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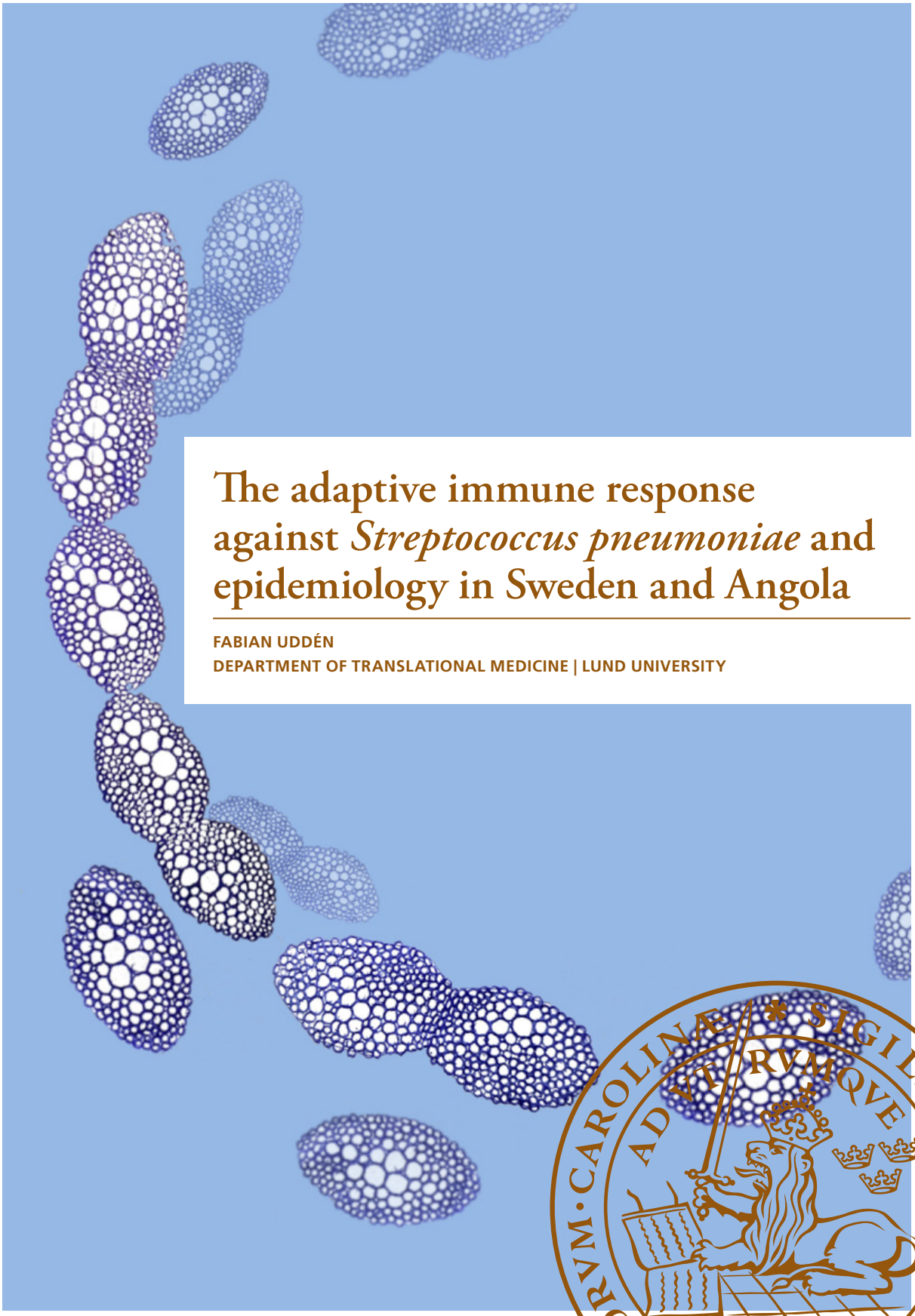

The adaptive immune response against *Streptococcus pneumoniae* and epidemiology in Sweden and Angola

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LUND UNIVERSITY

DOCTORAL DISSERTATION

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Abstract

Streptococcus pneumoniae, the pneumococcus, is a frequent colonizer of the human nasopharynx and a common cause of acute otitis media (AOM), community acquired pneumonia (CAP), and invasive pneumococcal disease (IPD; *i.e.*, mainly bacteraemia and meningitis). A structurally diverse capsule covers the bacterium and determines the over 100 pneumococcal serotypes, which vary in invasiveness and association with antimicrobial resistance. The human adaptive immune response against the pneumococcus is partly dependent on anti-capsular antibodies that facilitate phagocytosis through opsonizing bacteria. The pneumococal conjugate vaccines (PCVs) are based on capsule polysaccharides and are used in paediatric immunization programmes, leading to serotype replacement in the population.

