28 countries. In Sweden the incidence was 6.1% in 2017, and consequently among the lowest on a global scale, but represented an increase compared to 2012. Between 20-40% of *S. pneumoniae* are resistant to macrolides and more than 20% to clindamycin. Resistance to fluoroquinolones is low but increasing[152]. Macrolide non-susceptibility was found in 4.8% of reported isolates in Sweden and has been decreasing recent years [153].

The pneumococcal vaccines

Until 2009 the only vaccine available in Sweden against the pneumococcus was the Pneumococcal Polysaccharide Vaccine (PPV23, Pneumovax[®]). It contains purified capsular antigen from 23 serotypes and provides protection against IPD[154]. In Sweden, PPV23 is recommended for children over 2 years of age and adults with medical conditions that increases the risk of serious pneumococcal infection and in people ≥ 65 years[155]. However, the efficacy of PPV23 is lower in

immunosuppressed individuals, in adults with chronic diseases and has poor protection against pneumonia in the elderly[156]. Children younger than 2 years of age are unable to mount an effective antibody response to pure polysaccharides. Thus, in the two age groups at highest risk of infection the vaccine is suboptimal. The poor immunogenicity in infants is associated to the T-cell independent b-cell response to polysaccharides. The USA was the first country to introduce a new generation Pneumococcal Conjugated Vaccine (PCV) in 2000, immunogenic in infants[157]. Seven polysaccharide antigens (4, 6B, 9V, 14, 18C, 19F and 23F) were conjugated to a diphtheria carrier protein, thereby inducing a T-cell dependant immune response. The direct impact on IPD in children was impressive[158]. Adding to the effect was the “herd immunity” or indirect impact in older age groups[159]. PCV7 was later replaced by several new, higher valent PCVs. In the southern county of Skåne PCV7 was introduced in 2009 and replaced in 2010 by a 10-valent, Protein D-conjugated vaccine (PCV10; adding serotypes 1, 5, 7F). In 2013 a 13-valent CRM197 conjugated vaccine replaced PCV10 (PCV13; adding serotypes 3, 6A, 19A).

Impaired immune response to polysaccharide vaccines

Pneumovax[®] has been associated with an attenuated antibody response upon revaccination. Following PCV7 immunization, a hyporesponse has been demonstrated in the context of prior pneumococcal carriage in the nasopharynx[160–162]. The phenomenon was described by Poolman *et al.* as hyporesponsiveness (or immune tolerance) which “...refers to the inability of the individual to mount an immune response after repeated vaccination or colonization that is less than the same magnitude of immune response induced after primary vaccination...”. They found evidence for hyporesponse in vaccine trials with serotype 3 in PCV formulas[76]. This fact made GSK to exclude serotype 3 in the future PCVs.

Hyporesponse to capsular polysaccharides has been described for meningococcal vaccines as well. The biological basis is not fully understood, but depletion of a specific memory B-cell pool (i.e., clone) has been proposed[163]. Already in 1955 Felton and Ottinger demonstrated that large doses of pneumococcal polysaccharide can act as a paralyzing agent on the adaptive response in mice. In vaccine trials, excessive concentrations of polysaccharide have been found to reduce the antibody response[164]. There is evidence for genetic causes to variations in antibody responses. Genetic variants in the MHC region have been associated to diverse immune responses to vaccines, including PCV7[165]. Musher *et al.* reported hereditary unresponsiveness to PCV in a Jewish community[166].

PCV effects on IPD in Sweden

The introduction of PCV in the child immunization programme in Sweden has so far been a success. Total incidence of IPD has declined from 17.1 to 13.0 per 100,000 citizens. The decrease was most pronounced in children <2 years of age and in older children. Internationally, herd immunity has been repeatedly found to protect the elderly[167,168] but investigations in Sweden found no such indirect effects in people aged >65[169,170].

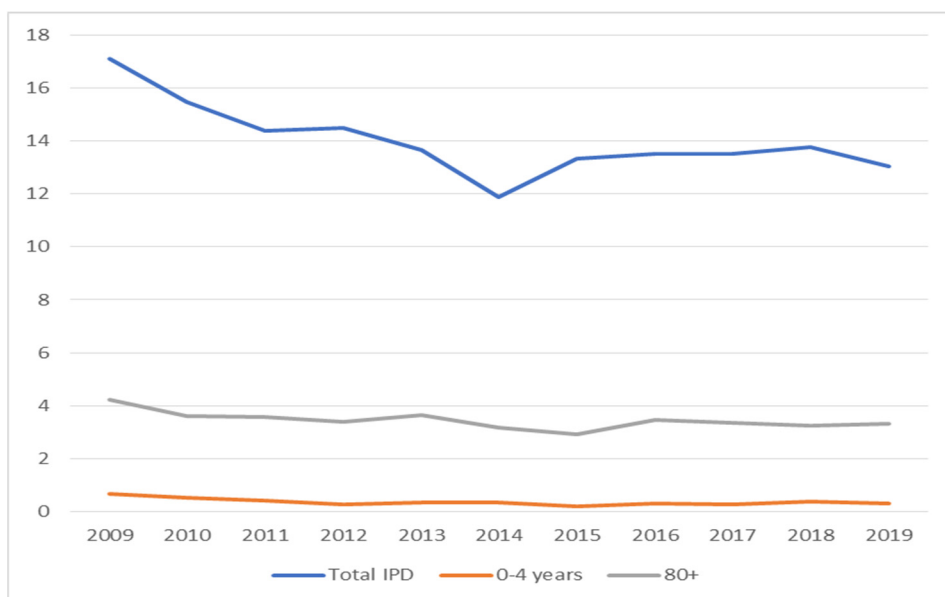


Figure 5. Incidence of IPD in Sweden during a ten year period following introduction of PCV in 2009. Source: National Health Board of Sweden (Folkhälsomyndigheten).

