Invasive Pneumococcal Infections

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ABSTRACT

Streptococcus pneumoniae is a major cause of disease, ranging from uncomplicated respiratory infections to severe invasive pneumococcal disease (IPD), including bacteraemic pneumonia, septicaemia with unknown focus and meningitis. Case fatality rate (CFR) remains high and antibiotic resistance is increasing globally. *S. pneumoniae* is surrounded by a polysaccharide capsule that can be divided into more than 90 immunologically different serotypes. Vaccination may reduce morbidity and mortality due to IPD. Two vaccine types exist: pneumococcal polysaccharide vaccine (PPV-23) and pneumococcal conjugate vaccines (PCV-7, 10, 13). The former contains 23 serotypes, but does not work in small children, whereas the latter also protects children below two years of age, but includes only 7, 10 and 13 serotypes, respectively.

The aim was to explore the epidemiology of IPD before the introduction of PCV-7 in the Swedish childhood vaccination programme, in January 2009: serotype distribution, antimicrobial susceptibility and potential vaccine coverage among isolates causing IPD; mortality, case fatality rate and incidence of different IPD manifestations related to age and risk groups; the impact of serotype and genotype on manifestations and outcome; and finally, long-term changes in the epidemiology during 45 years.

Consecutive isolates and clinical data from 836 adults and children with IPD were collected in the Västra Götaland region (VGR) and Halland during 1998-2001. Serotype and antibiotic susceptibility were tested. Clonal complex (CC) was determined for these isolates together with 424 IPD isolates from adults and children in Stockholm using pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). Clinical data for all 2977 IPD episodes in VGR during 1996-2008 were retrieved from hospital notes. Prevalence data for predisposing factors were included from patient registries and recent publications.

Of 836 strains, 42%, 70%, 75% and 94% belonged to serotypes included in PCV-7, -10, -13 and PPV-23, respectively. Decreased susceptibility was uncommon, and largely confined to certain clones and serotypes, especially those included in PCV-7. Serotypes 1 and 7F were most common; they infected younger patients with less underlying disease and caused lower CFR than other serotypes, whereas 19A caused higher CFR. Clonal distribution differed between adults and children. CC306 (all serotype 1), caused lower CFR among adults than six other CCs. The relation between serotype and CC was complicated; clinical characteristics differed between some CCs within the same serotype and between some serotypes within the same CC; it was often difficult to determine whether these differences were related to serotype, CC or both.

The annual incidence of IPD was 15/100,000 and varied largely with age and underlying disease. It was highest at extremes of age and in patients with myeloma (2238/100,000), followed by chronic lymphatic leukemia, haemodialysis, lung cancer, HIV, rheumatic diseases, chronic obstructive pulmonary disease and diabetes mellitus. In contrast, it was not elevated among asthma patients. When compared with data from previous studies during 45 years, the incidence increased threefold and the CFR dropped from 20 to 10% for all IPD, whereas the incidence remained stable (1.1/100,000/year) and the CFR dropped from 33 to 13% for meningitis.

In conclusion, incidence and CFR have changed considerably over time and vary widely between different age and risk groups. CFR is also influenced by serotype and genotype. These factors have to be considered during planning and evaluation of vaccination against pneumococci.

LIST OF ARTICLES

I   Berg S, Trollfors B, Persson E, **Backhaus E**, Larsson P, Ek E, Claesson BE, Jonsson L, Rådberg G, Johansson S, Ripa T, Kaltoft MS, Konradsen HB.
Serotypes of Streptococcus pneumoniae isolated from blood and cerebrospinal fluid related to vaccine serotypes and to clinical characteristics.
Scand J Infect Dis. 2006;38 (6-7):427-32.

II   **Backhaus E**, Berg S, Trollfors B, Andersson R, Persson E, Claesson BE, Larsson P, Ek E, Jonsson L, Rådberg G, Johansson S, Ripa T, Karlsson D, Andersson K.
Antimicrobial susceptibility of invasive pneumococcal isolates from a region in south-west Sweden 1998-2001.
Scand J Infect Dis. 2007;39 (1):19-27.

III   Browall S*, **Backhaus E***, Karlsson D, Naucler P, Berg S, Luthander J, Eriksson M, Spindler C, Ejdebäck M, Trollfors B, Andersson R, Henriques Normark B. (*=equal contributions)
Pneumococcal clonal type determines disease outcome.
Manuscript, submitted.

IV   **Backhaus E**, Berg S, Andersson R, Ockborn G, Malmström P, Dahl M, Trollfors B.
Invasive Pneumococcal Disease: Incidence, Mortality and Underlying Diseases.
Manuscript.

CONTENTS

ABBREVIATIONS

AOM	Acute otitis media
CC	Clonal complex
CFR	Case fatality rate
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CSF	Cerebrospinal fluid
ELISA	Enzyme-linked immunosorbent assay
ermB	Erythromycin resistance methylase B
IPD	Invasive pneumococcal disease
MALDI-TOF	Matrix assisted laser desorption/ionization - time of flight
MBL	Mannose binding lectin
mefA	Macrolide efflux A
MGUS	Monoclonal gammopathy of unknown significance
MIC	Minimal inhibitory concentration
MLST	Multi-locus sequence typing
MM	Multiple myeloma
OD	Optical density
OPA	Opsonophagocytic assay
OPSI	Overwhelming post-splenectomy infection
PBS	Phosphate-buffered saline (a medium)
PCR	Polymerase chain reaction
PCV	Pneumococcal conjugate vaccine
PFGE	Pulsed field gel electrophoresis
PNSP	Penicillin non-susceptible *Streptococcus pneumoniae*
PPV	Pneumoccal polysaccharide vaccine
PRR	Pattern recognition receptors
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
ST	Sequence type
TLR	Toll-like receptor

INTRODUCTION

History of the Pneumococcus

The pneumococcus is one of the most important pathogens. It is primarily a human pathogen, although transmission from humans to animals held in captivity causing both colonization and disease has been reported in horses, rodents, ferrets and rhesus monkeys [1-3]. The finding of previously unknown virulent clones of pneumococci in wild chimpanzees is probably also the result of an anthroponosis (which is the opposite of a zoonosis) [4]. This finding among our closest relatives may, however, invite to speculations about the earliest history of the pneumococcus; maybe its ancestors accompanied the first evolutionary steps of hominids towards mankind? Phylogeny for certain pneumococcal strains can, however, only be traced a few decades back [5]. Due to the rapidly changing pneumococcal genome, anything beyond that remains a speculation. The pathogen was not isolated until 1881, when the French microbiologist Louis Pasteur [6] and the US scientist George Sternberg independently of each other isolated, cultured and described the bacterium [7]. In the following decade, its position as a major cause of pneumonia, meningitis and death was established. In 1902, Neufeld showed that patients and animals developed immunity to specific pneumococcal strains, which could be observed in the laboratory as the Quellung reaction [8]. This method has remained the standard method for serotyping pneumococci since then. During the Spanish flu in 1918 most deaths were caused by secondary pneumonia [9], most of them probably caused by pneumococci. The influenza virus, however, was to remain unknown for another decade at the time, and the disease was generally believed to be caused by Pfeiffer's bacillus, later called *Haemophilus influenzae* [9]. During the desperate struggle against the overwhelming pandemic, vaccines directed against several other bacteria were tested, and some whole-cell inactivated pneumococcal vaccines were shown to be effective in reducing death due to influenza [10]. The number of known pneumococcal serotypes grew gradually during the first decades of the twentieth century, and by the end of the thirties, 32 immunologically distinct serotypes were known. The development of specific immune sera for treatment of pneumococcal disease had already begun in the 1880s, and when the Second World War started, several regimens using both horse, rabbit, chicken and human sera were described [11]. After the introduction of sulphonamides, and a few years later penicillin, serum treatment was largely forgotten. In parallel, the introduction of the first polysaccharide vaccine, directed against four pneumococcal serotypes in 1945 [12], was also overshadowed

by the introduction of penicillin, which lead to a remarkable decrease in mortality and morbidity due to pneumonia. Two polysaccharide vaccines were licensed but as a result of the success of penicillin, they were withdrawn after some years [13]. Actually, the first steps towards a conjugate vaccine had already been taken some years earlier, in 1931, when Avery showed that a serotype III polysaccharide retained its immunogenicity when conjugated to a protein [14]. The same Avery was going to be famous some decades later, when he and his co-workers showed that DNA is the carrier of genetic information (using the pneumococcus, of course). As the founder of genetics, he has been named "the most deserving scientist ever not to receive the Nobel Prize" [15].

In the 50s, it became gradually clear that antibiotic treatment alone could not eliminate the entire burden of pneumococcal disease; in 1964, case fatality rate in bacteraemic pneumonia was shown to remain as high as 25% despite adequate treatment [16]. The notion, that penicillin alone could not solve the problem was underlined by the finding of the first penicillin resistant pneumococcus in 1967 [17]. Therefore, the development of pneumococcal vaccines was restarted, resulting in a 14-valent polysaccharide vaccine which was introduced in 1976 [18]. It was later replaced by a 23-valent vaccine in 1983. Unfortunately, polysaccharide vaccines are not immunogenic in small children, and therefore, a 7-valent conjugate vaccine was developed. After its introduction in the U.S. in 2000, and later in other industrialized countries, a decline in severe pneumococcal disease was observed both in this vulnerable group and among non-vaccinated adults through herd effects [19]. Since then, conjugate vaccines protecting against 10 and 13 serotypes, respectively, have been licensed, offering protection against a larger proportion of pneumococcal infections.

Today, pneumococcal disease still remains a leading cause of death among children worldwide, causing more than 800 000 deaths per year among children less than 5 years old [20]. The highest mortality rates per 100 000 children are estimated to occur in Afghanistan and in several African countries. Since the populations in India and China are so much bigger, although the mortality rates are estimated to be lower, the total number of deaths in children under five due to pneumococcal infections in these countries is estimated to be large enough for them to enter the top ten list, as shown in figure 1. India is leading this hideous top list with 142 000 children who are estimated to die from pneumococcal disease each year, followed by Nigeria with 86 000. In industrialized countries, although the problem is of another order of magnitude, great differences in incidence between different groups in the

population, where marginalized indigenous people [21, 22] are especially at risk. Large scale programmes are currently undertaken in developing countries, to make pneumococcal conjugate vaccines available to the children which are at highest risk, and thereby hopefully reducing the global burden of pneumococcal disease during the next decades [23]. Pneumococcal disease also remains a major cause of mortality and morbidity in adults. When the proportion of elderly and people living with chronic diseases is increasing, the incidence of severe pneumococcal infections is also likely to increase.
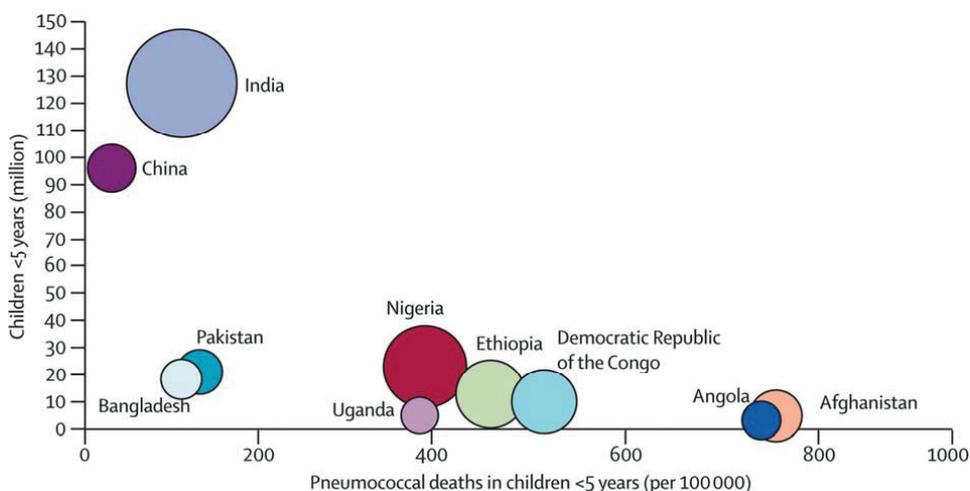
Figure 1. The ten countries with the highest number of estimated pneumococcal deaths in children aged 1–59 months. Bubble sizes indicate the number of pneumococcal deaths. From O'Brien et al Lancet 2009 [20]. Reprinted with permission from Elsevier Inc.

Microbiology, Pathophysiology and Clinical Aspects

Basic Characteristics and Identification

Pneumococci are Gram positive, non-motile, non-sporeforming, coccoid bacteria. Like many other streptococci, they are aero tolerant (facultative) anaerobes. They lack the enzyme catalase, which is required to neutralize the large amounts of hydrogen peroxide produced by the bacteria, and they therefore need to be cultured on media with capacity to neutralize hydrogen peroxide, for example blood agar. Hydrogen peroxide is oxidizing haemoglobin to green methaemoglobin, which is observed as greenish haloes around the bacterial colonies on blood agar; a phenomenon called alpha haemolysis [13]. Under anaerobic conditions they

switch to beta haemolysis caused by an oxygen-labile haemolysin [24]. Growth is enhanced by incubation in 5% carbon dioxide. On Gram stain, pneumococci often appear in pairs, and were therefore formerly named *Diplococcus pneumoniae*, although single cells and small chains also appear. Later, they were found out to be members of the streptococcus family, and the name was changed to *Streptococcus pneumoniae* in 1974. In the routine laboratory, the presumptive diagnosis of pneumococci is based on recognition of typical colony morphology on blood agar. Two different morphologies occur, depending on the amount of capsule which is produced by the strain. Most common are round smooth umbilicated colonies, 0.5-1.0 mm in diameter, whereas heavily encapsulated strains, especially serotype 3, form mucoid dome shaped colonies with a diameter of up to 5 mm.

Pneumococci are distinguished from other alpha haemolytic streptococci based on colony morphology and sensitivity to optochin (ethylhydrocupreine) and bile (they undergo lysis when natriumdeoxycholate is added to the culture) [13].

The Capsule

The most important virulence factor of *S. pneumoniae* is the polysaccharide capsule providing protection against both antibody dependent and independent immunity, especially opsonisation and phagocytosis by neutrophils. Nearly all pneumococci are surrounded by a polysaccharide capsule, but un-encapsulated strains occur occasionally. They are known to cause outbreaks of conjunctivitis [25], but are rarely found in nasopharyngeal colonization and invasive disease. Based on immunological properties of the capsule, pneumococci are divided into 46 serogroups. Of those, 20 are further divided into 2 – 4 serotypes, while 26 serogroups only have one serotype. Ninety-three immunologically unique serotypes have so far been described [26-28].

Serotype Specific Properties

Some serotypes are virtually never found among patients with invasive pneumococcal disease (IPD), some are found in both IPD and carriage, and some almost only in IPD patients. Comparisons between isolates from carriage and disease have shown that different serotypes have different capacities to cause invasive disease or carriage [29, 30]. Some of these differences have been linked to the chemical structure of the capsule [31]. The serotypes with highest invasive disease potential (i. e. 1, 5, 7F) have been suggested to act as primary pathogens, causing invasive disease predominantly in previously healthy individuals, with

lower case fatality rate (CFR) than other serotypes. Other serotypes (such as 19F, 23F) are more often found in carriage and behave more like opportunistic infections causing infections in compromised hosts and with a higher CFR [32]. A Danish study of almost 19000 IPD patients, with serotype data from surveillance together with data from the civil registry and diagnosis registers, showed that the risk to die from IPD was higher in nine different serotypes compared to serotype 1 [33]. Data from that study were also included in a meta analysis focusing on the risk to die from bacteraemic pneumonia among adults related to serotype, showing basically the same results as the Danish study, because it was much larger than all the other studies together [34]. It has, however, been shown that host factors seem to be more important than serotype in determining the severity and outcome of IPD. Therefore, it is possible that some serotypes with a higher degree of virulence, from a pathophysiological point of view, are connected with a lower CFR, because they afflict younger and healthier persons.

Genetic Properties of Pneumococci

Pneumococci are highly promiscuous, in the sense that they are easily transformed and may incorporate foreign DNA from other pneumococcal strains and from other species. Genes are not only transferred between different pneumococci; for example, resistance genes seem to have been transferred from closely related alpha streptococci, such as *S. oralis* and *mitis* [35]. Croucher et al. performed a whole genome sequencing of 240 isolates from the same clone, and they found that the high degree of genetic heterogeneity could be explained by a high frequency of recombinational events, especially in resistance genes and among genes coding for capsular polysaccharides [5]. This is the major reason why pneumococci are successful in evading our attempts to defeat them with antibiotics, and this may also help them to circumvent the effects of vaccination in a long term perspective. Therefore, not only serotype distribution (see paper I) and antimicrobial resistance (see paper II) have to be monitored in order to understand the epidemiology of pneumococcal infections; also the genetic content (the genotype) needs to be characterized by molecular methods (see paper III).