In Paper I and II, the opsonic activity of paired sera collected before/during and after pneumococcal infection from 80 patients was assessed. In many cases no improvement of opsonic activity was detected after IPD (60%) or CAP (35%), indicating a non-functional adaptive immune response. This outcome was associated with bacteraemia and low-invasive serotypes that produce a thick capsule. Furthermore, opsonic activity and anti-capsular antibody concentrations only partly correlated. The results confirm previous findings in smaller studies and support the hypothesis that a high load of capsular polysaccharides may hamper the immune response.

In Paper III and IV, pneumococci isolated from clinical respiratory tract samples in Skåne County 2-4 years after the transition from PCV10 to PCV13 were characterized (n=2,131). Serotypes covered by PCV13 comprised 17% of isolates, mostly representing serotypes 3 (9%) and 19A (5%) which still circuated in the population. A decrease of PCV13 serotypes was noted compared with the PCV10 period, with exception of serotype 3 for which no herd effect was indicated. Most non-PCV13 serotypes, with some exceptions, contributed modestly to IPD in the county compared with their prevalence in respiratory tract samples. Among non-PCV13 serotypes, non-susceptibility to different antibiotics was predominantly detected in a limited number of serotypes, *i.e.*, 6C, 15A, 23B, 24, and 35B. Additionally, a majority of the most multiresistant strains belonged to known internationally spread lineages that are known to cause IPD (17/25) why their presence in the population is a concern.

In Paper V, a study of pneumococcal carriage was performed among children aged 4-12 years (n=940) in Angola, 4 years after the introduction of PCV13. Pneumococci grew in nasopharyngeal cultures from 35% of children and 41% of isolates exhibited PCV13 serotypes. Non-susceptibility to penicillin (40%), tetracycline (21%), and trimethoprim-sulfamthoxazole (83%) was common. The results demonstrated that no evident PCV13 herd effect was established in Angola, which is likely due to low vaccine coverage and the limited time that PCV13 has been used.

Lastly, in Paper VI pathogens associated with chronic suppurative otits media (CSOM) from 152 patients in Angola were studied in depth. In total, 534 isolates were found in middle ear samples, representing 87 different species, demonstrating that a very wide range of bacteria may be involved in CSOM. The most common bacteria were *Proteus* spp. (15%), *Pseudomonas aeruginosa* (13%), and *Enterococcus* spp. (9%). High resistance rates were detected against chloramphenicol (43-100%), while the numbers were lower for quinolonoes (6-31%), supporting the use of the latter antibioitc for topical treatment of CSOM.

Key words: Acute otitis media, adaptive immunity, animicrobial resistance, chronic suppurative otitis media, invasive pneumococcal disease, nasopharyngeal colonization, opsonophagocytic assay, pneumococcal conjugate vaccine, pneumococcal serotypes, pneumonia, serotyping, *Streptococcus pneumoniae*

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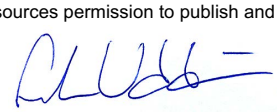
Signature 

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To my family, who might read it with limited interest

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II. **Uddén F**, Ahl J, Littorin N, Strålin K, Athlin S, Riesbeck K. Corrected and Republished from: A Nonfunctional Opsonic Antibody Response Frequently Occurs after Pneumococcal Pneumonia and Is Associated with Invasive Disease. *mSphere*. 2020;5(6):e01102-20.

III. **Uddén F**, Rünow E, Slotved HC, Fuursted K, Ahl J, Riesbeck K. Characterization of Streptococcus pneumoniae detected in clinical respiratory tract samples in southern Sweden 2 to 4 years after introduction of PCV13. *Journal of Infection*. 2021;83(2):190-196.