The most common serotypes in year 2017 was serotype 3 (15 %), 22F (10 %), 19A (9 %), 8 (8 %), 9N och 6C och 12F (5 % each)[22].

Serotype replacement

Following introduction of PCVs the serotypes included in the vaccines have been effectively reduced as agents in IPD [171]. Increases in disease caused by non-vaccine serotypes (NVT) (“serotype replacement”) have subsequently offset some of these reductions. PCV7 serotype 6B appears to offer cross-protection against serotype 6A, which is not included in the vaccine. However, such cross-protection has not been observed for 19F and the non-PCV7 serotype 19A [172]. Notably, following PCV7 introduction, the incidence of infections caused by multiresistant 19A has increased. Surveillance programs suggest that NVT are on the rise in the

post PCV7/PCV10/PCV13 era. The worldwide increase in prevalence of serotypes such as 11, 12, 15, 22F, 23A, 23B, 33F, and 35B attest to this [120,173–175].

In asymptomatic carriers the prevalence of NVTs has increased substantially and the net change in carriage prevalence has thus, as a result, been small or absent. The increase observed in NVT caused IPD has in most investigated populations been smaller than the increase in NVT carriage. A possible explanation for this is a lower-case fatality rate among IPD causing NVT:s. Furthermore, the protective efficacy against serotype 3 induced by the 13-valent vaccine has been demonstrated to be very limited[118].

PCV affects polymicrobial carriage and disease

Studies on an effect of PCV on polymicrobial carriage are scarce and with varying results on *H. influenzae* or *M. catarrhalis* carriage[176–178]. However, in a randomised controlled trial of PCV7 on Dutch infants, van Gils *et al.* observed decreased carriage of *M. catarrhalis* in the immunized group compared to controls [179], and in a long-term observational study persistently higher colonization rates were found for *H. influenzae* after PCV introduction [180]. In parallel, Olwagen *et al.* found that PCV vaccination is associated with a higher prevalence of *H. influenzae* [181]. In contrast to carriage, *H. influenzae* has replaced *S. pneumoniae* as the most prevalent pathogen causing OM after introduction of PCV [104,106,182]. It is possible that changes in aetiology represents relative numbers, since recent findings have suggested a decreased absolute incidence of all three species in complicated OM [183].

Objectives

The objectives of the research underlying this thesis were as follows:

- To study the effects of PCV on the interspecies dynamics of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in preschool children with respiratory tract infection.
- To detect changes in serotype distribution related to the introduction of PCV
- To assess the association between serotype and sepsis severity.
- To investigate the antibody response in adaptive immunity after IPD and pneumonia.

Material and Methods

Study design

- Paper I: A retrospective cohort study of association between serotype and sepsis severity in patients with invasive pneumococcal disease in Skåne County between 2006 and 2008.
- Paper II: A retrospective observational study of serotype distribution in prospectively collected upper respiratory tract samples before and after introduction of PCV in the child immunization program in southern Skåne.
- Paper III: A retrospective observational study of the epidemiology of the three most common upper airway pathogens before (years 2004-2008) and after (2009-2017) the introduction of PCV in southern Skåne.
- Paper IV: A retrospective cohort study using convenience samples of acute and convalescent serum from IPD patients. Pneumococcal killing is measured in an opsonophagocytic assay and compared between serotypes.
- Paper V: A retrospective cohort study of the antibody response in prospectively collected sera from patients with pneumonia, with or without sepsis.

General methods and definitions

Vaccine schedule

In the southern county of Skåne in Sweden, immunization with the 7-valent PCV Prevenar® was initiated in January 2009 and was administered on a 2 + 1 schedule at 3, 5 and 12 months of age. It was replaced by PHiD-CV10 (Synflorix®; PCV10) in June 2010, which was also administered to children who had received one or two doses of PCV7. PCV10 was replaced by PCV13 in 2014. A high coverage was obtained from the start and among children born in 2009 the coverage was 97.5 % at 2 years of age.

Sepsis definition

In order to determine disease severity and septic shock the Sepsis 2 criteria was used. Sepsis 2 is a modified version of the first comprehensive definition first published in 1992[97] (It has since the study was performed been replaced by sepsis 3). Sepsis 2 is based on the conception of SIRS (Systemic inflammatory response syndrome).

SIRS is met with ≥ 2 of

- Pulse >90 /minute
- Respiratory rate >20 /minute
- WBC $>12 \times 10^9/l$ or $<4 \times 10^9/l$ or >10 percent immature forms
- Body temperature $>38^\circ\text{C}$ or $<36^\circ\text{C}$

SEPSIS: Verified or probable infection together with SIRS

SEVERE SEPSIS: Sepsis criteria fulfilled together with either hypotension, hypoperfusion or organ dysfunction.

SEPTIC SHOCK: Severe sepsis criteria met together with sepsis-induced hypotension persisting despite adequate fluid resuscitation.

Criteria for hypotension, hypoperfusion or organ dysfunction:

HYPOTENSION: Systolic blood pressure ≤ 90 mm HG or mean arterial pressure (MAP) ≤ 70 mm HG

HYPOPERFUSION: B-Lactate >3 mmol/L or 1 mmol over the upper normal limit, alternatively base excess ≤ 5 mmol/L.

ORGAN DYSFUNCTION:

Renal: Oliguria $<0,5$ ml/kg/hour for at least two hours despite adequate fluid resuscitation.

Respiratory: $p_a\text{O}_2/\text{FiO}_2 <33$ or <27 if the lung is the focus of infection.