A fundamental question is: is there a difference in virulence between different clones (lineages of genetically related bacteria) that carry the same capsular type? Serotype 1 may serve as an illustration for this discussion. A very high CFR was seen during an outbreak of meningitis caused by serotype 1 in Ghana [36]. Was this because those patients suffered from

more underlying diseases? No. Or was it because they did not have the same intensive care facilities as in the developed world? Or was there a selection bias, because only the most severely ill were admitted? Or are there also differences in virulence between different lineages (clones) of pneumococci expressing the same capsular polysaccharide, in this case serotype 1? All these factors may contribute to the high CFR that was observed in that study, including the finding, that there may be a difference in virulence between different clones of serotype 1 pneumococci. This is furthermore underlined by the finding, that the major virulence factor pneumolysin is not functional in certain clones of serotype 1 (see next section). The same question, whether clonal type affects disease outcome, can be posed for all other serotypes as well, and it is still under debate. In mice infection models, it has been shown, that virulence differs between isolates from different clones with the same capsular type [37]. Whether clonal type really affects human disease remains to be shown (see paper III).

Non-capsular Virulence Factors

If not only serotype affects virulence, which non-capsular factors may also influence virulence? The capsule is surrounding a thick cell wall composed of peptidoglycans, teichoic acids and lipoteichoic acid, which are important during induction of the innate immune response. Both pili and several different classes of surface proteins that promote adherence to the respiratory epithelium are attached to the cell wall. One of them, pneumococcal surface protein A (PspA), is required for full virulence. It is thought to reduce complement activation, both the classical and the alternative pathway [38]. Pneumococcal surface protein C (PspC) is also a major virulence factor. Teichoic acid of the pneumococcus is also called the c-polysaccharide (c-ps). Despite the resembling name, it is not the same as the capsular polysaccharide. It is almost invariably expressed by isolates of *Streptococcus pneumoniae*, and it is involved in pathogenesis, mainly by inducing innate immunity. Antibodies against c-ps are commonly detected in patients with pneumococcal pneumonia [39], and therefore, this polysaccharide early gained interest as a potential target for a serotype independent vaccine, but antibodies against it did not turn out to be protective. C-ps reacts strongly with one of the major proteins which is produced by the host during the acute phase of inflammation. This protein is therefore named the C-reactive protein (CRP). The presence of CRP has been shown to reduce lethality among mice infected by *S. pneumoniae*, but this protective function does not seem to require binding of CRP to the c-polysaccharide, so the mechanism of that

interaction seems to be more complex than that [40]. The c-polysaccharide is used as a target for antigen detection tests, see the diagnostic methods chapter.

Autolysin, LytA, is an enzyme involved in remodelling of the cell wall structure during cell division. It can also induce lysis of bacteria in the stationary phase, and when they are exposed to antibiotics, for example penicillin. During lysis, both cell wall fragments and several virulence factors are released. Most important of them is pneumolysin, a cytotoxin which is stored in the cytoplasm and is released when pneumococci undergo lysis and die [41]. It induces pores in cholesterol rich membranes and leads to lysis of human cells, induction of pro-inflammatory cytokines as well as activation of complement, thereby promoting invasion. It also influences evasion of human dendritic cell responses [42]. Maus et al. showed that administration of purified pneumolysin in the lungs of mice gave a lung injury similar to pneumonia, and that this injury was the result of direct toxic effects of pneumolysin on the alveolar-capillary barrier rather than effects of resident and recruited phagocytic cells [43]. It is therefore intriguing, that serotype 1 strains belonging to one of the most common sequence types (ST 306) according to MLST, have been shown to express a non-haemolytic and therefore non-functioning pneumolysin [42, 44] causing less apoptosis but a stronger inflammatory response in dendritic cells than haemolytic alleles. Whether the absence of the lytic function of this major virulence factor also affects outcome or clinical picture in patients is not known yet. This question will be further discussed in paper III. A range of other toxins are also produced. Hydrogen peroxide, further discussed in the colonization section, can be regarded as a toxin because of its toxic effect on respiratory epithelium. Hyaluronidase is another important virulence factor degrading connective tissue, involved in the crossing of the blood-brain barrier during the pathogenesis of meningitis. Pneumococci also produce an enzyme degrading NET's produced by neutrophils [45] (see host defence section).

Colonization of the Nasopharynx

Pneumococci are spread via direct contact with secretions from carriers, via saliva [46] or are inhaled via an aerosol, where after they colonize the nasopharynx. They usually cause an influx of neutrophils, often resulting in a mild rhinorrhea without other symptoms, which promotes spread to other hosts, but they are most often not cleared by the immune system until days to months later [47]. The main reason for this is that the capsule protects the pneumococcus against killing by neutrophils. The length of the carriage period depends both

on bacterial and host factors. Some serotypes are generally carried for long periods whereas others usually persist only for a short time. Both age and immune status of the host influence carriage duration; especially children below two years of age carry pneumococci for much longer periods than adults. Also the risk of a subsequent development of disease varies greatly depending on both host and bacterial factors. Because of its dual nature, being both a commensal and a pathogen, pneumococci can be classified as commensal pathogens [48].

The pneumococcus is highly adapted to survive in the nasopharynx in many ways: although catalase negative, this anaerobe tolerates the oxygen rich environment because both exported and cytosolic proteins contain less amino acids (cysteine) that are vulnerable to erroneous oxidization [49]. Furthermore, its production of hydrogen peroxide is high enough to inhibit growth of more sensitive competing bacteria, like *Staphylococcus aureus* [50] and also to cause damage to host tissues [47]. The high amounts of hydrogen peroxide also cause oxidative damage to the DNA of the pneumococcus itself, which is partly overcome by its high ability to take up and integrate DNA from the environment. It also produces an array of enzymes facilitating its nasopharyngeal life style: one enzyme makes it less vulnerable to degradation by the lysozym of the mucus [48], other enzymes reduce the activity of complement components, C-reactive protein and secretory IgA, thereby reducing opsonisation and hence inhibiting clearance by neutrophils [47]. Other enzymes allow the pneumococcus to retrieve nutrients from the environment and to expose adhesive molecules on the epithelium [51]. Adhesion to the respiratory epithelium is promoted by a number of molecules, for example pili [52-54]. It has been shown that a gene coding for a pilus made certain pneumococci more successful in an animal model of carriage, and also that a certain clone expressing pili had been spreading very successfully among humans [55]. Furthermore, pneumococci also form biofilms, consisting of a layer of bacteria and extracellular matrix, making them less vulnerable to attacks from the immune system [56]. And finally, they adapt to the environment by displaying two different phenotypic variants, by changing the thickness of the polysaccharide capsule: the transparent colony variant, with a smaller amount of capsular polysaccharide per cell, is dominating in the colonizing state, thereby promoting adhesion [48], whereas the opaque variant, with a larger amount of capsular polysaccharide, is more commonly seen when they are in the blood stream, when they need to be protected against opsonophagocytotic killing [47].

Interactions with Other Pathogens

In the nasopharynx, pneumococcal strains compete with other pneumococci with other serotypes occurring there at the same time [57], and with other species, like alpha streptococci, *Staphylococcus aureus* and *Haemophilus influenzae*. The interactions are not only the question of a fight about food and accommodation; pneumococci also benefit from their capacity to internalize genes from other species. Furthermore, several viruses have a profound effect on disease development. There is firm evidence that much of the morbidity and mortality during influenza pandemics, for example in 1918 [10] and in 2009 [58], was due to pneumococcal pneumonia. Also in the absence of pandemics, peak incidence of invasive pneumococcal disease usually follows the peak incidence of seasonal influenza [59]. Influenza virus facilitates pneumococcal adherence and invasion in the lungs through several mechanisms: both unspecific epithelial damage and specific effects of viral neuraminidase facilitate the binding of the pneumococcus to receptors in the epithelium. Furthermore, the virus induces the expression of such receptors. The viral infection also leads to a modified immune response, partly through interferon gamma, leading to a decreased capacity of alveolar macrophages to clear pneumococci [60]. There are also interactions with other viruses, for example respiratory syncytial virus. It has been shown that pneumonia patients co-infected with virus and bacteria seem to develop more severe disease than those with a bacterial aetiology alone [61] (see figure 2).

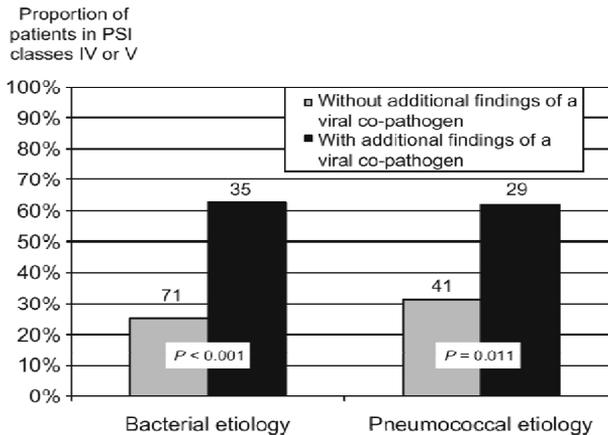**Figure 2.** Comparison of the proportion presenting with high pneumonia severity index (PSI), among adult patients with pneumonia, due to any bacteria or pneumococci, as single pathogens or together with viral co-pathogens. The figures above the bar charts represent the number of patients with known aetiology established with PCR and/or culture. Reprinted with permission from N. Johansson et al., SJID, 2011; 43: 609–615.

Development of Disease

Development of pneumococcal disease is a rather rare event compared to carriage. Severe infections, leading to death of the host, are not promoting spread of the organism, and could therefore be regarded as a mistake by the bacteria from an evolutionary point of view. In contrast, in cases where pneumococci cause less severe disease, leading to cough or nasal secretions, development of disease promotes spread of the organism and is therefore "motivated" also regarded from an evolutionary perspective. It is easier to understand the various virulence factors, when keeping in mind that they have evolved as adaptations to survival in the hostile environment of the nasopharynx.

An overview of the development of pneumococcal disease is shown in figure 3. From the nasopharynx, pneumococci may spread via existing anatomical channels to other parts of the upper airways and cause mucosal disease. Most common are infections in the middle ear, otitis media, and in the paranasal sinuses, sinusitis. Pharyngitis, tonsillitis and epiglottitis also occur. Pneumococci may also be inhaled in the lower airways, where they may cause bronchitis or pneumonia. In 20-30% of all culture verified cases of pneumococcal pneumonia, pneumococci are also found in the blood [13].
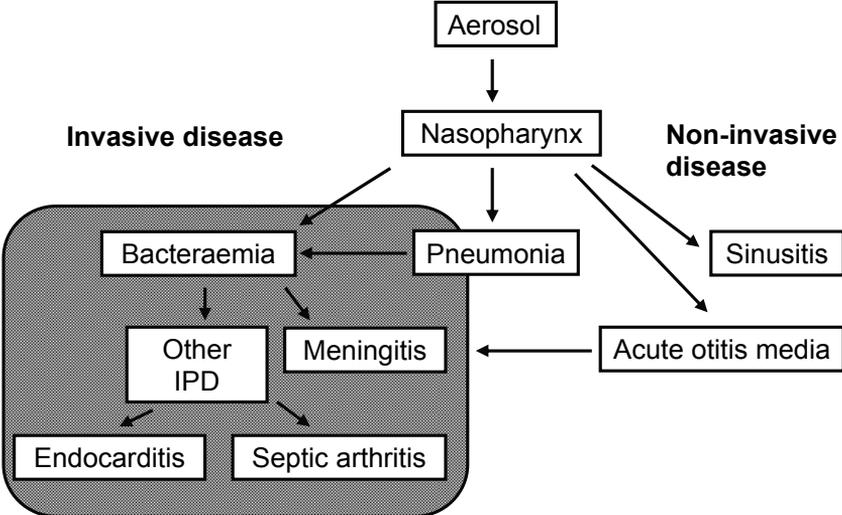

Figure 3. Development of invasive and non-invasive pneumococcal disease.

Infectious episodes where pneumococci are isolated from blood or other normally sterile body sites are defined as invasive pneumococcal disease (IPD). The most common IPD manifestation is bacteraemic pneumonia, where pneumococci have managed to cross the respiratory epithelium of the lower respiratory tract. Sometimes bacteraemia is found without a detectable primary focus. In those cases, the bacteria are believed to have crossed the mucosal barrier in the nasopharynx instead. Both the upper and lower respiratory epithelium is crossed through a process called transmigration. One of the mechanisms involved is the binding of pneumococci to the receptor for one of the key mediators in the inflammatory response, the platelet activating factor (PAF) (see figure 4) 1. When pneumococci multiply on the mucosal surface, neutrophils migrate into the alveolus. 2. During the attempts of alveolar macrophages and neutrophils to kill the pneumococci on the luminal side of the mucosal surface, large amounts of cytokines are released. These cytokines activate epithelial cells to express PAF receptors on their surface (PAFR). 3. The phosphorylcholine and lipoteichoic acid on the pneumococcal surface have the capability to attach to the PAF-receptor. The epithelial cell internalizes the receptor-bound pneumococcus, which is subsequently transferred across the stroma and through the endothelium of the blood vessel, to finally be released in the blood in the capillary on the other side of the mucosal barrier.
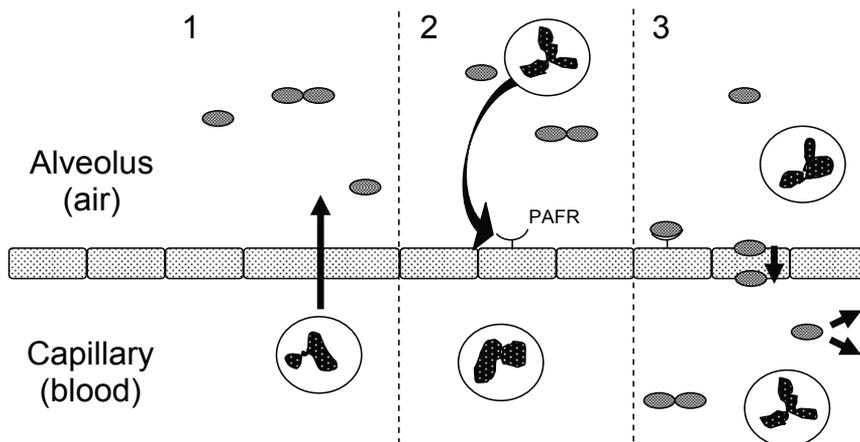

Figure 4. Very simplified scheme of how pneumococci use the platelet activating factor receptor (PAFR) for transmigration over the mucosal surface. See text for details.

Probably, in most cases, the bacteria are then rapidly killed by the defence forces waiting for them in the blood, mainly opsonisation by complement factors and antibodies, followed by phagocytosis by myeloid cells. If the bacteria are not recognized by the immune defence, or manage to grow fast enough to overwhelm its capacity, either because they are extra virulent, or because the immune system of the host is impaired, they multiply and give rise to a blood stream infection [62]. Pneumococci may then spread to distant locations in the body though the bloodstream, causing various secondary manifestations, for example osteitis, septic arthritis, endocarditis or abdominal infections. When transition to further compartments occurs, this process has similarities with the transmigration over the respiratory epithelium.

If the pneumococci manage to cross the blood-brain barrier and multiply within the cerebrospinal fluid (CSF), they give rise to meningitis. Spread to the CSF occurs either via the bloodstream, most commonly from a pulmonary focus, or by extension of an infection in the middle ear or in the paranasal sinuses.

Host Defence Mechanisms and the Consequences When They Fail.

The first line of defence against pneumococci entering the nasopharynx is the barrier function of the epithelium and the innate immune system, which also includes many functions of the respiratory epithelium, covered with mucus loaded with various antibacterial substances, such as IgA, antibacterial peptides and defensins. In the lungs, the cilia of the respiratory epithelium transport the mucus including bacteria through coordinated movements away from the alveoli, which is illustrated by the fact that patients with dysfunction of this system, as in cystic fibrosis or chronic obstructive pulmonary disease (COPD), run an increased risk for pulmonary infections, including pneumococcal pneumonia.