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VI. **Uddén F**, Filipe M, Reimer Å, Paul M, Matuschek E, Thegerström J, Hammerschmidt S, Pelkonen T, Riesbeck K. Aerobic bacteria associated with chronic suppurative otitis media in Angola. *Infectious Diseases of Poverty*. 2018;7(1):42.

Abbreviations

AOM	acute otitis media
AU	arbitrary units
CAP	community-acquired pneumonia
CBP	choline-binding protein
CC	clonal complex
ChoP	phosphorylcholine
COPD	chronic obstructive pulmonary disease
CPS	capsular polysaccharide
CRP	C-reactive protein
CSOM	chronic suppurative otitis media
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EUCAST	The European Committee on Antimicrobial Susceptibility Testing
GBD	Global Burden of Disease Study
GPSC	global pneumococcal sequence cluster
Hib	*Haemophilus influenzae* type B
HIV	human immunodeficiency virus
Ig	immunoglobulin
IPD	invasive pneumococcal disease
IQR	interquartile range
LMICs	low- and middle-income countries
MALDI-TOF MS	matrix-assisted laser desorption ionization – time of flight mass spectrometry

MDR	multidrug resistant
MIC	minimum inhibitory concentration
MLST	multi-locus sequence typing
NET	neutrophil extracellular trap
NIP	national immunization programme
NT	non-typeable
NTHi	non-typeable *Haemophilus influenzae*
NVT	non-vaccine serotype
OPA	opsonophagocytic assay
OR	odds ratio
PAMP	pathogen-associated molecular pattern
PBP	penicillin-binding protein
PCR	polymerase chain reaction
PCV	pneumococcal conjugate vaccine
Ply	pneumolysin
PMEN	Pneumococcus Molecular Epidemiology Network
PNSP	penicillin non-susceptible pneumococci
PPV	pneumococcal polysaccharide vaccine
PRR	pattern recognition receptor
ST	sequence type
STGG	skimmed milk-tryptone-glucose-glycerine
Th17	IL-17 producing helper T-cells
TLR	Toll-like receptor
WGS	whole genome sequencing
WHO	World Health Organization
XDR	extensively drug resistant

Populärvetenskaplig sammanfattning (Summary in Swedish)

Pneumokocker (*Streptococcus pneumoniae*) är en viktig orsak till de vanligt förekommande luftvägsinfektionerna öroninflammation och lunginflammation. Bakterierna kan också orsaka allvarliga infektioner såsom hjärnhinneinflammation och blodförgiftning vilka samlas under begreppet ”invasiv pneumokocksjukdom” (IPD). Det är främst små barn och äldre individer som drabbas och globalt sett orsakar pneumokocker en mycket stor sjukdomsbörda. Infektion orsakad av pneumokocker föregås alltid av bärarskap i svalget varifrån bakterierna kan spridas vidare till mellanörat eller lungorna, men de flesta fall av bärarskap leder inte till sjukdom utan pågår i några veckor till månader utan att ge symtom. Barn i förskoleåldern bär ofta på pneumokocker och utgör den huvudsakliga reservoaren för arten i befolkningen varifrån den kan spridas till andra åldersgrupper.

Pneumokocken är täckt av en kapsel som består av olika sockerarter och dessa är mycket viktiga för bakteriens sjukdomsframkallande förmåga genom att skydda den mot vårt immunförsvar. Kapselns struktur avgör vilken serotyp en viss stam tillhör och hittills har ca 100 olika serotyper upptäckts. Dessa skiljer sig åt avseende association till olika sjukdomar och antibiotikaresistens, samt geografisk förekomst. Specifika antikroppar riktade mot kapseln bildas av immunförsvaret och är viktiga för att förhindra infektion. Sådana antikroppar uppkommer vid naturlig exponering för bakterien, men även efter vaccination med de konjugerade pneumokockvaccinen (PCV) som innehåller sockerarter från olika serotyper och används i barnvaccinationsprogrammen. Vaccinen förhindrar även bärarskap av de inkluderade serotyperna varför vaccination av barn leder till ett skifte av serotyperna i befolkningen. Detta leder till att PCV också skyddar ovaccinerade åldersgrupper till viss del. Dock tenderar de serotyper som i stället blir vanliga, och i de flesta fall är mindre farliga för barn, att orsaka sjukdom hos äldre individer.