Hematologic: Blood platelets $<100 \times 10^9$ or INR $<1,5$ or APTT >60 s

CNS: acute alteration of mental state, for example confusion

Hepatic: S-bilirubin >45 $\mu\text{mol/L}$

Culturing and serotyping

Cultures, identification and antimicrobial susceptibility tests were performed at the Clinic of microbiology at Skåne University Hospital according to national guidelines (see reference in Paper I). Culturing of *S. pneumoniae* was completed on sheep blood agar plates overnight in 5% CO₂ incubators at 37°C. Initial serotyping into serogroups (e.g. 19, 23 etc.) was performed with a gel diffusion method with antisera from Statens Serum Institute (SSI; Copenhagen, Denmark). Further serotyping of subtypes (e.g. 19F, 23B etc.) was performed using the *Quellung* reaction with antisera (SSI). The *Quellung* reaction is based on Neufeldt's method (see previous chapter) in which serotype specific anti-capsular antibodies bind to the bacterial surface, causing the capsule to swell and the bacteria to immobilize and agglutinate. The reaction is visible in microscope and is the gold standard method for pneumococcal capsular serotyping[184]

Invasiveness of serotypes

Serotypes differ in their ability to cause invasive disease and in prevalence of nasopharyngeal colonization. Brueggeman *et al.* studied the invasive disease potential of different *S. pneumoniae* serotypes in children. The authors concluded that some serotypes (including 3, 6B, 15B/C, 19F, and 23F) conveyed a lower risk for invasive disease, and were more frequently isolated as colonizing bacteria than other serotypes (including 1, 4, 7F, 14, and 18C)[19]. These results have been confirmed by other groups [185,186]. Low invasive serotypes are associated with higher case-fatality rates and disease in immunocompromised patients, acting as “opportunistic” bacteria, whereas highly invasive serotypes more often infect healthy, immunocompetent individuals, acting as primary pathogens.

Statistical methods

In Paper II and III yearly estimates of prevalence (including 95% confidence intervals) for the respective bacterium as well as co-culture combinations (Paper III) were calculated. Prevalence for the total pre-vaccine and post-vaccine period were estimated and prevalence ratios were statistically compared. The population sizes in the studied geographical area, comprising of yearly numbers of children ≤ 6 per year in Paper III and the whole population in Paper II, were accessed from Statistics Sweden. Considering that outcome data were counts, univariate negative binomial regressions (since Poisson regressions were overdispersed) were performed to assess crude prevalence ratios comparing the pre- and post-vaccine periods. However, since data suggested a trend of general reduction of nasopharyngeal cultures over time in Paper III, and in order to better assess the association between increasing PCV vaccine coverage rates and the respiratory tract bacterial carriage

dynamics, multivariate negative binomial regressions using yearly bacterial counts as outcome variables and yearly vaccine coverage rates as the main predictor were performed. This analysis was adjusted for population as well as the total number of yearly nasopharyngeal cultures drawn. As a complementary analysis, ratios of positive samples in relation to total referrals in the pre- and post-vaccine period were compared using Fischer's exact test.

In Paper I, serotype dependent disease severity in IPD was determined in two different ways. The predefined classifications into low, intermediate and highly invasive serotypes were used. Generalized Fisher's exact test was used to compare binary data between the three groups and Kruskal Wallis test for ordinal data. In addition, serotypes were compared one by one to serotype 14 as a reference based upon previous research by Brueggeman *et al.*[19].

In Paper IV three groups (decreased antibody response, nonresponse and increased antibody response) were compared using the Chi square test for categorical variables and the Kruskal-Wallis test for continuous variables. Moreover, multivariate logistic regressions were performed to assess the association between the type of antibody response and clinical as well as bacterial predictors. In Paper V results were compared between any two groups using the Mann-Whitney U test and between any three groups using the Kruskal-Wallis *H* test. To test for equality of proportions between groups, the Pearson chi-square test or, if any cells had an expected count of less than five, Fischer's exact test was used.

Study specific methods and hypothesis

Paper I

Our hypothesis was that serotypes differ in their capacity to cause septic shock, a lethal clinical condition with approximately 40% mortality[187]. The county of Skåne in southern Sweden including 1.2 million inhabitants was the study area. Included in the study were 513 adult patients with IPD in the pre-vaccine era 2006-2008. The pneumococcal vaccine coverage was low in this community[100].

Serotype, co-morbidity and sepsis severity were determined. The following data were collected from the patients' medical records: medical condition upon admission, age, gender, admission to an ICU, 28 day- and one year mortality. Co-morbidities were noted when a diagnosis was specified in the records, and the diagnoses included were divided into these categories: heart, lung, haematological, and autoimmune diseases, liver and renal failure, diabetes mellitus, cancer, splenectomy, and HIV. Access to medical records and lab data and helped to characterize the disease severity in each case. Sepsis shock was determined according to the accepted definition at the time, see above. Serotypes were compared to serotype 14 as a reference and grouped according to their invasive potential.

Paper II

Our research question was: will PCV cause a shift in pneumococcal upper respiratory tract infections, from PCV serotypes to those not included in the vaccine? In order to detect changes in serotype distribution cultures from the nasopharynx, middle ear fluid and sinus cultures referred from physicians to a single laboratory for microbiological analysis were stored and serotyped. The effect of PCV on IPD is frequently reported, but the impact on upper respiratory tract infections in a clinical setting is less documented. We compared 2 years before (2007/2008) with 3 years after (2011–2013) initiation of the immunization campaign in 2009. Clinical isolates of *S. pneumoniae* from the upper respiratory tract identified at the local Clinical microbiology lab (Malmö, Sweden) were included and serotyped. This catchment area was unchanged during the study years, while the population increased from 473,245 to 526,092 inhabitants. Sixty-seven percent were referred from outpatient clinics and 33 % from hospital inpatient wards. According to regional guidelines, upper respiratory tract cultures are recommended in patients with recurrent AOM, treatment failure of AOM, community-acquired pneumonia (CAP) in adults and complicated respiratory tract infections, but can also be obtained in other clinical situations at the discretion of the referring physician [19]. Routine culturing from nasopharynx in adults with suspected pneumonia admitted to hospital was included in the guidelines in 2009.

Patients with a positive pneumococcal culture in addition to concurrent typical symptoms of AOM according to the description of the referring physicians were identified. According to current guidelines, which do not recommend culturing on uncomplicated URTI, the identified URTIs in our cohort were prolonged or complicated cases.