The basic principle of the innate immune response is that a molecule specialized in recognition of intruders sends a signal initiating various effector mechanisms. On the epithelial surface, as well as in all other parts of the body, the pattern recognition receptors (PRR) of the innate immune system identify highly conserved molecular patterns of bacteria. PRRs can be secreted, as for example the C-reactive protein (CRP) that is released during the inflammatory response and binds to the pneumococcal cell wall and activates complement [13]. Mannose binding lectin (MBL) is another PRR, with capacity to initiate the lectin pathway of the complement system. It has the capacity to bind to pneumococci, but it is still

under debate whether MBL deficiency is really a risk factor for death in patients with severe pneumococcal infections [63]. Other PRR's are bound to the surfaces of macrophages and other host cells. Toll-like receptors (TLRs) are important in detection and initiation of immune responses to pneumococci, illustrated by findings that mice lacking some of them are more susceptible to pneumococcal infection. TLR deficiencies have not been found in humans, but both mice and children with deficiencies in the intracellular signalling pathways downstream from TLRs, MyD 88 and IRAK4, have been shown to be more susceptible to pneumococcal disease. In contrast, CD14, which is another PRR, is probably used by pneumococci to spread from the respiratory tract using a mechanism similar to the one previously described for the PAF-receptor. Mice without this receptor seem to be protected against dissemination of the infection [64], but a human correlate to that finding has unfortunately not been found yet. Macrophages on the epithelial surface manage to engulf and kill small amounts of pneumococci. After phagocytosis, pneumococci (and other bacteria) inside the phagolysosome are recognized by intracellular PRR's, nucleotide binding oligomerization domain (NOD) receptors, leading to activation of the macrophage and production of cytokines, attracting more cells of the immune system, and initiating the inflammatory cascade. Effective killing, however, requires opsonization with complement factors (especially the classical pathway) or antibodies [62]. Therefore, patients with complement or antibody deficiencies run an increased risk to develop severe pneumococcal disease. When an epithelial cell binds pneumococci with PRRs on its surface, the production of both cytokines and antimicrobial peptides is increased. The most important cytokines are tumour necrosis factor α (TNFα) and interleukin 1 (IL-1). Inhibition of these mediators during anti-inflammatory treatment of patients with autoimmune diseases therefore leads to increased risk for severe pneumococcal diseases.

Many of the effector mechanisms initiated by the signals from PRRs are executed by the neutrophils, which usually accumulate in high numbers at the site of infection, for example in pneumococcal pneumonia. They act through phagocytosis and release of antimicrobial substances, including neutrophil extracellular traps (NET's), which are scaffolds consisting of DNA and antimicrobial peptides that are able to trap pneumococci. Unlike most other bacteria, pneumococci are not killed by NET's, because they produce a certain protective enzyme [45].

The second line of defence is the adaptive immune response. Both B-cells and T-helper (CD4) cells are involved in this process, illustrated by the fact that both patients with B-cell dysfunctions and AIDS run an increased risk to develop severe pneumococcal infections. Since the pneumococcal polysaccharide capsule consists of large repetitive molecules that are difficult for macrophages to digest and present to lymphocytes in the major histocompatibility (MHC) class II receptors on their surface, the T-cell independent route of B-cell activation dominates. In untreated patients, specific antibodies appear on the fifth or sixth day of illness [13], leading to a more effective opsonophagocytotic clearance of pneumococci than was achieved with the more unspecific mechanisms of innate immunity. The importance of specific antibodies directed to the polysaccharide capsule in the defence against IPD is illustrated by the effectiveness of vaccination with capsular polysaccharides [65-67] and of serotherapy [11]. Probably antibodies directed to other, non-capsular species-specific antigens, are also involved in protection against IPD [68]. Anti-capsular antibodies do also reduce carriage, illustrated by studies of immune responses among adult carriers [69] and by the effects of vaccination on serotype distribution among carriers [70]. The decline in both carriage rates and IPD incidence that occurs naturally after the second year of life is however not possible to explain only by increases in the levels of antibodies against capsular polysaccharides, indicating that serotype independent immunity involving CD4+ T-helper cells is probably more important [71]. There are data indicating that this protection against both carriage and non-invasive disease rather is due to cell-mediated immunity [68].

If the pneumococci have managed to reach the blood, the most effective killing of the opsonised bacteria (with IgG antibodies or the complement factor C3b on their surface) takes place in the marginal zone of the spleen, where the blood passes slowly through an area filled with B-lymphocytes and macrophages. Patients without a spleen, either due to congenital asplenia or splenectomy, or loss of splenic function secondary to disease, run an increased risk to develop pneumococcal septicaemia in general and overwhelming post-splenectomy infection (OPSI) in particular. It has been speculated, that this risk may be lower after posttraumatic splenectomy, because functional splenic cells sometimes are disseminated in the abdominal cavity after a splenic rupture, a condition called splenosis [13].

A third line of defence is the blood brain barrier protecting the brain against invading organisms such as *Streptococcus pneumoniae*. Patients with a deficient blood brain barrier due to CSF leakage, either secondary to trauma, such as scull base fractures or neurosurgery

[72], or primary due to scull base malformations such as fibrous dysplasia [73], run an increased risk to develop recurrent pneumococcal meningitis.

Risk Factors for Invasive Disease

The list of factors predisposing for the development of invasive pneumococcal disease is very long, beginning in the neonatal period and leading all the way to senility. Most recommendations listing target populations for vaccination, for example from Sweden [74] and from the US [75, 76], are repeating the same list with minor variations. High and low age are major determinants whether an individual is at risk to develop IPD or not. The risk is increased below two years of age, and prematurity (defined as <32 weeks) and birth weight below 1500 gram both add additional risk. Age above 65 years is a well-known risk factor for IPD, and the risk is increasing with age within this risk group. Immunosenscense leads to a decline in both innate and acquired immune functions [77]. Furthermore, various deficiencies of the innate and acquired immune systems, including antibodies and cellular immunity, as well as damages to the blood brain barrier and asplenia are also important risk factors, which have been discussed in the previous chapter. The importance of co-infections has also been mentioned in the chapter about interactions with other pathogens. In addition to the previously discussed risk factors, a number of chronic conditions have been shown to predispose for IPD. In children, Mb Down, cystic fibrosis and nephrotic syndrome are important. In adults, cardiovascular, pulmonary, liver, renal, neurologic and autoimmune diseases together with diabetes mellitus are all important risk factors, as well as smoking and alcohol abuse. Socioeconomic factors are important. Poverty predisposes for IPD through many ways; malnutrition, crowding, polluted indoor air and patients' delay. It has also been shown that living together with a toddler who is in day care is predisposing for IPD among adults [71, 78]. These issues are further discussed in paper IV.

Diagnostic Methods

A correct diagnosis of pneumococcal disease leading to targeted antibiotic treatment is of dual importance. For the patient it improves outcome, and for society it reduces the increasing antibiotic resistance caused by inadequate or unnecessary antibiotic use. Furthermore, a correct diagnosis is crucial for surveillance, especially in the follow up of vaccination programmes. Identification by culture has remained the cornerstone in the diagnosis of

pneumococcal disease since the 1880s. In invasive disease, when the pathogen is isolated from a normally sterile site, mainly blood, CSF or synovial fluid, the matter of causality is clear. In non-invasive disease, the finding of pneumococci in specimens from non-sterile sites is not as conclusive in determining the aetiology, and must be related to the clinical context. The specificity of the finding of pneumococci in the nasopharynx of a child is low, since carriage is common, but specificity is high if they are isolated from sputum from an adult with pneumonia. A major problem with cultures in both severe and non-severe disease is the low sensitivity. Sometimes the cultures are negative because the patient has received antibiotics prior to culture. On some occasions, the cultures remain negative despite optimal sampling of specimens. One reason for this is that pneumococci are fastidious, in the sense that they have complicated nutritional requirements. Furthermore, autolysins may be activated during growth in culture, leading to the death of the bacteria. In these cases, a Gram stain from the blood culture bottle often yields distorted bacterial structures appearing as fluffy Gram negative short rods. In community acquired pneumonia, the diagnostic yield may be increased using transthoracic needle aspiration. This invasive procedure is not used in the clinical routine because the risk of complications, but it is useful in studies. For example in a Spanish study, in one third of the pneumonias with negative blood and sputum cultures, pneumococci could be cultured from transthoracic needle aspirates [79].

Because of the low sensitivity of culture, several new diagnostic techniques have been developed, that either detect antigen or nucleic acids. They all share the drawback, that they cannot test the antimicrobial susceptibility, and must therefore be seen as complements to culture. A main target for antigen determination is the c-polysaccharide, which is species specific and independent of serotype. Its potential as a complement to culture to get an aetiological diagnosis in patients with pneumococcal pneumonia has been evaluated using several methods on both sputum and saliva [80-84], and serological methods measuring antibodies against it have also been evaluated [85]. The c-polysaccharide is today mainly detected in urinary antigen tests, that are useful to diagnose pneumococcal pneumonia among adults, especially when adequate sputum samples cannot be collected or when the patient has received antibiotics before admittance [86]. The commercially available Binax NOW antigen test can also be used on CSF, as a valuable complement to culture in establishing an aetiological diagnosis in meningitis patients [87]. Other antigens, for example pneumolysin and capsular polysaccharides, have been evaluated as targets for antigen detection, but they are not used in the laboratory routine today.

Recently, an abundant mass of studies have been published using various polymerase chain reaction (PCR) tests, either pneumococcal specific or multiplex methods on various specimens, for example sputum [70], bronchoalveolar lavage [88], nasopharyngeal specimens [89], transthoracic lung aspirates [90] blood [91] and CSF [92]. The most widely established molecular typing method is based on 16 S rRNA gene sequences. Unfortunately, *Streptococcus pneumoniae* shares 99% of this gene sequence with *Streptococcus mitis* and *oralis* [93]. The specificity of those findings has therefore been questioned. Several other targets have been evaluated [94]. One of them, the superoxide dismutase gene sodA, seems to allow a more precise species determination [95]. Recently, a 313-bp part of the recA-gene was used successfully for identification of *Streptococcus pneumoniae* to species level, because of a lower degree of interspecies homology together with the finding of signature nucleotides specific for *S. pneumoniae* within the fragment [96]. The development in this field thus moves rapidly towards more specific and reliable methods. One consequence is that the proportion of pneumonias with known aetiology has increased. These new findings have confirmed that *Streptococcus pneumoniae* is the most common causative agent of pneumonia, alone or together with viruses [97].

New diagnostic tools, especially Matrix-assisted laser desorption/ionization – time of flight (MALDI-TOF) mass spectrophotometry, with capacity to analyze large molecules, such as DNA, proteins, peptides and polysaccharides, is now entering the routine diagnostics. It provides reliable species determination for a vast number of clinically relevant species much faster than the old phenotypic methods [98]. Unfortunately, there is a considerable risk for misinterpretation of pneumococci and closely related alpha streptococci [99], disqualifying its routine use for this group of bacteria in the clinical laboratory.

Serological analysis of paired sera is another useful complement to the above mentioned methods, in order to increase the diagnostic yield. It is mainly motivated in studies, but it has limited value in the clinical context. Serotype determination of invasive pneumococcal strains has no value in the clinical routine diagnostics, but it is crucial in surveillance for evaluation of pneumococcal vaccine effects. The Quellung method, based on a method from the 19th century [8], has remained the gold standard [100]. Although latex agglutination kits [101] have simplified the process to a certain extent, serotyping is still expensive and time-consuming. Therefore new PCR-based serotyping methods have been developed, that will

probably make the necessary serotype data more accessible when large scale vaccination programmes are launched, especially in developing countries [102]. Also high-throughput sequencing technologies and/or MALDI-TOF are promising future alternative pneumococcal serotyping methods [102].

Treatment of Pneumococcal Infections

Penicillin is the treatment of choice for both severe and non-severe pneumococcal infections, unless resistance or hypersensitivity forces the physicians to choose other alternatives. Cephalosporins and carbapenems are usually highly active alternatives in severe disease. Pneumococci are naturally resistant to aminoglycosides, such as gentamycin, but when administered together with penicillin, a synergistic effect exists in vitro at least in penicillin non-susceptible *Streptococcus pneumoniae* (PNSP) [103]. This is the reason why the combination is recommended for the treatment of endocarditis caused by PNSP. Macrolides are also usually effective against a majority of pneumococci, and are the first alternative to beta lactam antibiotics in the treatment of pneumococcal pneumonia, although resistance is an increasing problem. Although they do not show any synergistic effect with penicillin in vitro [104], the combination of penicillin and macrolides improved outcome remarkably compared to penicillin alone in a study of mice with both pneumococcal pneumonia and influenza [105]. The authors suggest that "the robust inflammatory response that occurs during severe influenza virus infections is magnified by subsequent bacterial super infections, and is further exacerbated by β-lactam-mediated lysis of bacteria". The hypothesis that treatment leading to a lower inflammatory response may result in better outcome is interesting. Whether this combination is better than penicillin therapy alone in humans with severe pneumococcal pneumonia with or without influenza infection, is the subject for an ongoing discussion across the Atlantic Ocean. Combination therapy with macrolides and penicillin against pneumococcal pneumonia is not recommended in the Swedish guidelines for treatment of community acquired pneumonia in adults [106]. This discussion is not to be confounded with the well-established routine to combine macrolides with beta lactam antibiotics in the initial empiric treatment of severe pneumonia, when "atypical" pathogens like legionella, mycoplasma and chlamydophilae have to be included in the spectrum. Fluoroquinolones, tetracycline and co-trimoxazole are also usually effective (see paper II), but should be regarded as second or third line drugs in the treatment of pneumococcal infections with known aetiology.

Antimicrobial Resistance

Resistance to penicillin is due to genetic changes leading to an altered structure of penicillin binding proteins (PBP's), making them bind penicillin with lower affinity. Some of these resistance genes have probably been transferred from closely related alpha haemolytic streptococci [35]. Penicillin non-susceptible *Streptococcus pneumoniae* are categorized as either indeterminate (I) or resistant (S) to penicillin, according to the minimum inhibitory concentration (MIC) level. Penicillin resistant isolates are sometimes also cephalosporin resistant. It has been difficult to correlate penicillin resistance to increased disease severity or lethality in pneumonia, until a meta-analysis of 10 studies managed to show increased case fatality rate among patients with pneumonia caused by PNSP [107], although concerns regarding the influence of confounding and publication bias still remained [108]. In contrast, treatment failures and delayed sterilization of CSF has been reported when meningitis caused by PNSP (MIC >0,5 mg/l) has been treated with penicillin or cephalosporin monotherapy [109]. In these cases, combination therapy with a cephalosporin in combination with vancomycin is given.

Two mechanisms of resistance to macrolides dominate: In macrolide efflux, a protein encoded by the mefA gene causes resistance to macrolide compounds only, for example erythromycin, by pumping them out of the bacteria. Target-site modification is mediated by an erythromycin ribosomal methylase (erm) that reduces binding of macrolide, lincosamide, and streptogramin B (MLS) antibiotics to the target site in the 23S rRNA of the 50 S subunit. The phenotypic expression of MLS resistance can be inducible or constitutive. When inducible MLS resistance occurs, the strains seem to be erythromycin resistant but clindamycin sensitive when tested separately, but if clindamycin and erythromycin disks are placed on the same plate 25 mm apart, a D-shaped clindamycin zone occurs. This is because clindamycin resistance is induced in the area closest to the erythromycin disk, thereby enabling the bacteria in that area to grow closer to the clindamycin disk. Those strains are reported as R to clindamycin with MIC 256 mg/L irrespective of the measured value on the MIC-scale, because there is a high risk of treatment failure if a patient infected by pneumococci with this resistance mechanism would be treated with clindamycin. In contrast, the risk of treatment failure is less well-documented among infections caused by strains with efflux-mediated resistance [110]. Some macrolide resistant strains cannot be classified as either efflux or

methylation. In these cases, resistance is due to ribosomal mutations [111]. Resistance to Trimetoprim-sulfamethoxazole and quinolones are mediated by point mutations.

Epidemiology

Epidemiology of Carriage

Pneumococci are frequently members of the normal flora of the upper respiratory tract. They can be found in 30-70% of the nasopharynges in pre-school children [57, 112-114]. Young age, day care centre attendance and having young siblings are associated with higher carriage rates among children. Duration of carriage is shorter in adults [112], and the carriage frequency among them is lower. In one study, 4% of adults were shown to be carriers [57]. Duration of carriage also varies largely between different serotypes. Pneumococcal disease, both invasive and non-invasive, is thought to be preceded by a carrier state.