Paper III

The objective was to study effects of PCV introduction on the interspecies dynamics of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* in the respiratory tract of pre-school children in Skåne county, Sweden. All nasopharyngeal culture samples from children ≤ 6 years of age from the Clinical laboratory of southwestern Skåne collected 2004-2017 were analysed. Pre-vaccine prevalence of single as well as co-cultures of the three species were included (years 2004-2008) were compared to post vaccine prevalence (2009-2017). The population of children ≤ 6 year-old in the area increased from 33.640 individuals in 2004 to 50.603 in 2017. During this period 14.473 nasopharyngeal cultures were analysed. A subset of the *S. pneumoniae* isolates had been stored and serotyped in Paper II (n=1747), which enabled an analysis of associations between individual serotypes and co-cultures of *M. catarrhalis* or *H. influenzae*. Local guidelines for upper respiratory tract culture described for Paper II above applied. Apart from information on bacterial findings patient characteristics such as age, sex, date of culture, site of culture were available. The population in the catchment area was derived from Statistics Sweden.

Paper IV and V

As previously described, Pneumococcal polysaccharide vaccines may elicit a hyporesponse under certain conditions. There is limited knowledge, however, on the type of specific antibody response in individuals with pneumococcal infections.

To investigate the dynamics of naturally acquired anti-pneumococcal antibodies in relation to an episode of infection we performed two studies using OPA to determine functional antibodies before and after disease. In Paper V, opsonic antibody activity was studied with paired acute-phase and convalescence sera obtained from 54 patients with pneumococcal community-acquired pneumonia (CAP). Results were compared with clinical characteristics and anti-capsular immunoglobulin (Ig) concentrations, information that had been published previously by Athlin *et al.*, one of the contributing authors of the paper[188]. The patient sera were obtained from a subsample of patients from a cohort at Örebro University Hospital in 1999-2002. Our subsample included only patients infected by serotypes in PCV13.

In order to explore serotype-dependent differences in Paper IV, the antibody response to IPD sera from patients in the Örebro University cohort was used together with a convenience sample from Biobank Skåne. In all, 40 pre/acute-IPD and post-IPD sera, was tested in immunological assays (Figure 6). Among them, 20 paired sera had been collected in acute phase upon admission to hospital and at 5 weeks convalescence. Another 20 paired sera were acquired 2-24 months before and 6-60 months after disease. Furthermore, we sorted pneumococcal serotypes into two groups according to invasive potential. Twenty paired sera from patients infected by low invasive serotypes were included (serotype 3, $n = 12$; 19F, $n = 4$; 23F, $n = 4$), and for comparison, 20 paired sera from patients infected by highly invasive serotypes (serotype 7F, $n = 11$; 14, $n = 5$; 1, $n = 2$; 4, $n = 2$). Patient medical records were available for review of age, sex, co-morbidities, clinical condition upon admission to hospital, laboratory data and microbiology findings.

The functionality of antibody titers in both papers was determined by a single serotype Opsonophagocytic assay (OPA). The human promyelocytic cell-line HL60 was differentiated into neutrophils by propagation with addition of 0.8% dimethylformamide (DMF) for 5–6 days before use in the OPA. To prevent bacterial clumping, the opsonization and phagocytosis steps were performed with microtitre plates on a mini orbital shaker (700 rpm). Patient sera were tested in two separate experiments in duplicates against the homologous serotype that had caused IPD. OPA titers were defined as the serum dilution that killed 50% of bacteria compared to controls containing no patient serum but cells only. Depending on the results patients paired sera exhibited increased, decreased or unchanged pneumococcal killing.

In Paper IV, we utilized a WHO standardized Enzyme-Linked Immunosorbent Assay (ELISA) for determination of capsular antigen IgG concentration. Serotype 22F specific capsular polysaccharide and cell-wall polysaccharide (both from SSI)

were used for blocking of unspecific binding, and 007sp was used as reference serum[189]. A ratio between pre-/acute-IPD and post-IPD was calculated for each patient. In Paper V, ELISA results from a previous study on the cohort was re-analyzed[188].

To exclude bias of IgG deficiencies in both papers sera was tested for levels of IgG and IgG2 in an in-house ELISA utilizing rabbit anti-human IgG and IgG antibodies (DARKO).

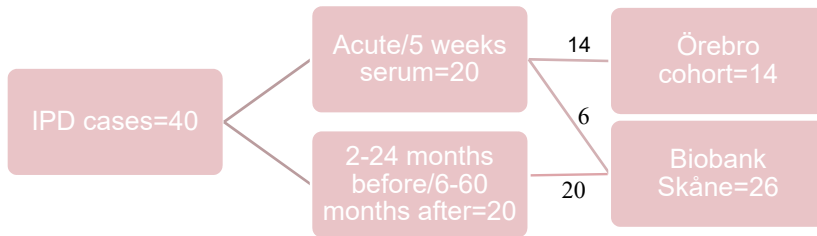


Figure 6. Paired sera from 40 cases of IPD were included in Paper IV. Sera was collected at different time points in relation to IPD, as illustrated. Either acute and 30 days after or several months before and after.

Results

Paper I

A total of 551 patients were included in this analysis with a median age of 66 years (range 0–101 years). In all, 513 pneumococcal isolates were serotyped. The most abundant serotypes were 14 (12.5%) and 7F (12.2%). The majority of patients suffered from pneumonia (86%), and serotypes 14, 7F, 4, 9V, and 3 represented 49% of the isolates.