Epidemiology of Pneumococcal Disease

Pneumococci cause a wide range of diseases in humans. They are the leading cause of uncomplicated mucosal infections, such as otitis media, sinusitis and conjunctivitis, but may also cause lobar pneumonia, with or without bacteraemia, septicaemia with unknown focus and meningitis. A wide range of risk factors exists, as discussed previously. Incidence and case fatality rate due to invasive pneumococcal disease varies widely between different areas. For example, an increased rate of pneumococcal bacteraemia has been observed in Sweden during recent decades [118-120]. Often these differences can be explained by differences in risk factors including age, comorbidities and socioeconomic factors. Differences in incidence and case fatality rate between countries at a comparable socioeconomic level [115], and between different time periods in the same country [29, 116], might also be the result of an unequal distribution of clones and serotypes with different virulence, both over time and geographically. Such differences should, however, be interpreted with caution , because of selection bias in studies, and because of the impact of different blood culturing practices. If fewer patients are blood cultured, incidence seems lower. When only more severe cases are blood cultured, case fatality rate (CFR) is higher. Also resistance data are dependent on culturing practices; if only patients with treatment failures are cultured, the proportion of resistant strains is likely to be very high. With all this in mind, it is easily understood that both

incidence and CFR is reported at different levels in different studies also in developed countries. However, it remains a fact that, despite culprits in terms of selection bias, case fatality rate due to IPD remains 10-20% in most studies from the developed world [33, 115], and even higher in the developing world [36, 117]. IPD epidemiology is now going to be discussed with respect to serotype, genotype, antimicrobial resistance and influence of vaccination.

Changes in Serotype Distribution and Incidence

Before interpreting the changes in serotype distribution caused by vaccination, it is necessary to know about the fluctuations that occur naturally. Harboe et al. analyzed serotype distribution among invasive pneumococcal isolates during 70 years in Denmark, and found that the proportion caused by some serotypes (such as 14, 7F and 23F) seemed to be stable over time, whereas the proportion of others (such as 1-5) appeared to fluctuate [33]. The pattern of these fluctuations had changed, from peaks every 2-3 years before the 1960s to peaks every 7-10 years thereafter. The reason for this change is not known, but probably more widespread use of antibiotics contributes. The emergence and spread of new clones with increased virulence are probably also of major importance but not much studied. Before the era of antibiotics, outbreaks of IPD predominantly caused by the serotypes with fluctuating incidence were common, but they still occur, especially in settings like military camps, prisons and among marginalized populations [36, 46, 121, 122]. There are data suggesting that changes in the serotype distribution of invasive isolates over the past 40 years have occurred also in Sweden [123, 124].

There are also geographical differences in serotype distribution [125]. The most pronounced difference is the high frequency of serotype 5 in the southern hemisphere, while this serotype is uncommon in the northern hemisphere. Another important difference is that serotype 1 seems to be more common in Africa and Asia than in Europe and North America.

Profound changes in the serotype distribution among isolates causing IPD have followed the introduction of PCV-7 in several countries. This issue will be discussed in the conjugate vaccine section and in paper III.

Molecular Epidemiology

Not only the serotype distribution, but also genotype distribution varies among invasive isolates [126-129]. A clone is a lineage of genetically related bacteria of the same species, for example *Streptococcus pneumoniae*. The high capacity of the pneumococcus to include genetic material from other pneumococci in its own genome, which has been discussed previously, also includes the genes encoding the polysaccharide capsule. Therefore, strains with the same serotype may belong to different clones, and strains belonging to different clones may have different capsular types. Serotypes with a high propensity to cause invasive disease (1, 7F) seem to be more genetically related, with only a few clones, while serotypes less likely to cause IPD (19F, 23F) are more diverse [126]. The explanation for this is probably that strains carried in the nasopharynx for longer periods have more opportunities to find suitable partners for horizontal gene transfer, and are therefore more genetically diverse, whereas for example serotype 1 strains, that are only carried for short periods, are genetically homogeneous, since they do not have such excellent mating opportunities. Genes encoding antimicrobial resistance are often clonally related, with a few resistant clones dominating globally.

The clonal distribution among both carried and invasive pneumococcal strains has changed dramatically as a consequence of widespread PCV-7 vaccination. Serotype replacement is known to have occurred [130]. This has been due both to expansion of pre-existing clones of non-vaccine types and to serotype-switching events, where clones that used to have a vaccine type capsule, emerge with a non-vaccine type capsule. If the expanding clones have other virulence factors than the capsule, this might threaten the success of vaccination in a long-term perspective [22, 130, 131]. Whether clonal type is related to outcome, patient characteristics or disease manifestations has been difficult to show [32, 132, 133]. This matter will be further discussed in paper III.

Epidemiology of Antibiotic Resistance

The rapid spread of isolates of pneumococci with decreased susceptibility to several antibiotics, especially penicillin, is a universal problem [134]. The rate of antibiotic resistance varies with age, serotype and geographic area. Resistance is more common among isolates from carriage and non-invasive disease compared to invasive disease [135]. There seems to be a relation between the prevalence of PNSP and consumption of antibiotics both in the population and in the individual [136, 137]. Certain serotypes, in particular those which are

poorly immunogenic in small children, and therefore carried longer periods, are more often resistant to antibiotics than others. Since some of these serotypes were also included in PCV-7, a decline also in the incidence of IPD caused by PNSP was noted after introduction in the childhood vaccination programme in the US [138]. However, the degree of resistance varies largely between different clones within the same serotype, and there is evidence that old well-known resistant clones have emerged in the post-vaccine era, carrying non-vaccine capsules following serotype shift events [134].

Sweden has a favourable situation compared to other countries. However, in the late 1980s, the proportion of PNSP carriers increased among children in day-care centres in south Sweden [139]. Despite an intervention programme and decreased antibiotic prescribing, the proportion of PNSP has remained high [140]. However, no increase in antibiotic resistance was observed among IPD isolates [141]. This subject will be further discussed in paper II and in the supplement of paper III.

Prevention of Pneumococcal Disease

Pneumococcal disease can be prevented in many ways: reduction of risk factors, hygienic and socioeconomic interventions, antibiotic prophylaxis and passive or active immunization. Interventions that reduce the prevalence of some of the well-known risk factors for IPD, for example alcohol abuse, tobacco smoking, crowding, less exposure to open fire and malnutrition, would certainly lead to a reduction in IPD incidence. Some of those factors are, however, closely related to socioeconomic factors that need long-term strategies and profound changes in society to be influenced. The impact of other risk factors may be reduced by treating the underlying disease, it has for example been shown that highly active antiviral therapy (HAART) against HIV infection leads to a reduction in IPD incidence [142]. Furthermore, a high level of hygiene is important to prevent dissemination of pneumococcal disease, especially in extra vulnerable patients. In outbreak situations, hygienic restrictions may have a direct effect to reduce the number of cases, as for example was the common practice to share drinking bottles stopped during an outbreak among military in southern Israel [143]. It was however not possible to calculate the impact of this intervention since the recruits also got both antibiotics and vaccination, which is the recommended way to deal with outbreaks of pneumococcal disease [121]. Antibiotic prophylaxis is also used for prevention in selected high risk groups, for example patients after splenectomy or some patients with

myeloma with very low levels of immunoglobulins. Long term prophylaxis carries a risk for development of antibiotic resistance and is therefore most often not a good option. In some patients with low gamma globulin levels, either due to a primary immune deficiency or secondary to advanced HIV-infection or haematological malignancy, gamma globulin substitution has been shown to reduce but not to eliminate the risk to develop pneumococcal infections [144], but due to the high costs, this treatment can only be offered to a very limited number of patients. Therefore, the main and only option for large-scale prevention of pneumococcal disease remains active immunization through vaccination. Because of "les liaisons dangereuses" between the influenza virus and the pneumococcus described above, influenza vaccination has a potential to reduce the incidence of pneumococcal disease, and vice versa, pneumococcal vaccination has been advocated as an important method to prevent morbidity and mortality caused by both pandemic and seasonal influenza [145]. We are now only going to discuss the two groups of pneumococcal vaccines that are available; polysaccharide vaccines and conjugate vaccines.

Pneumococcal Polysaccharide Vaccines

A prospective, randomized, double blind study of a 13-valent polysaccharide vaccine given to young South African gold miners showed a 76-92% protective effect against presumptive pneumococcal pneumonia in the 70s [18]. This study has been criticized because the drop out was not reported and analyzed. Although a large American study thereafter failed to show efficacy [13], a 14-valent vaccine was subsequently introduced on the market. It was later replaced by the 23-valent pneumococcal polysaccharide vaccine (PPV-23) which is available today. It contains purified capsular polysaccharides from 23 of the most common serotypes isolated from patients with invasive disease. Several observational studies have indicated good protective effectiveness against both IPD and pneumococcal pneumonia, especially among certain risk groups and the elderly, and the vaccine is therefore recommended by WHO and CDC [76, 117]. The results from clinical trials and meta-analyses of clinical trials have, however, been conflicting, especially regarding protection against pneumococcal pneumonia. This is partly due to the heterogeneity and great variations in the quality of the studies, and for example one meta analysis in 2009 that also took these factors into account could not find evidence that the vaccine is effective against pneumonia or death, not even in the risk groups for whom the vaccine is currently recommended [146]. Most authors do however believe that the vaccine protects against IPD among adults, including the authors of a

Cochrane report from 2009 [147], who found "strong evidence of PPV efficacy against IPD with no statistical heterogeneity" based on a meta analysis including 22 studies with 110 000 participants. They too could not find evidence that the vaccine could reduce rates of all-cause pneumonia or all-cause mortality, and furthermore, efficacy appeared to be poorer in adults with chronic illness. Evidence for protection against IPD in the risk groups for whom the vaccine is recommended came mainly from non-RCTs.

Two main principles for the laboratory evaluation of the immune response after vaccination have been developed: quantitative and qualitative methods. Quantitative antibody responses in adults are measured with ELISA (Enzyme-linked immunosorbent assay). Although the level of antibodies correlates to some extent to the risk for pneumococcal pneumonia after vaccination [148], it is not necessarily a reliable measurement of the function of the immune response. Therefore, qualitative methods have been developed, measuring the level of antibody-mediated killing of pneumococci by phagocytes using opsonophagocytic assays (OPA) [149]. It has been shown that the quality of the immune response seems to correlate to the quantity of antibodies for most but not all serotypes among healthy adult carriers [150], and when it comes to predict the protective effect of vaccination, quantitative measurements are insufficient and have been replaced by OPA as the standard method. OPA is important for the evaluation of candidate vaccines and required for the licensure of new pneumococcal conjugate vaccine formulations, which will be discussed below.

PPV gives rise to a T-cell independent immune response, and is therefore not evoking memory B-cells and as a result, there is no booster response on repeated vaccination. Another consequence of the T-cell independent mechanism of action is that it is not immunogenic in children younger than 2 years, and the immunogenicity in elderly individuals and in immunodeficient patients is variable [66, 146]. Thus, the vaccine has low efficacy in those, who need it most. There are also reports that the immune response, both measured as the quantity and quality, gets lower from subsequent vaccinations, a phenomenon called hypo responsiveness. The 23-valent vaccine is recommended for certain risk groups and persons above 65 years of age in Sweden [74]. The risk groups will be further discussed in paper IV. Since sales statistics for vaccines are not openly available in Sweden, in contrast to all other pharmaceutical products, it is difficult to calculate the possible impact of vaccination on the incidence of IPD, but it is probably quite low.

Pneumococcal Conjugate Vaccines

The pneumococcal conjugate vaccines (PCV) were developed to circumvent the inability of PPV to evoke an immune response in children below two years of age. The basic principle is that the capsular polysaccharide is conjugated to a carrier protein, which will be recognized by T-helper cells that will orchestrate the subsequent immunological response, including to production of antibodies also directed to the polysaccharide. Thereby the vaccine is also immunogenic in children below two years of age, as well as in certain adult risk groups with impaired capacity to respond to pure polysaccharides. Furthermore, the vaccine has the capacity to induce an immunologic memory and a booster response when the vaccine is administered at a later occasion. Because it is technically complicated to link such proteins and polysaccharides to each other, only a limited number of serotypes could so far be represented in such a vaccine. The conjugation technique has been successful in creating more immunogenic vaccines with higher protection against many other diseases e.g. *Haemophilus influenzae* type b, *Neisseria meningitides* groups A, C, W-135 Y and *Salmonella typhi*.

The first PCV, Prevenar ®, contained polysaccharides from 7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F.) known to be common causes of IPD among children in North America [151]. It was introduced in the childhood vaccination programme in the United States in 2000, and later in several other countries. Before introduction, vaccine efficacy had been shown to be 97% against vaccine type IPD and 35% against x-ray verified pneumonia irrespective of aetiology in the Northern California Kaiser Permanente study [151]. Vaccine efficacy was also shown against acute otitis media (AOM) in a Finnish study [152]. The incidence and the overall mortality due to IPD in both vaccinated children and unvaccinated adults has declined markedly in the USA following PCV-7 introduction [19] (see figure 5). Also the number of hospitalizations due to pneumonia and the number of antibiotic prescriptions against AOM decreased following vaccine introduction. Although conclusions regarding causality should be made with caution based on results from observational studies, it seems very likely that PCV-7 vaccination has had a major impact on these changes. In fact, the sustained herd effects on unvaccinated adults, that have been observed in the USA after PCV-7 introduction, were much larger than any clinical trial could have foreseen [145].
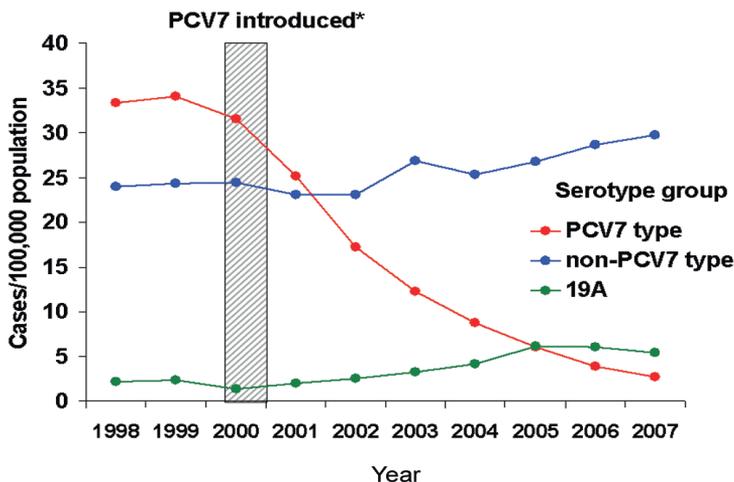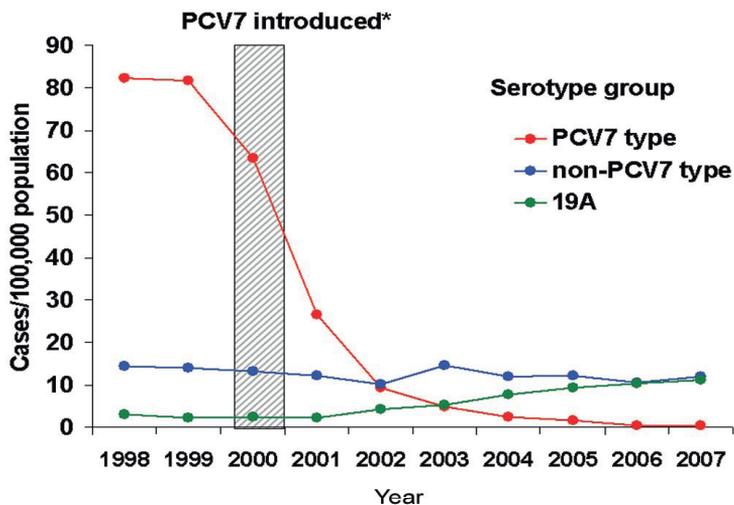
Figure 5. Changes in invasive pneumococcal disease incidence by serotype group among children aged <5 years (above) and adults aged 65 years (below), 1998–2007. *Seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in the United States for routine use among young children and infants in the second half of 2000. Reprinted with permission from T Pilishvili, J Infect Dis. 2010 Jan 1;201(1):32-41.

A Cochrane review 2009 concluded that "PCV is effective in preventing IPD, X-ray defined pneumonia, and clinical pneumonia among HIV-1 negative and HIV-1 positive children less than two years". They also concluded that the impact was greater for vaccine type-IPD than

for all serotypes-IPD, and for X-ray defined pneumonia than for clinically diagnosed pneumonia [153].

The main matter of concern during the success story of general vaccination with PCV-7 in the US and also in other countries, has been an increase of both invasive disease and carriage caused by non-vaccine types (NVTs), especially 19A [19, 131, 154]. Probably, these serotypes fill an ecological niche previously occupied by vaccine types, but antibiotic pressure may also have influenced the serotype distribution by promoting spread of resistant clones [21]. Some changes in the disease pattern after introduction of PCV-7 have also been suggested to be secondary to the impact on serotype distribution following vaccination [155]. Figure 5 also shows that the increase of IPD caused by NVTs due to serotype replacement has not been of the same order of magnitude as the decrease in vaccine type IPD, so overall IPD incidence has remained very low compared to the pre-vaccination in the US [19]. Furthermore, rates of pneumococcal meningitis have also decreased among both children and adults since PCV7 was introduced in the US. The effect of the vaccine has remained, although an increase in meningitis caused by non-PCV7 serotypes, including strains non-susceptible to antibiotics, has been observed [156].

PCV-7 was approved for marketing in Sweden 2003, i.e. after the study period in papers I-III, and the use was very limited and restricted to certain risk groups during the last years of study IV. It was finally included in the general childhood vaccination programme in January 2009, directly after the end of the study period in paper IV. Following this introduction, a decline in the proportion of IPD that is caused by vaccine serotypes has been noticed among children <2 years old (see figure 6) in Sweden.
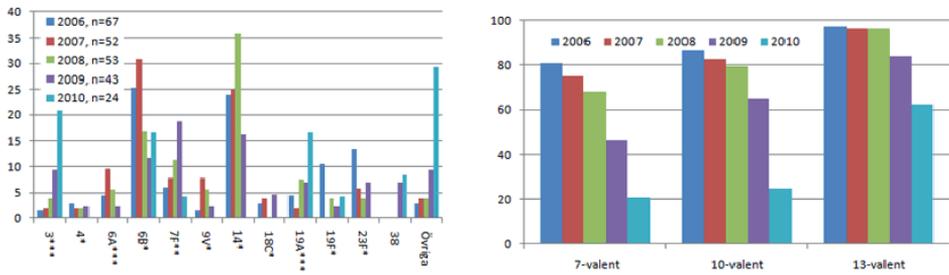
Figure 6. Serotype distribution and potential vaccine coverage among isolates causing invasive pneumococcal disease in children younger than 5 years in Sweden during 2006-2010. A decline in vaccine type IPD seems to have occurred after introduction of PCV-7 in the general childhood vaccination programme.
Serotypes marked * are included in all PCVs, ** in PCV 10+13 and *** in PCV-13.
Reprinted with permission from the Swedish centres for disease control (SMI).

A main problem with PCV-7 is that the serotypes included in the vaccine are not corresponding to the serotype distribution in the developing countries with the highest disease burden. Especially serotypes 1 and 5, known to have a high invasive disease potential, are more common in developing countries. Therefore, higher-valency vaccines were developed. Two double-blind placebo controlled randomized trials of a nine-valent conjugate vaccine were performed among children younger than one year in Africa. This investigational vaccine contained the same serotypes as PCV-7 together with serotypes 1 and 5. Both studies showed efficacy against radiologically confirmed pneumonia and invasive pneumococcal disease [157]. The study from Soweto showed that this was true for both HIV positive and negative children [157], and the study from The Gambia even showed significant efficacy against all-cause admissions and all-cause mortality [158]. These two studies clearly showed that pneumococcal vaccination has a huge potential to influence childhood morbidity and mortality in developing countries, leading to the great efforts that are made today in order to make pneumococcal conjugate vaccines accessible to the children who need them most [23].

The polysaccharides in PCV-7 and the investigational PCV-9 are conjugated to the carrier protein CRM 197, which is basically a modified variant of diphtheria toxin. A study from the Philippines tested an 11-valent conjugate vaccine, with 4 serotypes conjugated to diphtheria toxoid and 7 serotypes conjugated to tetanus toxoid. It showed efficacy against radiologically confirmed pneumonia in children younger than 2 years of age but no efficacy against clinical pneumonia. This vaccine, produced by Sanofi Pasteur, never reached the market.

New PCV's providing protection against 10 and 13 serotypes, respectively, have recently been approved for marketing, and they have markedly increased the potential vaccine coverage in most countries. These vaccines have now (2011) largely replaced PCV-7 in Sweden. In the 10-valent vaccine (Synflorix®), serotypes 1, 5 and 7F have been added to the serotypes represented in PCV-7. In PCV-10, eight of 10 serotypes are conjugated to a new carrier, the protein D from non-typeable *Haemophilus influenzae* (H.i.). Serotypes 19F and 18C are conjugated to diphtheria and tetanus toxoids, respectively, since they were shown not to be sufficiently immunogenic when conjugated to protein D in a 11-valent study vaccine that was studied before the licensing of PCV-10 [152]. The vaccine response to serotype 3 was also poor in that study, so this serotype was not included when PCV-10 was later registered. Because this was not known when paper I was written, we discussed the potential coverage of that investigational vaccine in that article. The efficacy of the study vaccine against AOM caused by *Streptococcus pneumoniae* was shown to be 52%, and efficacy against AOM caused by H.i. was 35%. These results were then extrapolated to PCV-10, which was registered for prophylaxis in children less than 5 years old against otitis media caused by *Streptococcus pneumoniae* (but not H.i.). Efficacy against IPD has not been shown in any clinical trial for PCV-10, nor for the preceding study vaccine. Instead, the immune response, measured as the geometric mean concentration (GMC) of antibodies with OPA, was shown to be non-inferior compared to PCV-7 for 5 of 7 serotypes, and above a certain cut-off for all 10 serotypes. According to recommendations from the WHO, this is sufficient evidence to predict the efficacy of a conjugate PCV against IPD, and therefore, PCV-10 is also licensed for prophylaxis against IPD in children less than five years old (www.fass.se).

In PCV-13 (Prevenar 13®), all serotypes from PCV-10 together with serotypes 3, 6A and 19A have been conjugated to CRM197. The licensure of this vaccine did not require new clinical trials measuring vaccine efficacy against IPD, pneumonia or otitis media, so the registration was only based on studies showing that it did not cause more side effects than PCV-7, and that it evoked functional antibodies above a certain level (measured with OPA) to all included serotypes. For each PCV-7 serotype, the GMC of functional antibodies was comparable to the results of PCV-7. PCV-13 was licensed in Europe for use as a single dose in adults > 50 years on November 3 2011. This indication was also based on studies in measuring the OPA titres induced by the vaccine instead of clinical trials measuring protective efficacy against IPD or other pneumococcal disease manifestations (www.FASS.se). Large randomized control trials comparing PPV and PCV-13 are under way

[159]. Since there is no defined OPA level of antibodies that is regarded as protective against IPD in adults, the most important outcome parameter in the clinical studies preceding licensure was to compare the OPA-titres evoked by PCV-13 to those evoked by PPV-23. The immune response to PCV-13 was non-inferior to all 12 serotypes that are included in both vaccines, and significantly better for all except serotypes 3 and 14. The titre against serotype 3 was lower, and the titre against serotype 14 was higher compared to other serotypes, after administration of both vaccines. The specific titre against serotype 6A, which is unique for PCV-13, was shown to be four-fold higher post- compared to pre-immunization, and since it was at a comparable level with all other serotypes, it was regarded as protective. In patients with highly elevated risk to develop IPD, both PPV-23 and PCV-13 can be given. In those cases, PCV-13 is always given before PPV-23, regardless of previous vaccination with PPV-23 [160].

Serotype 3 merits a comment: it has a special status among serotypes both due to its virulence, (see serotype section) and to its poor immunogenicity. It was excluded from the investigational 11-valent PCV that preceded the present 10-valent conjugate [150, 152] and the OPA – levels in the present PCV-13 and in PPV-23 against serotype 3 are lower than for all other serotypes in that vaccine. This is almost ironic, since the first conjugate vaccine ever described, in 1931, contained only serotype 3 [14].

In conclusion, PCV-13 might be a good alternative to PPV-23 in some adult risk patients with well-known poor response to PPV-23, considering the advantages and shortages of both PCVs and PPV-23, which are listed in table I. Whether OPA-levels are sufficient to predict clinical efficacy in adults against pneumococcal disease is still a matter of discussion [160]. If the large clinical trials that are under way manage to show reductions in pneumococcal pneumonia, IPD incidence and/or mortality due to pneumococcal infections, more widespread use may be recommended in adult risk populations in future.

Table I. Comparison between PPV-23 and PCVs. Adopted from Frenck RW, Yeh S., Expert Opin Biol Ther. 2011 Nov 10. [Epub ahead of print].

Type of vaccine	Advantages	Shortcomings
Polysaccharide	Long experience (licensed in 1983) Broad serotype coverage Efficacy for preventing IPD Low cost	T-cell-independent mode of action Decline in antibody levels in 3 -- 5 years No booster response at re-vaccination Poor immunogenicity in young children and high-risk adults No effect on nasopharyngeal carriage Unclear effectiveness in preventing pneumonia
Conjugated	T-cell-dependent mode of action Theoretically better immunogenicity than PPV Good efficacy in young children against IPD Moderate effect against pneumonia (PCV-7) Reduces nasopharyngeal carriage Indirect effect (herd immunity)	Limited serotype coverage (PCV-7). Better for PCV10 and PCV13 Only limited data on efficacy in adults Serotype replacement after introduction in children (PCV-7) High cost

AIMS

The aims of the present study were:

- to survey the serotype distribution of pneumococci isolated from blood and cerebrospinal fluid (CSF) in a region of Sweden prior to general childhood vaccination with PCV-7 (I),

- to study serotype coverage of pneumococcal vaccines (I),

- to investigate possible differences in risk factors, manifestations and prognosis between different serotypes (I),

- to investigate the pattern of antimicrobial susceptibility among all invasive pneumococcal isolates in the same area during four years (II),

- to study the impact of the genotype on the development and disease outcome of IPD, in particular between different genotypes within the same serotype (III),

- to study if decreased haemolytic capacity in serotype 1 strains correlated to outcome (III),

- to see if decreased susceptibility to one or several antibiotics was related to serotype or clinical characteristics (II),

- to study the molecular epidemiology and resistance mechanisms of antibiotic resistant strains causing IPD (III),

- to investigate incidence, risk factors, manifestations, mortality, disease severity and sequelae, for all patients with IPD in a region of Sweden during 13 years before introduction of PCV-7 in the general childhood vaccination programme in January 2009 (IV),

- to identify the persons with the highest potential benefit from vaccination (IV),

- to estimate the relative risk to fall ill with and eventually die from different IPD manifestations for different age groups and for patients with specific underlying diseases (IV),

- to explore long-term trends in the epidemiology of IPD in the same area during 45 years (IV).

MATERIALS AND METHODS

Definitions

Invasive pneumococcal disease (IPD) is defined as an infectious episode where pneumococci could be isolated from a normally sterile body site, for example blood and/or cerebrospinal, synovial, pleural, pericardial or vitreal fluid or pus extracted under sterile conditions from a deep-seated abscess. Pneumonia was defined as an x-ray verified infiltrate or post mortem diagnosis.

A **clone** is defined as a group of genetically related strains of the same bacterial species, and hence **clonality** means the genetic relationship between strains.

Incidence is defined as the number of new cases due to a certain disease in a population during a defined time period, most often a year. It is commonly described as the number of cases per 100.000 inhabitants per year. **Case fatality rate** (CFR), also called lethality, is the risk to die during an episode of a certain disease. We used the common definition of death within 30 days from the time when the cultures were taken. **Mortality** is defined as the number of deaths during a defined time period, most often a year, in a certain population caused by a certain disease [161]. Hence, mortality is the function of incidence and case fatality rate. The term "mortality" is however often used in a less stringent way, when actually CFR is meant. In paper III, we partly adhere to this praxis.

Prevalence is the number of persons in a certain population that are living with a certain disease at a certain time point. This is a useless measure when describing short-lasting diseases like IPD, but it is necessary when it comes to the epidemiology of chronic diseases, for example when estimating the number of persons living with a disease predisposing for IPD. Prevalence is the function of the **duration** of a disease and its incidence. The duration is the time from onset of a disease to death or recovery.

Patient Populations

Isolates

In papers I and II, we used isolates of pneumococci from blood, CSF and other sterile sites that were collected prospectively from the six laboratories of clinical bacteriology in the counties of Västra Götaland and Halland from 1998 to 2001. These laboratories served all 13 hospitals in the two counties. Isolates were identified as pneumococci by standard procedures such as colony morphology, alpha haemolysis on blood agar and optochin susceptibility [100]. They were stored in broth medium at -70° Celsius until further analyses were performed. In paper I, altogether 839 pneumococcal strains were collected of which 836 could be revived after storage. Of these, 827 could be revived for analysis in papers II and III. In paper III only the isolate from the first episode in each patient during the study period was included, whereas all episodes were included in papers I and II. In study III, isolates from altogether 768 adults and 52 children <18 years from study I and II were analyzed together with 357 isolates from adults during 1993-1995 and 1999-2000 and 74 isolates from children <18 years from 1998-2001 from the Stockholm area. Only one isolate from each infectious episode was included in studies I-III.

Clinical Data

Patients were identified through the microbiological laboratories and clinical data were obtained retrospectively from individual hospital notes following a standardized protocol, which was basically the same in studies I, II and IV. In papers I and II, hospital records were found for 827 and 818 patients, respectively. For the remaining nine patients, date of culture, source of isolate, age and gender were known. In study IV, only clinical data for patients that were registered inhabitants in the Västra Götaland region were included, in order to make a population-based analysis possible. All patients with growth of pneumococci in blood, CSF or other sterile body fluids during the years 1996-2008 were included. Clinical data regarding patients for whom isolates had been available for analysis in study I, and who were registered inhabitants in Västra Götaland, were retrieved from that study. Data were retrieved from medical records for all other patients during those 13 years, including all patients, except one, for whom clinical data had been missing in studies I and II. In total, clinical data were available for 2977 out of 2980 patients with IPD during 1996-2008 in study IV. Data concerning precise dates of death or emigration were collected from Statistics Sweden (SCB).

In paper III, clinical data for all 827 patients in study II were included. In addition to them, clinical data for patients from the Stockholm area were included. The adult patients from Stockholm came from a previously published study [32] whereas data concerning the children from Stockholm had not been published earlier. Data had been collected prospectively in 252 adults from Stockholm, and retrospectively for all other patients. Duration of hospitalization and intensive care unit (ICU) stay were recorded from hospital records for all patients <18 years. Data concerning underlying diseases were missing for one child and 34 adults. In eight of these, focus was known. All adult patients from Stockholm, who had been hospitalized within 30 days before admission, had been excluded from the study. This discrepancy between the two study populations was not considered to have any major impact on the results. All other clinical data had been collected using very similar protocols. The two databases were merged evaluating the cases one by one, and in the very few cases where there was a question regarding classification of underlying disease, clinical data were recollected from hospital notes.

Laboratory Methods

Serotyping

Serotyping of the isolates was performed by the capsular swelling test using 46 group, type and factor serum samples. All pneumococci belonging to a group (including >1 type) were examined by the capsular reaction test with type and factor specific antisera [100]. The analyses of the isolates in study I, II and the majority of study III were analyzed at the World Health Organization Collaborating Centre for Reference and Research on Pneumococci at Statens Serum Institut, Copenhagen, Denmark, and the remaining isolates in study III were analyzed at Smittskyddsinstitutet, Stockholm, using the same method.

Resistance Determination

Minimum inhibitory concentrations (MIC) were determined for penicillin G, erythromycin, clindamycin, tetracycline, moxifloxacin and trimethoprim-sulfamethoxazole (cotrimoxazole) for all isolates in study II using an Epsilometer test (Etest®) The principle is as follows: a plastic strip that is impregnated with an antimicrobial agent, in an increasing concentration from one end of the strip to the other, is placed on a layer of bacteria on an agar plate. The drug then diffuses into the agar, producing an exponential gradient, leading to an elliptical

zone of inhibition on the plate. The concentration at the point where the ellipse meets the strip is the MIC of the drug, and can be easily read since it is printed on the strip. The testing was performed according to the instructions of the producer (www.abbiodisk.com), and according to guidelines and breakpoints of the Swedish Reference Group for Antibiotics (SRGA) at the time of the study, with one exception: values in between the two-fold dilutions were recorded in order to give a higher resolution when analyzing data. This method was checked by two independent readers in a pilot study showing a perfect match between readings. The values were rounded off to the next upper two-fold dilution before susceptibility categorisation. Since study II was performed, the SRGA recommendations concerning both media and some breakpoints have changed. Therefore, some of the strains that were classified as sensitive (S), indeterminate (I) or resistant (R) in paper II in 2006 had changed classification when paper III was written in 2011, see Table II. These minor changes did not have any impact on the conclusions in study II and III, but they may serve as an illustration, that the question, whether a strain is resistant or not, may be a matter of which definition is used. For example, the MIC breakpoints for cotrimoxazole at the time of study II referred to the sum of the two substance's concentrations in the ratio 1:19, so they had to be divided by 20 and then rounded off to the next upper twofold dilution step before susceptibility categorisation. At the time of study III, the definition had changed, and the official breakpoint referred to the trimethoprim component of the combination, which corresponds to the values printed on the E-test strips. MIC's for the isolates from Stockholm in study III were determined using either E-test or a gel diffusion method described elsewhere [162].