Previous research has identified serotype 3 as associated with higher case fatality in IPD. Our study supported this finding and added that serotype 3 was found to be associated to septic shock, a lethal clinical condition with a mortality rate of about 40%[187]. In our study, serotype 3 had significantly more often septic shock (25%, odds ratio (OR) 6.33 [95% confidence interval (CI) 1.59-25.29]), higher mortality (30%, OR 2.86 [CI 1.02-8.00]), and more often co-morbidities (83%, OR 3.82 [CI 1.39-10.54]) when compared to serotype 14. Serotype 19F was associated with a significantly higher percentage of patients that were admitted to the ICU (OR 6.50 [CI 1.90-22.25]) compared to serotype 14. This is in accordance with the knowledge that serotype 19F is one of the most encapsulated serotypes and is associated with a high risk of mortality [115]. The broad CI:s suggest that the inference is based limited numbers of cases.

Furthermore, we found that low invasive serogroups (3, 6, 8, 15, 19, 23 and 33) caused IPD in older and sicker patients. The median age and percentage of patients with underlying co-morbidities were 72 years and 79%, respectively, for serogroups associated with low invasiveness, 68 years and 61%, respectively, for serogroups with intermediate invasiveness, and, finally, 62 years and 48%, respectively, for serogroups with high invasiveness. These findings are in concordance with previous observations[111]. The number of isolates for each serotype was relatively low, *i.e.*, 23 serotypes represented 10.1% of the isolates. Another limitation is the difficulty to assess sepsis severity retrospectively. All clinical data may not be accounted for in the medical records and larger prospective clinical and laboratory studies are required to study serotypes one by one, and to determine whether some serotypes are more virulent and cause more severe clinical disease than others.

Table 2. Characteristics of Patients with IPD Shown by Infecting Serotype and Statistical Comparison in Relation to Infection with Serotype 14. Bold characters indicate statistical significance.

Serotype (n)	Septic shock		28 day mortality		Admitted to the ICU		Any co-morbidity		Median age	
	No (%)	OR (CI)	No (%)	OR (CI)	No (%)	OR (CI)	No (%)	OR (CI)	Years (range)	p-value
14 (n =60)	3 (5)	1	8 (13)	1	8 (13)	1	35 (58)	1	70 (20–94)	-----
1 (14)	0 (0)	-----	0 (0)	-----	0 (0)	-----	4 (29)	0.31 (0.09-1.07)	49 (32–68)	<0.001
3 (36)	9 (25)	6.33 (1.59-25.3)	11 (30)	2.86 (1.02–8.00)	8 (22)	1.86 (0.63–5.48)	30 (83)	3.82 (1.39–10.5)	75 (40–94)	0.530
4 (55)	6 (11)	2.32 (0.55–9.79)	3 (55)	0.37 (0.09–1.49)	7 (13)	0.95 (0.32–2.81)	30 (55)	0.92 (0.44–1.92)	66 (20–91)	0.550
6A (20)	0 (0)	-----	3 (15)	1.15 (0.27–4.82)	0 (0)	-----	14 (70)	1.78 (0.60–5.28)	73 (34–91)	0.669
6B (24)	1 (4)	0.83 (0.08–8.36)	1 (4)	0.28 (0.03–2.39)	3 (12)	0.93 (0.22–3.84)	18 (75)	2.29 (0.80–6.59)	73 (34–97)	0.760
7F (65)	7 (11)	2.29 (0.56–9.31)	6 (9)	0.66 (0.22–2.03)	12 (18)	1.47 (0.56–3.89)	33 (51)	0.74 (0.37–1.50)	63 (19–95)	<0.001
8 (18)	2 (11)	2.37 (0.36–15.4)	2 (11)	0.81 (0.15–4.2)	2 (11)	0.81 (0.16–4.22)	11 (61)	1.20 (0.41–3.53)	64 (49–86)	0.051
9V (41)	4 (10)	2.05 (0.43–9.71)	4 (10)	0.70 (0.20–2.5)	8 (20)	1.58 (0.54–4.61)	27 (66)	1.42 (0.65–3.39)	69 (41–97)	0.381
18C (16)	2 (12)	2.71 (0.41–17.8)	4 (25)	2.17 (0.56–8.40)	4 (25)	2.17 (0.56–8.40)	14 (88)	5.35 (1.12–25.6)	70 (37–85)	0.199
19A (7)	0 (0)	-----	0 (0)	-----	1 (14)	1.08 (0.11–10.2)	6 (86)	4.59 (0.52–40.5)	70 (34–91)	0.845
19F (16)	3 (19)	4.38 (0.79-24.2)	4 (25)	2.17 (0.56–8.39)	8 (50)	6.50 (1.90-22.2)	13 (81)	3.31 (0.85–12.8)	64 (24–91)	0.395
23F (25)	0 (0)	-----	0 (0)	-----	1 (4)	0.27 (0.03–2.29)	21 (84)	4.1 (1.23–13.1)	73 (23–101)	0.835

Paper II

The aim of this 5-year observational study was to investigate changes in serotype distribution in URTI by *S. pneumoniae* following the sequential introduction of PCV7 and PCV10. We found an overall decrease in the prevalence of pneumococcal isolates examined and an increase in non-vaccine serotypes as agents of disease.

Our study was initiated in 2007 and continued through 2013. Clinical isolates of *S. pneumoniae* from the upper respiratory tract (from nasopharyngeal swabs, middle ear fluid and sinuses) identified at the local Clinical microbiology lab (Malmö, Sweden) were included. Upper respiratory cultures before PCV ($n=1566$) positive for *S. pneumoniae* were serotyped and compared to 1707 cultures after (years 2011-2013). The proportion of pneumococcal serotypes covered by PCV10 decreased from 45 to 12% ($p<0.001$). Non PCV10 serotypes increased from 49 to 80% ($p<0.001$). This serotype replacement was in concordance with previous research. In spite of the increase in non-vaccine serotypes post PCV we observed an overall decline of 35% in *S. pneumoniae* positive cultures. Furthermore, the number of pneumococcal positive middle ear secretion cultures decreased by 32% ($p<0.001$).

Paper III

We analysed a total of 11,810 positive cultures from the nasopharynx of children age ≤ 6 years. We found that the prevalence of clinical nasopharyngeal cultures of *M. catarrhalis* in addition to *S. pneumoniae* decreased in children following sequential introduction of PCV7, PCV10 and PCV13. *S. pneumoniae* decreased by 65.5% (16.6 to 5.7 per 1,000 person-years; $p<0.001$), whereas *M. catarrhalis* and *H. influenzae* decreased by 52.6% (21.7 to 10.3 per 1,000 individuals; $p<0.001$) and 47.61% (13.7 to 7.3 per 1000 individuals; $p<0.001$), respectively. In multivariate negative binomial regressions adjusted for yearly numbers of samples taken, *S. pneumoniae* and *M. catarrhalis* were significantly negatively associated with increasing vaccine coverage proportions (adjusted prevalence ratio: 0.17, $p<0.001$ and aPR=0.48, $p<0.001$, respectively) while *H. influenzae* (aPR=0.75, $p=0.17$) was not. The absolute incidence rates of *H. influenzae* decreased but not when adjusted for the total number of samples taken. Importantly, at the end of the surveillance period, *M. catarrhalis* and *H. influenzae* were more common than *S. pneumoniae* in cultures from nasopharyngeal swabs. The absence of any decrease in the proportion of *H. influenzae* and the increase of *H. influenzae* pure cultures in the multivariate model suggests an insignificant influence of PCV on the prevalence of this bacterial species. A positive association between pneumococcal vaccine serotypes in general and two individual vaccine serotypes (19F; $p<0.001$ and 23F; $p=0.043$) in particular

in polymicrobial growth with *M. catarrhalis* is a novel observation not previously seen. This phenomenon needs to be verified and mechanistically examined in future studies.

Due to the retrospective nature of the study we identified the possible confounding factors age and penicillin susceptibility in pneumococcal isolates but no differences between serotypes in *M. catarrhalis* co-colonizations were found.

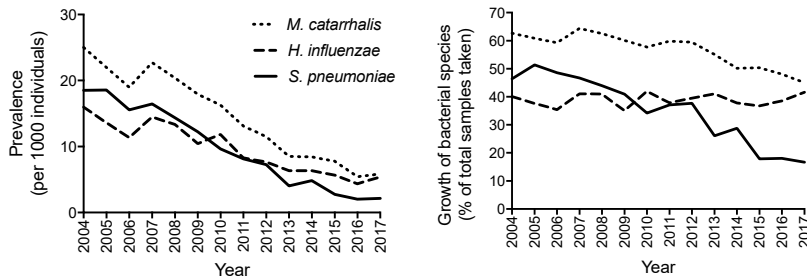


Figure 4. Prevalences and proportions of the three main pathogens detected in the upper respiratory tract of children 0-6 years of age having clinical symptoms of upper respiratory tract infections.

Paper IV

Patients were diagnosed with pneumonia (38/40 cases), meningitis or ethmoiditis (1 case each). Median age was 59.5 years (range 14–91 years) and 55% ($n = 22$) were women.

Variable OPA responses were observed between serotypes. According to the antibody responses detected, patients were categorized either as having a functional antibody response as judged by titres in OPA (increasing titres, $n = 16$, Figure 3), a nonresponse (no change in titre, $n = 18$), or a decreased response (reduced titres in OPA; $n = 6$). The majority of patients ($n = 24$; 60%) thus had a non-functional antibody response, that is, either a decreased or nonresponse.

In total, only 4 of 20 cases (20%) with low invasive serotypes (3, 19F, and 23F) developed a functional antibody response as compared to sera from patients that had been infected with highly invasive pneumococcal serotypes (1, 4, 7F, and 14; $p = 0.022$). Twelve out of 20 cases (60%) developed an increased antibody response in this group.

In a multivariate logistic regression model with functional responses collapsed to a dichotomous response variable (functional antibody response vs. non-functional antibody response), infection by a low invasive serotype (3, 19F, and 23F) was the only predictor significantly associated with a non-functional antibody response, adjusted for old age and disease severity ($p = 0.015$). Time of sera collection post-

IPD was also determined as it might influence the quality or quantity of antibodies, but was not associated to the results in OPA. We determined total IgG or IgG2 in pre-/acute-IPD sera by ELISA. Values according to local clinical guidelines for measurement of deficiencies in IgG (6.7–15.5 g/L) and IgG2 (1.15–5.7 g/L) were used as reference. All titres were above the lower reference limits of Ig concentrations.