Table II. MIC breakpoints according to SRGA in study II and III, and changes in classification.

Compound	MIC breakpoints***		Isolates that changed SIR classification between study II and III
	2005 (study II)	2010 (study III)	
Penicillin G	0,06/1*	0,06/1*	none
Cefotaxime	0,12/1	0,5/2	12 isolates classified as I in study II were S in study III
Erythromycin	0,5/0,5	0,25/0,5	16 isolates classified as S in study II were I in study III
Clindamycin	0,5/2	0,5/0,5	none
Tetracycline	2/2	1/2	3 isolates classified as S in study II were I in study III
Co-trimoxazole	16/32**	1/2**	none
Moxifloxacin	0,5/0,5	0,5/0,5	none

SIR classification: S=susceptible, I=indeterminate, R=resistant
*interpretation: 0,06/1 means S ≤ 0,06, R > 1
**Defined as the sulphamethoxazole concentration in 2005 and as the trimethoprim concentration in 2010 (1:19)
***according to the Swedish reference group for antibiotics (SRGA)

Molecular Methods

Molecular Typing Methods

Altogether 1251 isolates were analyzed using a PFGE method that was adapted from a previously described procedure [163, 164]. The chromosomal DNA was cleaved with a restriction enzyme, separated on an agarose gel, stained with ethidium bromide and then analyzed using Bionumerics version 6.01 (Applied Math). Clones were defined by using previously described criteria [164]. PFGE clones with more than 10 isolates were categorized into 18 different eBURST groups. MLST was performed on selected strains from each dominant PFGE pattern [165]. Seven housekeeping genes were sequenced and assigned allele numbers using the pneumococcal MLST database (http://www.mlst.net). The obtained allele numbers formed an allelic profile, which was assigned a sequence type (ST) and compared with other profiles in the MLST database. Phylogenetically related ST's are grouped in clonal complexes (CC). MLST was performed on 346 isolates. Based on these MLST results, all isolates in the same PFGE clone were considered to belong to the same clonal complex.

Haemolytic Pneumolysin Assay

Among 153 serotype 1 isolates, 150 could be revived for analyzing haemolytic pneumolysin. Pneumococci were grown over night on blood agar plates at 37°C and 5% CO2, re-suspended in 3 ml PBS to OD600=0.5. The cell wall was lysed by vortexing and 50µl bacterial suspension was added to 50µl human blood suspension in phosphate buffer saline containing 1mM dithiothreitol. Tubes were incubated for 30 min and haemolytic activity was considered to be functional when the content became transparent, and non-functional when an erythrocyte sedimentation occurred. All observations were compared to TIGR-4 control strains with either known functional or non-functional pneumolysin genes, derived from a study by Littmann et al. [42].

Detection of Erythromycin Resistance Genes (supplement paper III)

Macrolide resistance genes (ermA, ermB, and mefA) were identified by polymerase chain reaction (PCR), in all 65 strains classified as erythromycin resistant, together with 138 randomly chosen strains. In addition, positive and negative controls were run concurrently with the isolates in the study.

Statistical Methods

The two-tailed Fisher´s exact test and the χ2 –test were used to compare proportions when appropriate. Median ages were compared with the Mann-Whitney U test. No corrections for multiple comparisons were performed in study I and II. Mean populations, annual incidence rates, CFR and mortality rates were calculated using Excel 2003. P-values < 0.05 were regarded as significant.

All statistical analyses in Study III were performed using STATA version 11.0. Adults (>18 years) and children were analyzed separately. A log binomial regression model was used to assess the association between clonal complex and age, co-morbidity, disease manifestation and severity of disease [166]. Severity of disease was assessed using 30-day mortality for adults and ICU stay >24 hours for children since mortality was a rare event among children. For adults, the clonal complex (CC)306 was used as reference category since it was a common type among adult IPD patients, had the lowest 30-day mortality and was highly homogenous (all CC306 isolates belonged to serotype 1). Only clonal complexes with more than 25 isolates were included in the analyses of adults. The association between clonal complex and age was assessed using one-way ANOVA with Bonferroni method to account for multiple comparisons. Analyses assessing the association between clonal complex and disease manifestation and 30-day mortality among adults were adjusted for age (>65 years) and co-morbidity using

Charlson Index score, where patients were categorized as having score 0, 1-2 and >3 [167]. For children, CC191 was used as reference category since it was a common type among children, had the lowest absolute risk of ICU stay and contained one or more events for all intended comparisons. For children, four clonal complexes with nine or more isolates were included in the models. Due to limited numbers adjustment for potential confounders could not be performed and these results should be interpreted with caution. In 2x2 comparison Chi Squared test and Fisher's test were used as appropriate. P-values ≤0.05 were regarded as significant.

In study IV, confidence intervals for relative risks were calculated using CIA version 2.1.2, all other statistical analyses were performed using SPSS version 19.0.0. The total number of blood cultures in each laboratory and the number of inhabitants in the region for each year

were included in a linear model, in order to assess if there was an increasing number of blood cultures per inhabitant during the period. Differences in CFR between men and women were assessed using a logistic regression model, controlling for possible confounders (age, any comorbidity, alcohol abuse and cardiovascular disorders). Relative risks were calculated as univariate analyses. A multiple logistic regression model was constructed with death within 30 days as outcome and asthma, age, sex and comorbidity as explanatory variables. The comorbidity variable was constructed with respect to all predisposing factors other than asthma. Student's T-test was used to compare means.

RESULTS AND DISCUSSION

Serotype Distribution and Potential Vaccine Coverage (Paper I)

In total, 836 pneumococcal isolates causing invasive pneumococcal disease in children and adults were included. Serotype distribution varied with age, as shown in table III. The most common serotypes were 1, 7F, 9V, 14, 4 and 12F. These serotypes together with serotypes 3 and 6A+6B have been among the most common in other studies from northern Europe but their rank order differs with time and place of origin [116, 123, 168-170]. The proportion of serotype 1 had increased remarkably compared to a previous study performed 30 years ago including a part of the same area as the present study [123]. This increase was due to the spread of a new clone [128]. This corresponds to a Danish study of long-term trends in serotype distribution [116] where the incidence of serotype 1, together with serotype 3 seemed to fluctuate since the 1970s with peaks every 7-10 years. In studies from Denmark and Norway from the same period, type 1 was also found to be the most frequent serotype [169, 170], whereas it was not the most frequent serotype in the Stockholm area at the same time [32]. Type 3, which was the most common type 30 years ago [119] ranked only as number 7 in the present study.

Table III. Serotype distribution in different age groups, and in different vaccines.
[1] One patient was infected with both serotype 1 and 4. [2] Serotypes 6A and B were considered to be cross reactive at the time of the study, see text for details.

| Serotype | Age Group (Years) | | | | | Included in Vaccine | | | |
	0-4	5-19	20-59	≥60	All Ages	PCV-7	PCV-10	PCV-13	PPV-23
1	3	17	70	29 + 1[1]	119 + 1[1]		x	x	x
7F	4	4	49	33	90		x	x	x
9V	1	3	27	41	72	x	x	x	x
14	2	2	20	47	71	x	x	x	x
4	0	1	40	28 + 1[1]	69 + 1[1]	x	x	x	x
12F	0	0	23	26	49				x
6B	7	0	5	24	36	x	x	x	x
3	0	0	9	26	35			x	x
23F	0	1	10	23	34	x	x	x	x
8	1	0	13	16	30				x
9N	0	0	8	21	29				x
6A	3	1	3	21	28	(x)[2]	(x)[2]	x	(x)[2]
19A	1	0	4	21	26			x	x
22F	0	0	7	18	25				x
18C	2	3	7	10	22	x	x	x	x
19F	1	0	5	15	21	x	x	x	x
11A	0	0	3	11	14				x
23A	0	0	5	5	10				
10A	0	0	1	6	7				x
16F	0	0	5	2	7				
33F	0	1	2	3	6				x
5	0	0	0	0	0		x	x	x
Other PPV-23	0	0	2	2	4				x
Other non-PPV-23	1	0	11	16	28				
Rough	0	0	2	1	3				
All serotypes	**26**	**33**	**331**	**446**	**836**				

Of all strains, 42%, 70%, 75% and 94% belonged to serotypes included in PCV-7, -10, -13 and PPV-23, respectively (table III). At the time of the study, an 11-valent investigational conjugate vaccine was being developed, and the coverage for this vaccine was therefore described in the paper. Serotype 3 was, however, not included when PCV-10 was later marketed. At the time of studies I and II, serotypes 6A and 6B were considered to have sufficient cross-reactivity to be considered as cross-protective, and 6A was therefore included among PCV-7 types. It was later shown that strains formerly categorized as 6A actually belonged to two different serotypes, 6 A and C, and that probably only 6A is cross-reactive with 6B [28]. Since we have not been able to reanalyze the 28 isolates categorized as serotype 6A at the time of the study, this may have led to a slight overestimate of vaccine coverage. Besides cross protection between serotypes 6A and 6B is not complete.

CFR and Clinical Characteristics Related to Serotype (Paper I)

The main finding was that CFR was lower among patients infected with serotype 1 and 7F, 2/119 =2%, (p=0.0009) and 2/89=2%, (p=0.011), respectively, compared to patients infected by all other serotypes. The patients with these two serotypes were also younger than the patients infected with all other serotypes, and a significantly lower proportion of them suffered from underlying diseases. These two factors probably explain the low CFR. The finding that CFR due to these serotypes is low compared to other serotypes has later been confirmed by other European studies [32, 33, 133]. The proportion of pneumonia was also higher among patients infected by serotype 1, together with serotype 4, compared to all other serotypes, 111/119=93% (p<0.0001) and 61/69=88% (p=0.004), suffered from pneumonia, respectively.

Only serotype 19A had a higher CFR than the average of all other serotypes (7/26=21%, p=0.006). A large proportion of those patients were old and suffered from underlying diseases, but those differences were not significant. In contrast, the proportion of patients having risk factors was higher among patients suffering from disease caused by serotype 19F compared to all patients with non-serotype 19F. The same was true for serotype 6A. These serotypes have also been shown to be associated with higher CFR [33] than other serotypes.

After paper I had been published, Alanee et al. investigated the relation of disease severity and mortality with serotypes using another approach [171]. Instead of comparing the serotypes one by one, they were grouped in three categories, depending on their known ability

to cause carriage or invasive disease [29]. We therefore reinvestigated all data from 777 adults in paper I, and subdivided them as "invasive" (1, 7F; n=197), "paediatric" (6A+B, 9N+V, 14, 19A+F, 23A+F; n=304) and "other" serotypes (n=206). These results were published in 2009 [172]. Infections caused by "invasive" strains were more often seen in younger patients without concomitant conditions and had lower case-fatality rate than "paediatric" and other strains, which did not differ from each other (see table IV). The CFR was very low in infections caused by "invasive" strains, only 2% which partly was due to the lower age and absence of risk factors among these patients. The CFR was, however, lower also among patients with underlying diseases and/or high age that were infected by "invasive" serogroups compared to other serogroups (4/88 patients, 4.5%, P=0.047).

Table IV. Clinical characteristics of invasive pneumococcal infections caused by "invasive", "paediatric" and "other" (non-invasive, non-paediatric) serogroup strains.

Clinical Characteristic	All Serotypes No (%)	"Invasive" No (%)	"Paediatric" No (%)	"Other" No (%)	"Invasive" vs "Other" P-value
Age <65 years	416 (54)	135 (72)	132 (43)	149 (52)	<0.0001
No underlying disease	301 (39)	120 (64)	88 (29)	93 (33)	<0.0001
<65 years, no underlying disease	202 (26)	99 (53)	49 (16)	54 (19)	<0.0001
Pneumonia	591 (76)	167 (89)	209 (69)	215 (75)	<0.0001
Fatal outcome	76 (10)	4 (2)	37 (12)	35 (12)	<0.0001
All patients	777	187	304	286	

The finding, that both manifestation and outcome are related to serotype is not unique for the pneumococcus. It was early observed that *Haemophilus influenzae* type b caused meningitis in small children, in contrast to other capsulated and non-encapsulated *H.influenzae* [173, 174]. Vaccination with a conjugate vaccine against this serotype has therefore been very successful reducing meningitis and epiglottitis incidence due to this bacterium, and no serotype replacement has occurred. Among invasive infections caused by group B streptococci (GBS), serotype distribution is fluctuating over time and the distribution varies with age, but serotype does not seem to influence clinical manifestations [175, 176]. No GBS vaccine has reached the market yet.

CFR and Patient Characteristics Related to Genotype (Paper III)

The main finding of this study was that the CFR in adult patients infected with pneumococci belonging to six different clonal complexes (CCs) was higher compared to those infected with CC306, even after adjusting for age and comorbidities. Differences between clones with regard to disease manifestations and age among both adults (Table V) and children (Table VI), were also seen. The proportion of adult patients with underlying diseases was higher in eleven different CCs compared to CC306. It has previously been difficult to demonstrate such correlations [32, 133], due to the need for a very large study material. The only previously described correlations between clone and CFR correspond to our findings: Inverarity et al. showed lower CFR for ST306, which in our study comprised 82 of 112 CC306 isolates [133]. Both Sjöström and Inverarity showed increased CFR (if not adjusting for age) for ST 180, which in our study constituted the majority of the CC180 [32, 133]. Significantly higher CFR among adults for CC53, CC218, CC176, CC180 and CC 199 than for CC 306 has not, to our knowledge, been described before.

Since all **CC306** isolates belonged to serotype 1, we could not conclude whether the low CFR seen among adult patients infected with strains belonging to this CC was due to the genotype or the serotype. This implicates, that the finding in paper I, that CFR was lower and that pneumonia was more common among patients infected by serotype 1, could as well be explained by the genotype of those strains. As a matter of fact, the same could be true for other European studies that have shown lower CFR among patients infected with serotype 1 compared to other serotypes [33], since they did not analyze clonal type. There are data indicating that different serotype 1 clones might have a higher virulence in Africa, where CC217 is the most common CC with serotype 1 [177]. We did not have any CC217 in the present study, and no differences between CC306 patients and those infected with another serotype 1 clone, **CC304**. Neither could we find any differences regarding any characteristics between 102 patients infected by CC306 strains expressing a non-haemolytic pneumolysin compared to 47 patients infected with serotype 1 strains with functional pneumolysin. Since in vitro data have shown that isolates with non-haemolytic pneumolysin are more pro-inflammatory in cell cultures [42], a difference in outcome or manifestation could have been expected.

Table V. Clonal complex, disease manifestation and CFR in adult patients.

			Disease manifestation[β]						CFR	
Clonal Complex	Serotypes[a]	Patients no	Pneumonia no (%)	RR[δ]	Meningitis no (%)	RR[δ]	Sepsis[γ] no (%)	RR[δ]	no (%)	RR[δ]
CC191	7F, 14	114	100 (88)	0.92 (0.85-1.00)	6 (5)	2.11 (0.54-8.25)	7 (6)	6.13 (0.77-49.05)	5 (4)	2.08 (0.41-10.51)
CC306	1	112	107 (96)	1 (ref)	3 (3)	1 (ref)	1 (1)	1 (ref)	2 (2)	1 (ref)
CC156	**9V**, 14, 19F, 24A, 24F	111	90 (82)	**0.87** (0.79-0.96)	8 (7)	3.40 (0.91-12.38)	14 (13)	**11.31** (1.50-85.06)	11 (10)	4.24 (0.96-18.80)
CC205	**4**	90	81 (90)	0.95 (0.88-1.03)	2 (2)	0.95 (0.16-5.55)	6 (7)	6.27 (0.77-51.26)	5 (6)	2.56 (0.51-12.91)
CC124	**14**, 23F, 17F	80	64 (80)	**0.85** (0.76-0.96)	6 (8)	3.67 (0.94-14.41)	12 (15)	**12.54** (1.65-95.45)	8 (10)	3.86 (0.83-17.90)
CC53	**8**, 11A	53	42 (79)	**0.85** (0.73-0.99)	3 (6)	2.81 (0.60-14.00)	7 (13)	**10.77** (1.35-86.13)	8 (15)	**6.01** (1.31-27.60)
CC218	**12F**, 22F, 35B, 19V	50	32 (65)	**0.70** (0.57-0.86)	4 (8)	3.80 (0.88-16.44)	10 (20)	**17.54** (2.29-134.24)	7 (14)	**5.59** (1.19-26.20)
CC176	**6B**, 8, 23F, 33F, 6A	48	33 (70)	**0.75** (0.62-0.91)	6 (13)	**6.16** (1.58-24.00)	6 (13)	**10.69** (1.31-87.16)	10 (21)	**7.99** (1.80-35.50)
CC439	**23F**	41	33 (83)	0.90 (0.78-1.04)	2 (5)	2.51 (0.43-14.81)	5 (13)	**10.29** (1.22-86.46)	4 (10)	3.59 (0.67-19.08)
CC180	**3**	39	33 (85)	0.89 (0.77-1.03)	4 (10)	**4.56** (1.05-19.90)	2 (5)	4.47 (0.41-48.27)	9 (23)	**8.64** (1.93-38.68)
CC66	**9N**	36	16 (44)	**0.59** (0.42-0.82)	3 (9)	4.51 (0.94-21.58)	10 (34)	**24.36** (3.18-186.57)	6 (17)	**6.24** (1.30-29.89)
CC433	**22F**	32	22 (69)	**0.73** (0.57-0.92)	5 (16)	**7.36** (1.86-29.10)	7 (22)	**18.18** (2.30-143.86)	2 (6)	2.58 (0.38-17.70)
CC9	**14**, 19F, 12F	32	27 (84)	0.91 (0.79-1.07)	4 (13)	**5.44** (1.28-23.08)	4 (13)	**12.11** (1.40-104.54)	2 (6)	2.81 (0.41-19.16)
CC199	**19A**, 15B, 15C, 19F, 12F	28	20 (77)	0.83 (0.67-1.04)	2 (8)	3.80 (0.65-22.04)	4 (15)	**11.90** (1.37-103.32)	6 (23)	**8.09** (1.71-38.31)
CC490	**6A**, 6B	25	15 (58)	-	4 (16)	-	5 (19)	-	7 (28)	-
CC113	**18C**	20	13 (65)	-	2 (10)	-	2 (10)	-	1 (5)	-
CC460	**10A**, 35F	14	6 (55)	-	0 (0)	-	4 (36)	-	3 (27)	-
CC304	**1**	14	13 (93)	-	0 (0)	-	0 (0)	-	0 (0)	-
Other		186	112 (65)	-	13 (8)	-	38 (22)	-	29 (17)	-
Total		1125	859 (78)	-	77 (7)	-	144 (13)	-	125 (11)	-

[a] Serotypes in decreasing frequency. Dominating serotype (>50%) in bold.
[β] Patients can suffer from more than one manifestation.
[γ] Bacteremia with not detectable focal manifestation
[δ] Relative risk adjusted for age and Charlson Index Score, calculated on patients with complete clinical data (97.4% of all patients).

Table VI: Clonal complex associated to disease manifestation, underlying disease and intensive care unit stay in children.

			Disease manifestation[β]				Age <2 yrs		ICU >24 hrs		Died
Clonal Complex	Serotypes[b]	Patients no	Pneumonia no (%)	RR[δ]	Meningitis no (%)	RR[δ]	no (%)	RR[δ]	no (%)	RR[δ]	no
CC176	**6B**, 6A	24	7 (29)	0.62 (0.28-1.35)	11 (46)	1.45 (0.66-3.20)	19 (79)	**1.88** (1.07-3.31)	9 (41)	3.68 (0.91-14.93)	1
CC306	**1**	20	19 (95)	**2.01** (1.24-3.26)	0 (0)	N/A[v]	0 (0)	N/A[v]	3 (15)	1.35 (0.25-7.19)	0
CC191	**7F**, 14	19	9 (47)	1 (ref)	6 (32)	1 (ref)	8 (42)	1 (ref)	2 (11)	1 (ref)	0
CC124	**14**, 19A, 23F	9	4 (44)	0.94 (0.39-2.24)	6 (67)	2.11 (0.94-4.73)	7 (78)	1.85 (0.98-3.48)	5 (56)	**5.00** (1.19-20.92)	1
CC9	**14**, 19F	5	1 (20)	-	3 (60)	-	3 (60)	-	2 (40)	-	0
CC113	**18C**	5	0 (0)	-	2 (40)	-	1 (20)	-	1 (20)	-	0
CC199	**19A+F**, 15B+C	5	1 (20)	-	2 (40)	-	3 (60)	-	2 (40)	-	0
CC304	**1**	5	4 (80)	-	1 (20)	-	2 (40)	-	0 (0)	-	0
CC460	**35F**, 6A	4	0 (0)	-	4 (100)	-	4 (100)	-	0 (0)	-	0
CC439	**23F**	4	0 (0)	-	3 (75)	-	3 (75)	-	1 (25)	-	0
CC490	**6A**	3	0 (0)	-	1 (33)	-	2 (67)	-	1 (33)	-	0
CC53	**8**, 11A	3	1 (33)	-	3 (100)	-	2 (67)	-	2 (67)	-	1
CC156	**9V**	3	0 (0)	-	0 (0)	-	0 (0)	-	0 (0)	-	0
CC205	**4**	2	0 (0)	-	2 (100)	-	1 (50)	-	1 (50)	-	1
CC180	**3**	2	0 (0)	-	2 (100)	-	1 (50)	-	0 (0)	-	0
Other		13	4 (31)	-	5 (38)	-	6 (46)	-	4 (31)	-	0
Total		126	50 (40)	-	47 (38)	-	62 (49)	-	33 (26)	-	4

[a] and [β] see Table V.
[δ] Relative risk
[v] N/A: RR not applicable due to zero cases in the subgroup. Comparision between cc306 and cc191 using Fisher's Exact Test gives p=0.001 for age <2 years and p=0.008 for meningitis.

CC180 had the highest RR for death (8.6) among all CCs. All isolates belonged to serotype 3, which has been associated with higher CFR than other serotypes [33, 178]. In parallel with serotype 1 and 7F, it is impossible to say, if at least a part of its virulence is due to genotype instead of serotype. Furthermore, CFR was also higher among **CC199** infected patients, but since it was dominated by serotype 19 A, which was the only serotype with higher CFR than all other serotypes in paper I, it is again difficult to conclude whether serotype or clonal type is most important for the virulence.

To know whether serotype or clonal type is most important, we looked at CCs with two dominating serotypes, and serotypes with two dominating clonal types: **CC53** and **CC124** were dominated by two serotypes each. Among patients with CC53, those with serotype 11A were older than those with serotype 8. Among patients infected with CC124 strains, serotype 14 was more prone to cause pneumonia than serotype 23F, which was more often associated with septicaemia with unknown focus. These serotype-specific differences within the CCs might be due to different pathogenic properties of different capsules.

Serotype 14 was dominated by two CCs; the above mentioned **CC124**, and **CC9**. Among all patients infected with serotype 14, it was more common among those with CC124 to suffer from underlying diseases than among those with CC9. There also seemed to be a higher risk among CC124 patients to die, although this difference was not significant. Interestingly, most CC9 isolates were erythromycin resistant due to the presence of a mefA cassette, which may result in a bacterial fitness cost. Also serotype 23F was dominated by two CCs; CC124 and **CC439**. In similarity with serotype 14, patients with CC124 were more likely to suffer from septicaemia without an identified focus than those with CC439.

It is important to know if outcome or disease manifestations correlate to clone and not only to serotype because the currently used vaccines provide protection against serotypes, not clonal types. Serotype replacement is a well known phenomenon after introduction of PCV-7 in general childhood vaccination. If more virulent clones acquire non-vaccine capsules, and are able to spread, this may lead to changes in both incidence and disease panorama, which might threaten the success of vaccination in a long-term perspective [130, 131]. This study showed that properties shared among isolates of the same clonal type influence pathogenesis as well as the obvious impact of capsular types.

Antibiotic Resistance in IPD (Papers II and III)

Altogether 83/827 isolates (10%) in paper II and 112/1251 (9%) in paper III were I or R to at least one agent. Only 2.7% of all isolates in papers II and III were PNSP which is low compared to other countries. In both studies a majority of the PNSP isolates were I or R also to cotrimoxazole. Serotype 9V was the most common serotype among PNSP isolates in both studies. In study III, 41% if all PNSP had the clonal type defined as CC156. It was found to include the highest proportion of PNSP isolates (12%) and cotrimoxazole resistant strains (15%) compared to other CCs. ST156, which is the most common sequence type in CC 156, is known to be an internationally successful penicillin resistant clone worldwide [179].

Resistance to erythromycin was found in 30 isolates, of which 6 also were resistant to clindamycin. All 24 strains, which were R only to erythromycin and no other agent, belonged to serotype 14. Double disc diffusion showed a typical D-shaped zone indicating inducible resistance in only one strain. This corresponds well to the findings in study III, that erythromycin resistance was highly clonally related and that it was mainly mediated by the mefA mechanism. The risk for treatment failures due to erythromycin resistance is greater when it is caused by the ermB mechanism [180].

The number of strains reported as I or R to cotrimoxazole in study II was 17 and 31, respectively, which was a greater proportion than for the other studied compounds. When resistance determination was performed on a reference strain (ATCC 49619), correlation with the target values was found to be good for all agents except for cotrimoxazole that gave MIC values 0.5-1 two-fold dilution steps higher than the target values. The MIC distribution pattern gave the impression that the breakpoints cut off a part of the normal distribution curve. The number of true outliers on the MIC distribution curve was 21(2.5%). There are reports in the literature stating that the E-test is less reliable for determining cotrimoxazole resistance in *S. pneumoniae*, because the method gives too high MIC values [181]. Furthermore, MIC breakpoints for this drug were changed between the two studies. Therefore, the data for cotrimoxazole resistance should be regarded with caution.

Quinolone resistance in many bacteria, including *S. pneumoniae,* is a growing concern in many parts of the world. Quinolones are not recommended for treatment of respiratory infections in Sweden. The quinolone moxifloxacin, which is used widely abroad but not much

in Sweden, was included in study II in order to see if there was any resistance before introduction of this drug on the market. Only one strain was resistant to moxifloxacin (MIC 8 mg/L). This patient was receiving ciprofloxacin since 4 days before admittance against suspected pyelonephritis. This agrees well with previous studies that have shown that quinolones may induce resistance in pneumococci [182].

The proportion of strains with reduced antibiotic susceptibility varied largely with serotype. In study II, it was lower in serotypes 7F, 4 and 3 and higher in 14 and 19F which corresponds to previous studies [70]. Paediatric serotypes (6B, 9V, 14, 19F, 19A and 23F), are commonly carried for long periods in children and therefore exposed to more antibiotics. Since many of the serotypes commonly seen with resistance are included in the pneumococcal conjugate vaccines, it was not surprising to find that the proportion of strains with reduced susceptibility (I or R) to at least one agent among the vaccine types were 61/350 (17%) and 68/557 (12%) and 80/781(10%) for PCV-7 and PCV-10, respectively. Of 22 PNSP strains, 20 (91%) expressed serotypes represented in PCV-7. Therefore, widespread use of this vaccine might have a positive impact on the proportion IPD caused by PNSP.

Incidence, Manifestations and CFR Related to Age and Underlying Diseases (Paper IV)

Altogether 2980 episodes of IPD occurred in residents of the Västra Götaland region (1.5 million inhabitants) during the period 1996-2008. The mean IPD incidence was 15.1/100,000 inhabitants/year. The annual IPD incidence was highest among people ≥65 years (45/100,000), followed by infants 0-23 months (23/100,000). This is low compared to most countries prior to PCV vaccination [138, 183]. The low incidence in this age group might partly depend on blood culturing habits, since blood cultures are mainly performed in in-patients, whereas for example in a study from the US showing an annual incidence of 188/100,000 among children 2 years old or less prior to PCV vaccination, only 29% were in-patients [138].

Pneumonia was the most common manifestation, followed by septicaemia without a detectable primary focus and meningitis. A broad variety of other, more rare manifestations were observed. The distribution of manifestations varied greatly with age. Among infants (younger than 2 years), 32% of patients had meningitis compared to 5% among elderly (65 years and above), whereas pneumonia occurred in 25% of infants and 80% of elderly. The annual incidence of non-meningitic infections and meningitis was 14.1/100,000 and 1.1/100,000, respectively. The former was highest in infants, 7.0/100,000, and only 2.0/100,000 among elderly, whereas the latter was highest among elderly, 42.8/100,000 compared to 15.5/100,000 among infants. Of 180 meningitis patients who survived, 70 (39%) survived with sequelae. Neurologic sequelae, such as paresis, developed in 54 patients, and hearing loss in 30 patients; 21 suffered from both.

CFR in all IPD was 9.9%, which is low compared with other studies. This is interesting, since the proportion of elderly was found to be relatively high, and CFR increased with age. The male female ratio was 1:1, but CFR was higher in men (12%) than in women (9%), $p<0.01$, despite the fact that the mean age was higher for women than for men, 62.4 vs 59.0 years, $p<0.001$. Male sex was an independent risk factor after correcting for age and comorbidities. CFR also varied between manifestations; it was highest among patients with septicaemia with unknown focus, compared to all other manifestations, probably because a high proportion among these patients had underlying diseases.

The patient suffered from one or more underlying disorders in 67% of all IPD episodes. The risk to die within 30 days for patients with underlying disorders was 3.4 (2.45-4.81) times higher than for patients without underlying disorders. The highest relative risk to die was seen among patients with solid tumours (especially lung cancer), followed by patients with haemodialysis, cardiovascular disease and haematological malignancy, especially multiple myeloma (MM). The highest annual IPD incidence was seen among MM patients, 2238/100,000, which was 154 times higher than for persons without myeloma. The incidence in this group was even higher than previously described [184]. PPV-23 is poorly immunogenic in this group [185] and it remains to be shown if PCV is more effective. Patients with chronic lymphatic leukaemia, haemodialysis, lung cancer, HIV, SLE, asplenia, rheumatoid arthritis, COPD, and diabetes mellitus also had a high IPD incidence, in descending order, which is similar to previous studies [119, 186]. Patients with renal disease, especially those with haemodialysis, also had a markedly elevated risk to get IPD and to die from it.

Both CFR and incidence was significantly lower among asthma patients (RR=0.27 (95% CI 0.10-0.71) compared to all patients without asthma. With an estimated asthma prevalence of 8.3%, annual incidence among persons with asthma was 11.7/100,000, compared to 12.5/100,000 for persons without asthma. This finding stands in contrast to a study from Tennessee, where asthma was shown to be an independent risk factor for IPD in the age group 2-49 years [187]. Asthma is included US recommendations for PPV-23 as a result of that study [76]. However, there are some concerns regarding confounding of smoking and COPD in that study. The American study primarily comprised persons of low socioeconomic status enrolled in a Medicaid program, whereas the Swedish study is population based. It seems as if the asthma populations in the age group are different, illustrated by the fact that 8% of the American asthma patients had received long-term oral corticosteroids, whereas only 1 of 54 (2%) Swedish asthma patients in the same age group were treated with oral corticosteroids at the time of the IPD episode. Our data indicate that asthma without COPD is not an independent risk factor for IPD in a population with good availability of supportive asthma medication.

Regional Differences and Long-Term Changes (Paper IV)

Altogether 1083 IPD episodes occurred in the Greater Gothenburg area, giving an annual incidence of 12.9/100,000, compared to 1894 episodes and 16.8/100,000 in the surrounding region (p<0.001). CFR was 9% (93/1083) in Greater Gothenburg and 11% (201/1894) in the rest of the region (p=0.08). The lower incidence in the Greater Gothenburg area may partly be explained by the fact, that the proportion of patients ≥80 years was lower in Gothenburg (201/1083) compared to the region (426/1894, p=0.012). The annual incidence in this age group was, however, lower in Gothenburg compared to the region, 48 vs 71/100,000, (p<0.001). This might be due to a bias caused by different blood culturing practices. Another contributing factor for the regional differences is that 12% of the patients from greater Gothenburg were alcoholics compared to 5% in the region (p<0.001).

Data from greater Gothenburg from 1996-2008 were compared with data from two studies that were performed earlier in the same area using the same protocol [119, 120]. The annual incidence of all IPD increased from 5.3/100,000 and 10.3/100,000 in 1964-1980 and 1981-1995, respectively, to 12.9/100,000 in 1996-2008. A similar development has been observed in Denmark [116]. Age specific incidence among infants (<2 years) did not change significantly, whereas annual incidence among persons ≥65 years had increased from 11/100,000 in 1964-1980, and 30/100,000 in 1981-1995, to 40/100,000 in the present study. The male female ratio was 1:1 in 1996-2008, compared to 2.1:1 and 1.3:1 in 1964-1980 and 1981-95, respectively. The proportion of patients with alcohol dependency had remained stable at 12% since 1981-1995, compared to 27% in 1964-1980. This corresponds to a decrease in the prevalence of advanced alcohol dependency syndrome that has occurred since the 1960s (Sven Andréasson, personal message). CFR was 9% compared to 15% and 20% in 1964-80 and 1981-95, respectively. Mortality, was lower in the age groups 18-50 and 51-64 years in 1996-2008 compared to 1964-1981, whereas it had increased among patients aged 65 years and above, all p-values<0.05.