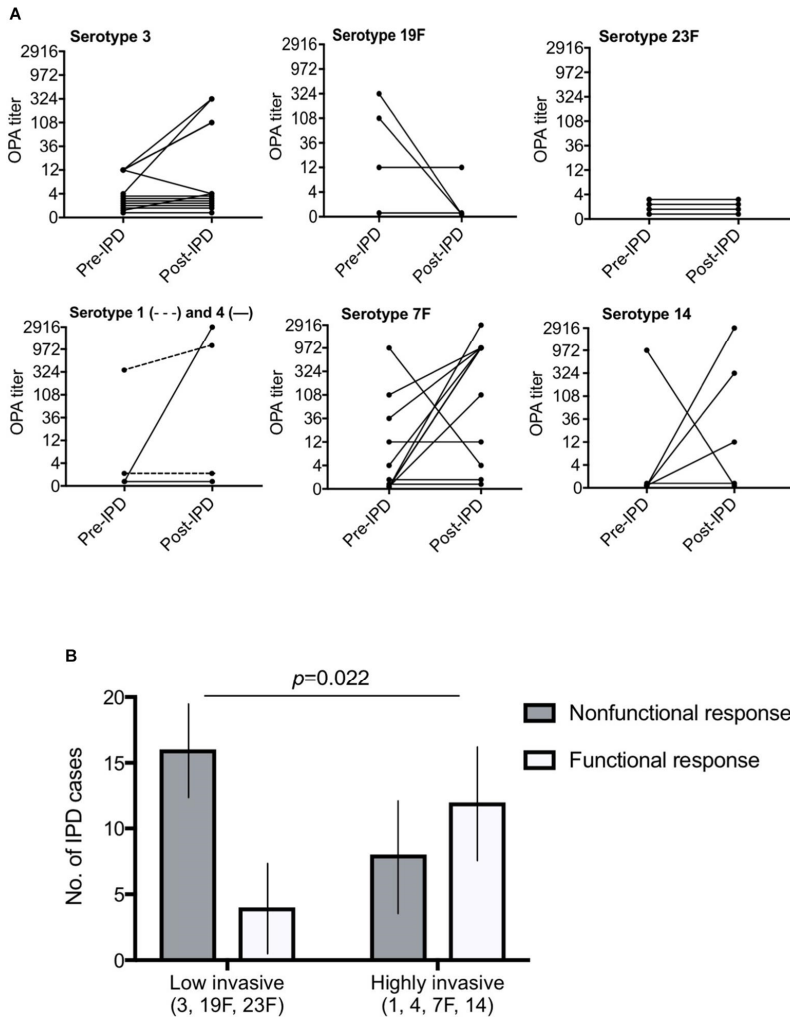


Figure 3. In **(A)**, 7 different pneumococcal serotypes were analyzed by OPA. The threshold for significant bacterial killing was defined as an OPA titer >1:8. Each line represents pre-IPD and post-IPD sera from one patient. A decreased response was defined by a decreasing curve, whereas an increasing curve and titers above the threshold indicates a functional antibody response. Undetectable titers or titers below the 1:8 titer threshold pre- and post IPD were nonresponsive. **(B)** Low-invasive serotypes (3, 19F, and 23F) were significantly associated with a nonfunctional antibody response (decreased or nonresponsive; Fisher's exact test $p = 0.022$) compared to highly invasive serotypes (1, 4, 7F, and 14). Invasive potential was defined according to a study by Brueggemann et al. (2004). Black vertical lines represent 95% confidence interval.

Paper V

More than a third of patients (35%) had a non-functional opsonic antibody response (characterized by a decreased convalescence serum OPA titre as compared to the acute-phase serum, or undetectable titres in both sera)

Remaining individuals exhibited either an increased convalescent OPA titre or detectable, but unchanged, titres at both time points. Cases of IPD was significantly associated to a non-functional convalescent antibody response, (53%; $p=0.019$). Moreover, as in Paper IV a poor correlation was found between anti-capsular Ig concentration and OPA titres.

During the acute-phase, undetectable OPA titres were significantly more common among patients younger than 65 years (68% vs. 39%; $p=0.032$). However, the lowest median age (61 years) was found in those with an increased convalescent OPA titre ($p=0.028$).

Discussion

Serotype as a risk factor for septic shock in IPD

IPD is one of the main causes behind childhood mortality. The serotypes most commonly involved are therefore the target of conjugated vaccines. There has been some debate on whether serotypes differ in their capacity to cause severe disease such as septic shock or death. Some studies suggest that host factors such as underlying disease and age are more important determinants than a particular serotype [190,191]. In contrast, certain serotypes are associated with more severe outcome and mortality even after adjustment for relevant host factors[192,193]. In Paper I we found evidence of serotype 3 as a risk factor for septic shock in IPD. The clinical outcome in IPD related to serotype is usually studied with case fatality rate as endpoint. However, this approach may be biased since serotypes with low case fatality rates infect younger and healthier patients and vice versa. Our hypothesis was that septic shock as a primary clinical outcome would give a more valid picture of the virulence since shock is a result of the immune response induced by the virulent microorganism in question. Due to the retrospective nature of this study incomplete data sets were occasionally found in the medical records of patients with less severe disease. The validity of data is difficult to assess in retrospect. Moreover, the number of isolates for each serotype was relatively low, and our results should be included in future meta-analysis or replicated in multicenter studies with more patients to determine whether some serotypes are more virulent and cause more severe clinical disease than others. Of interest, serotype 3 has been identified as a possible vaccine evader and the impact of PCV on serotype 3 caused IPD is questioned [169,194,195]. A possible route to control this microbe would be a protein or whole cell-based vaccine, that bypasses its highly virulent capsule.

Serotype replacement after PCV introduction

The serotype replacement observed in this study is in concordance with previous research [196]. The vacant niche left by eradicated vaccine type pneumococci is rapidly occupied by non-PCV10 serotypes such as 19A and 3. However, the increases are only relative as there was an overall reduction of *S. pneumoniae* in nasopharyngeal specimens. Cross-protection of capsule polysaccharides included in

the vaccine could have contributed to the overall reduction. Previous results from studies on PCV7 and PCV10, which both contain the serotype 6B capsule, have suggested significant cross-reactivity against serotype 6A, and subsequent protection from disease caused by this particular serotype, a finding supported by our results[197,198]. Pre-marketing studies suggested a 19F-19A cross protection[172]. No indication of such cross-reactive antibodies was found in our study. Instead, we found that the absolute and relative incidence of *S. pneumoniae* serotype 19A increased during the observation period, and together with serotype 3 comprised 18.8 % of all *S. pneumoniae*, 4 years after PCV introduction with PCV7 and then PCV10. Both serotypes 3 and 19A are included in PCV13, which raised the question of possible benefits for this vaccine in the study population. A more recent Swedish study demonstrated no PCV10 or PCV13 effect on serotype 3 in Sweden, but decreasing incidences of 19A in areas that have used PCV13. However, differences in overall impact of IPD incidences were not statistically significant irrespective of vaccine used[170]. Serotype 11A increased significantly, which is in accordance with international studies, that have also suggested expansion of an antibiotic non-susceptible clone[37,199]. Increased prevalence of 15B has been found in several post-PCV studies and capsular switching from 19A because of selective PCV13 pressure was suggested as an explanation[200]. However, our study revealed 15B increase in a PCV10 setting, suggesting other mechanisms also involved[76]. A switch between serotype 15B and 15C capsule takes place frequently in some isolates, but in our study 15C was uncommon suggesting few such events[201]. The challenge that serotype replacement present may be overcome temporarily with higher valent vaccines.

IPD induces a poor antibody response

Our results indicate that natural immunization by IPD in a majority of cases fail to develop functional antibodies. Instead of the expected increase in antibody mediated pneumococcal killing, no change or even a hyporesponse (a decreased killing) in the convalescent sera compared to the pre- or acute sera was found. The poor correlation between IgG concentration in ELISA and pneumococcal killing in OPA suggests that other factors than capsule-specific IgG concentrations are of importance. Furthermore, the non-functional antibody response seems to be serotype dependent. Serotypes known to be more heavily encapsulated and low invasive were in our study associated to a non-functional antibody response. However, less encapsulated serotypes induced a poor immune response in some cases. Factors such as immunosenescence, individual variations in host immune system and previous exposure to the antigen could possibly influence the results.

Based upon previous studies, a plausible theory, also put forward by Poolman *et al.*, is that the massive exposure to antigen by heavily encapsulated bacteria in IPD

depletes the antibody pool and possibly induces apoptosis of serotype-specific B-cells[76,163]. In vaccine trials, excessive concentrations of polysaccharide have been found to reduce the antibody response[164]. Another possible explanation is suggested by sepsis models in animals. The powerful immunological reaction observed in septic patients is followed by an immune suppressive state that might cause prolonged defects in humoral and cellular immunity, as demonstrated in mice[202,203].

Prior carriage of *S. pneumoniae* in the nasopharynx, as well as prior bacteremia has been associated with a hyporesponse to PCV and PPV23, respectively[160,204]. Ekdahl and collaborators suggested that IgG deficiency caused the poor antibody response in patients with prior IPD when immunized with PPV23. We excluded IgG deficiency as the cause in our cases. It cannot be excluded, however, that a nonfunctional antibody response induced by IPD resolves over time. In a recent vaccine trial, the hyporesponse induced in toddlers by a combined schedule of PCV7 and PPV23 was not sustained when the children were in preschool age[205].

In the present studies, serum concentrations of serotype specific IgG were determined, and a ratio between post and pre-IPD serum was calculated. Patients with a functional antibody response had higher IgG titer ratios, which may indicate that an antibody increase is necessary for efficient pneumococcal killing. Nevertheless, a divergence in IgG ratios and results obtained by OPA was found in several cases. These findings are also supported by a Japanese study, where infants immunized with PCV7 demonstrated protective IgG titers post-IPD, but all 17 patients had suboptimal responses in OPA. Low avidity of serotype-specific antibodies was suggested as the cause[206]. We tested the hypothesis that lower avidity of anti-capsular IgG antibodies post-IPD may contribute to the discrepancy in some patients that were nonresponsive in OPA in spite of a high IgG-ratio. We did not, however, find any changed avidity that could explain the discrepancies observed with high IgG ratios and a nonresponsive opsonophagocytosis (data not shown). Another possible explanation for diverging results between the serum tested in OPA and IgG ratios may be differences in levels of anti-pneumococcal polysaccharide IgM levels. Park *et al.* found that low levels of IgM in older adults contributed to a poor opsonization of pneumococci [207].

Our studies included only 40 and 56 patients respectively and only a few of known serotypes. To disentangle the relative importance of serotype and host factors for IPD antibody response, larger prospective investigations should be undertaken.

PCV effects on nasopharyngeal pathogens

Previous studies on effects of PCV on other nasopharyngeal pathogens than *S. pneumoniae* have produced mixed results. Some studies have not shown any PCV-dependent effect on *H. influenzae* and *M. catarrhalis* carriage [177,178,208,209], while other investigations have suggested a decreased carriage of *M. catarrhalis* [179] as well as higher colonization rates for *H. influenzae* [181,210,211]. *H. influenzae* has replaced *S. pneumoniae* as the most prevalent pathogen causing otitis media (OM) after introduction of pneumococcal conjugated vaccines (PCV) [104,106,182]. The change in rank could be in relative incidence and not in absolute numbers, and recent findings have suggested a decreased incidence of all three species in complicated OM [183]. Furthermore, *S. pneumoniae* and *H. influenzae* may be positively associated in colonization but negatively associated in URTIs [57]. Interestingly, Lysenko *et al.* reported that co-colonization with *H. influenzae* and *S. pneumoniae* in a murine mouse model resulted in a rapid clearance of the latter species after *H. influenzae*-dependent activation of phagocytosing neutrophils [58]. In contrast, *S. pneumoniae* has developed different strategies to clear *H. influenzae*, including the release of bactericidal hydrogen peroxide [32]. Since *S. pneumoniae* and *H. influenzae* were rarely found in specimens taken from the nasopharynx, our results together with available data suggest that there is an antagonistic relationship between *S. pneumoniae* and *H. influenzae* in URTI.

Our results suggests a negative effect on *M. catarrhalis* carriage in URTI. We further suggest that PCV-serotypes are associated to *M. catarrhalis* co-cultures. PCV introduction might therefore explain the negative impact on *M. catarrhalis*. *H. influenzae* incidence was less affected, if at all, and no associations between specific pneumococcal serotypes was found.

These findings are important in the evaluation of vaccine programmes. All three pathogens decreased in prevalence in absolute numbers. If our results are replicated the introduction of PCV can be expected to yield positive effects not only on IPD but on bacterial URTI in general.

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Streptococcus pneumoniae has caused immense suffering and death throughout history of man. Research on the bacteria has led to some of the most astonishing scientific discoveries of modern medicine. In spite of effective treatments and vaccines, *S.pneumoniae* is still one of our biggest microbiological enemies. In this dissertation the effects of pneumococcal conjugated vaccines in Skåne is evaluated and new findings on the virulence of the bacteria is presented.