The long-term changes in incidence and CFR for all IPD were probably largely influenced by increased blood culturing frequency and improved culturing techniques. Changing age distribution in society and better intensive care do probably also contribute. In contrast, meningitis incidence remained rather stable during 45 years, with a slight decline during the last period. The CFR in meningitis patients declined from 33 to 13%. Since there are no low

symptomatic cases of meningitis, the incidence and CFR are less influenced by culturing practices, since CSF cultures or at least blood cultures are almost always performed in these patients.

Limitations of Retrospective Data (Papers I-IV)

All clinical data for all patients in studies I-IV except a minor fraction of the adult population in study III were collected retrospectively. This is a limitation, since we are dependent on the documentation that occurred in the hospital at the time of the disease episode. Additional information concerning underlying diseases and sequelae were collected from hospital notes before and after the episode, when available, in order to get as complete information concerning each patient as possible. Data concerning exact dates of death were collected from population registries, in order to get completely reliable information whether the patient died within 30 days of admission or not. Additional data concerning permanent addresses of the patients in paper IV were also collected from these registries if they were missing in the medical record, in order to make the population based approach as reliable as possible. Length of stay was provided by hospital administrative systems.

Sweden has a major advantage compared to many other countries when retrospective studies are performed. Underlying diseases, disease manifestations and outcome are usually meticulously documented in hospital notes. Furthermore, the system with personal identification numbers makes it relatively easy to find all necessary information, which would be more difficult and imprecise if we only had to rely on name and age. This is the explanation for the extremely low dropout figure in study IV, where we were able to find clinical data for 2977 of 2980 episodes. Through this population based approach, selection bias was minimized. Patients not living permanently in the area were excluded from the analysis, to ensure reliable data on incidence, and to make the comparisons with the earlier studies as reliable as possible. There is, however, a risk to underestimate the incidence since some permanent residents probably experienced IPD episodes outside the study area during the study period. Those missing episodes were, however, probably few compared to the number of episodes that were excluded due to this reason, since it is common that residents from the neighbouring county south of Västra Götaland seek medical help in Gothenburg, but uncommon that patients move the other direction to seek medical help (this was confirmed through patient registries, data not shown). The potential bias of this factor is therefore probably very low.

Another limitation, linked to the retrospective approach, is that death is a blunt method to measure the disease severity. Many clinically important differences in virulence between different strains, regardless if we look at serotype or genotype, may be missed when we only look at this parameter. Disease manifestation may give some additional information concerning severity, and registering sequelae and complications also give valuable additional information. Furthermore, length of stay and ICU-care may also provide valuable information on severity. These parameters seemed, however, dependent on local routines in different hospitals, with great differences even within the same region. Whereas some patients, especially in the small hospitals, were kept in the ICU for observation the first hours after admission, in order to see if they were going to deteriorate or not, most patients that were admitted to the ICU in the large university hospital were already in a critical condition when admitted to the ICU. The threshold for the decision whether a patient was to be sent to the ICU or not also seemed to depend on the speciality of the ward. Infectious disease units seemed to keep the patients longer before sending them to the ICU. Not surprisingly, it seemed more common to observe children in the ICU before any physiological function deteriorated. Therefore, ICU-care for more than 24 hours was instead used as a marker for disease severity in study number III. Need for mechanical ventilation was also registered as a measurement of disease severity.

CONCLUSIONS

- Serotype distribution varies with age.
- PCV-7 had a coverage of only 42% among IPD isolates from 1998-2001.
- Potential vaccine coverage was much higher for PCV-10, PCV-13 and PPV-23.
- CFR due to infections caused by serotypes 1 and 7F was lower than infections caused by other serotypes, and higher in infections caused by serotype 19A.
- Not only serotype, but also clonal type has an impact on manifestations and CFR.
- Many differences were observed between clones with one dominating serotype, making it difficult to determine whether serotype or clonal type has the greatest impact.
- Clonal distribution differed between adults and children.
- Among isolates causing IPD, antimicrobial resistance was uncommon and clonally related.
- The potential vaccine coverage of PCV-7 was higher among isolates with decreased susceptibility.
- Antimicrobial resistance did not correlate with outcome or patient characteristics.
- Total and specific incidence rates and CFR vary widely between different age and risk groups.
- IPD incidence increased threefold from 5 to 15/100,000 inhabitants/year and the CFR dropped from 20 to 10% in a well-defined population during 45 years.
- Pneumococcal meningitis incidence remained stable but the CFR dropped from 33 to 13% during 45 years.
- Predisposing factors changed considerably over time before introduction of general vaccination.

FUTURE PERSPECTIVES

Widespread vaccination among children with conjugate vaccines against pneumococci will hopefully reduce the incidence and mortality among vaccinated children in Sweden, and hopefully, we will also see herd effects, with reduced incidence and mortality among unvaccinated adults. However, we cannot take these effects for granted, since the serotype distribution among disease causing isolates was different in Sweden compared to the US before vaccination. The herd effects that were observed following PCV-7 in the US may be more pronounced when PCV-10 or PCV-13 are used. Swedish infants are currently vaccinated with PCV-10 or PCV-13. Since these vaccines have been on the market for a much shorter period, no data on herd effects exist. An obvious question is if the known PCV-7 effects can automatically be translated since the polysaccharides in PCV-10 are conjugated to another carrier protein than in PCV-7 in contrast to PCV-13, which uses the same carrier as PCV-7. Furthermore, serotype replacement may also hamper the positive effects of vaccination. Therefore continuous surveillance is crucial, remembering the variable and sometimes unpredictable nature of the pneumococcus. There are many examples of new clones spreading rapidly in different populations.

In many cases, the spread of new clones has been promoted by a high selective pressure caused by widespread antibiotic use and misuse. Therefore, an important issue will be to retain the policy that antibiotic treatment should be reserved to those patients who really need it. This perspective is especially important in developing countries.

The implementation of pneumococcal conjugate vaccines in developing countries will hopefully lead to radically decreased pneumococcal death tolls among children. Pneumococcal vaccination has been said to be necessary if the fourth millennium goal should be achievable, i.e. to reduce the under-five mortality rate by two thirds between 1990 and 2015. Of course, pneumococcal vaccination campaigns have to be coordinated with the fight against malaria, TB, HIV, diarrhoea, measles and malnutrition.

It remains to be shown, if PCV-13 is effective in preventing pneumonia and invasive pneumococcal disease also in adults, but immunogenicity data have given promising results. The first step will be to use the vaccine among risk groups that are known to respond poorly to the existing polysaccharide vaccine. It is important to do clinical trials with PCV-13 in

selected adult risk groups in the near future, in order to further identify the potential benefits from this vaccine among the patients who run the highest risk to develop IPD. A broad-range use among elderly people has to wait until robust clinical data on efficacy exist.

Hopefully, the impact of serotype replacement will remain limited while we are waiting for future generations of pneumococcal vaccines. One possible development will be higher valency conjugates. More likely, the next step will be protein vaccines providing serotype independent protection against severe pneumococcal disease. Several promising vaccine candidates developed through a reverse genomics approach are currently being tested. Also a potential revival of whole cell vaccines, that could be cheaper and provide serotype independent protection, has been discussed. New adjuvants and administration routes are also being developed. When pneumococcal vaccination extends to an ageing population, certain aspects of immunosenescense have to be considered [77]. Before new vaccines arrive, however, the goal must be to use the existing ones as appropriate as possible.

Another development in the world of pneumococcal infections is the arrival of faster and more reliable diagnostic methods, which will have positive effects both for the patient, who will receive goal-directed antibiotics earlier, and for the society, with lower antibiotic pressure. New insights regarding the early identification and management of sepsis patients will hopefully contribute to bring IPD mortality down. These developments do not only concern Sweden and other industrialized countries, but hopefully, both reliable diagnostic methods and new insights regarding supportive care can be adapted to low-income settings.

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To do research is to make a career. According to my dictionary, the word career was imported from the French word "carrière", meaning "road, racecourse". I would rather like to associate my research with the other French word "carrière", meaning quarry, "where rocks are excavated." Not that the work with this thesis has been like forced labour, at least not most of the time. In contrast! I have been like a devoted geologist, searching a quarry for traces and layers providing greater context, finding precious stones here and there. But most of all, however, I found tons and tons of illness, misery and death. It is important not to forget all the individuals and all the tears behind the dry figures and tables in this thesis. My hope is that vaccination will eliminate the burden of invasive pneumococcal disease in the future, thereby forcing me to look for new quarries to excavate.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Streptococcus pneumoniae, eller pneumokocker på svenska, tillhör de viktigaste sjukdomsframkallande bakterierna. De är en ledande orsak till barnadödlighet i världen, och de orsakar också mycket sjukdom och lidande i andra åldersgrupper. Paradoxalt nog bär upp till hälften av barn under två års ålder pneumokocker innanför näsan utan att bli sjuka av dem, och ett par procent av befolkningen i högre åldrar likaså. Sjukdomspanoramat sträcker sig från okomplicerade övre luftvägsinfektioner, via lunginflammationer av varierande svårighetsgrad till svåra och ibland livshotande invasiva pneumokockinfektioner, vilket definitionsmässigt är de infektioner då man kan odla fram bakterierna från blod (blodförgiftning) eller ryggmärgsvätska (hjärnhinneinflammation). Pneumokockerna omges av en kapsel uppbyggd av sockerarter (polysackarider) som skyddar mot immunförsvarets angrepp. Det finns 93 olika kapseltyper, s.k. serotyper, varav vissa är vanligare vid bärarskap, och andra är vanligare som sjukdomsorsak. Det finns sedan länge så kallade polysackaridvacciner som minskar risken att insjukna med svåra infektioner orsakade av 23 serotyper, men dessa vacciner har ingen effekt på barn under två års ålder. För att kunna ge upphov till ett skydd hos dem har man utvecklat nya vacciner där man kopplat (konjugerat) utvalda sockerarter till ett äggviteämne, s.k. konjugatvacciner. Det första konjugatvaccinet, PCV-7, omfattande 7 serotyper, infördes i barnvaccinationsprogrammet i USA år 2000. I slutet av nittiotalet var frågan om också Sverige skulle införa detta i sitt barnvaccinationsprogram. För att kunna uppskatta nyttan av en eventuell vaccination ville vi veta hur stor del av de invasiva infektionerna både bland barn och vuxna som ingick i det aktuella vaccinet (I). Dessutom undrade vi veta om det fanns skillnader mellan olika serotyper avseende vem som drabbades och hur det gick för dem (I), samt om detta dessutom påverkades av genetiska egenskaper (III). I vilken utsträckning de var resistenta mot antibiotika var också viktigt att veta (II). Vi behövde också exakta data angående hur många som insjuknade och dog av invasiv pneumokocksjukdom i olika ålders- och riskgrupper (IV), dels för att veta vilka grupper som kan ha störst nytta av vaccination, dels för att i framtiden kunna utvärdera effekten av barnvaccinationen, som sedermera infördes i Sverige januari 2009. Vi jämförde också våra data med två tidigare studier från Göteborgsområdet, för att se hur epidemiologin hade ändrat sig under en 45 årsperiod.

För att besvara dessa frågor samlades alla pneumokockstammar som orsakade invasiv pneumokocksjukdom i Västra Götaland och Halland under åren 1998-2001. Dessa drygt 800

isolat serotypades och uppgifter om sjukdomsmanifestationer och bakomliggande sjukdomar hämtades ur journalerna för patienterna (I). Vi utförde resistensbestämning på bakterierna med en metod som ger mer detaljerad information än vad som erhålls i rutindiagnostiken (II). Arvsmassan hos de västsvenska stammarna tillsammans med drygt 400 stammar från barn och vuxna i stockholmsområdet undersöktes med genteknik för att se om sjukdomsbilden och risken att dö skilde sig mellan patienter som infekterats med pneumokocker som tillhörde olika genetiska släktled, så kallade kloner (III), samt undersökte om pneumokocker som tillhör en viss serotyp producerade en defekt variant av giftämnet pneumolysin. Vi letade reda på journalerna från ca 3000 patienter som insjuknat med invasiv pneumokocksjukdom under åren 1996-2008 och sammanställde data angående bakomliggande sjukdomar, sjukdoms-förlopp, komplikationer och död (IV).

42% av alla infektioner orsakades av pneumokocker med serotyper som ingår i PCV-7, vilket är en internationellt sett mycket låg siffra. Bland barn, som är den primära målgruppen för vaccinationen, var andelen vaccinserotyper något högre (I). Andelen serotyper som ingår de konjugatvacciner med skydd mot 10 respektive 13 serotyper, som under 2010 och 2011 har ersatt PCV-7, visade sig vara mycket högre (I). Den högsta andelen serotyper som teoretiskt skulle kunna förhindras hade det gamla polysackaridvaccinet, över 90% (I). Serotyper som ingår i PCV-7 var vanligare bland bakterier som hade nedsatt antibiotikakänslighet än bland fullt känsliga stammar, varför vaccination sannolikt skulle kunna bidra till att hålla tillbaka en framtida resistensutveckling (II). Det var ovanligt att antibiotikaresistenta pneumokocker orsakade invasiv sjukdom under den aktuella perioden (II). De resistenta stammar som dök upp tillhörde i huvudsak vissa kloner (III).

Dödligheten i infektioner orsakade av vissa serotyper var lägre än infektioner orsakade av andra serotyper, men en stor del av dessa skillnader kunde förklaras av att patienterna var yngre och att färre av dem hade bakomliggande sjukdomar (I). En av serotyperna orsakade högre dödlighet än de andra, men patienterna infekterade med den serotypen var också äldre och fler hade bakomliggande sjukdomar (I). En viss klon orsakade lägre dödlighet än ett flertal andra kloner, men bakterierna i denna klon tillhörde en av de serotyper som visats ha lägre dödlighet (III). Vi såg dessutom vissa skillnader avseende sjukdomsförlopp och utgång mellan infektioner orsakade av olika kloner med samma serotyp, vilket skulle kunna betyda att inte bara serotypen utan också andra faktorer i bakteriernas arvsmassa påverkar hur det går

för patienten (III). Produktion av ett ickefungerande giftämne verkade inte påverka förloppet, vilket var förvånande (III).

Trots god tillgång på sjukvård är det fortfarande ca 10% av patienterna med invasiva pneumokockinfektioner som dör inom en månad efter diagnos. Både risken att insjukna och risken att dö, samt vilken sjukdomsmanifestation patienten utvecklar, beror både på patienternas ålder och på eventuella kroniska sjukdomar (IV). Små barn och gamla hade högst risk att insjukna, och de gamla löpte störst risk att dö. Stora variationer sågs mellan olika bakomliggande sjukdomar, t ex var risken att insjukna 150 gånger högre för patienter med blodsjukdomen myelom än för dem som inte hade denna sjukdom. Patienter med KOL, hjärtkärlsjukdom, cancer samt njursjukdom var också hårt drabbade (IV). Risken att dö var högre för män än för kvinnor, även om man tog hänsyn till ålder och andra sjukdomar.

Under en 45-årsperiod hade antalet diagnosticerade fall tredubblats, från 5 till 15 per 100 000 invånare och år, sannolikt mest beroende på att man blododlar mer nuförtiden. Samtidigt hade risken att dö mer än halverats, trots att andelen gamla hade ökat kraftigt. Risken att insjukna med hjärnhinneinflammation var ganska konstant under hela 45-årsperioden, men däremot hade andelen som dog av detta mer än halverats. Antalet män i förhållande till antalet kvinnor som insjuknar med invasiv pneumokocksjukdom har gått från 2,1:1 till 1:1. Denna ökade jämställdhet kunde bara delvis förklaras av att andelen grava alkoholister som drabbas hade minskat jämfört med tidigare studier, och inte heller förklaras av en ändrad ålderssammansättning i befolkningen. Risken att dö var högre för män, trots att de var yngre än kvinnorna.

Sammanfattningsvis har vi med dessa studier kartlagt vilken sjukdomsbörda invasiva pneumokockinfektioner orsakar, hur stor andel som går att förebygga med olika vacciner, samt i vilken utsträckning sjukdomsförloppet påverkas av bakteriella egenskaper såsom kapsel och arvsmassa, och av patientegenskaper såsom ålder och samtidiga sjukdomar. Denna kunskap kommer att vara användbar både för att planera och utvärdera effekterna av barnvaccination och vaccination av vuxna riskgrupper, inte minst eftersom konjugatvaccin nu även kommer att börja användas på de vuxna riskgrupper som får otillräckligt skydd av polysackaridvaccin.

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