För att undersöka hur immunförsvaret mot pneumokocker påverkas av en allvarlig infektion analyserades funktionen hos antikroppar mot bakterien i blod från patienter med lunginflammation och IPD (artikel I och II). Resultaten visade att antikroppsfunktionen i många fall inte förbättrades som förväntat, eller till och med försämrades, efter tillfrisknande. Dessa fynd tyder på att en icke-funktionell immunrespons kan uppträda vid pneumokockinfektion, och detta mönster var vanligare vid IPD och kopplat till vissa serotyper. Den kliniska betydelsen av detta

är i nuläget okänd, men det skulle kunna innebära att vissa patienter är mottagliga för infektion med samma serotyp igen.

I artikel III och IV studerades pneumokocker som detekterats i luftvägsprover från patienter i Region Skåne under åren 2016-2018 för att undersöka effekterna av det vaccinbyte som skedde i barnvaccinationsprogrammet 2014. Då ersattes PCV10, inkluderande 10 serotyper, av PCV13 som täcker ytterligare 3 serotyper. Totalt sågs en halverad förekomst av de serotyper som täcks av PCV13 (17%) jämfört med en studie av perioden då det tidigare vaccinet användes. Dock var de två PCV13-serotyperna 3 och 19A fortfarande vanliga bland äldre barn och vuxna. Fortsatt omfattande spridning av just serotyp 19A i befolkningen kan utgöra ett problem då PCV13 åter byttes ut mot PCV10 nationellt 2019, eftersom det kan leda till ett ökat antal fall av IPD hos barn. De flesta serotyperna som inte täcks av vaccinen orsakade sällan IPD i förhållande till hur vanliga de var i luftvägsprover. En betydande nivå av nedsatt känslighet mot penicillin och andra antibiotika detekterades dock hos vissa av dessa. Slutligen undersöktes genetiken hos de mest multiresistenta bakterierna vilket visade att många av dem tillhörde välkända internationellt spridda stammar som förkommer vid allvarliga infektioner.

I artikel V undersöktes bärarskap av pneumokocker hos ovaccinerade barn i Angola 2017, ett land som är hårt drabbat av pneumokockinfektioner och där PCV13 infördes i barnvaccinationsprogrammet 2013. Nästan hälften (41%) av pneumokockerna som återfanns var serotyper som täcks av PCV13. Detta tyder på att någon flockeffekt mot dessa ej etablerats i Angola, vilket kan förklaras av den korta tid som vaccinet använts och att täckningsgraden av vaccin behöver förbättras. Resultaten från denna studie kan användas för jämförelser vid framtida studier som görs för att utvärdera vaccinationsprogrammets effekter. Den information om bakteriernas antibiotikaresistens som presenteras är även av värde för att stödja antibiotikabehandling av luftvägsinfektioner då begränsade data föreligger.

I Angola genomfördes även ett projekt (artikel VI) för att ta reda på vilka bakterier som orsakar kronisk suppurativ mediaotit (CSOM). Detta tillstånd är en komplikation till vanlig öroninflammation och innebär långvarig infektion i mellanörat med läckage av var från hörselgången. Uppemot 90 arter hittades i prover från mellanörat hos patienter med CSOM och i genomsnitt fanns det 3 olika arter i varje prov. Även om de vanligaste bakteriearterna i denna studie var sådana som ofta ses vid CSOM visar resultaten att ett mycket brett spektrum av bakterier föreligger. Många bakterier var resistenta mot olika antibiotika som används för att behandla CSOM, vilket kan bidra till att infektionen är svårbehandlad. Resultaten tydde dock på att antibiotikasorten ciprofloxacin är det bästa valet.

Sammanfattningsvis belyser denna avhandling en aspekt av immunförsvaret mot pneumokocker som inte studerats i stor utsträckning tidigare. Den bidrar med information om de pneumokocker som förekommer i Skåne respektive Angola vilket kan vara av värde för att förstå effekterna av PCV och för beslut om vaccinationsstrategier. Slutligen ger den nya insikter om vilka bakterier som kan orsaka CSOM och data om deras antibiotikaresistens i Angola.

Introduction

The discovery of the pneumococcus and its serotypes

> "Each of these little particles is surrounded at a certain focus with a sort of aureole which corresponds perhaps to a material substance"
>
> Louis Pasteur, 1881 [1]

Subsequent to injecting rabbits with human saliva in their separate experiments in 1880, both Louis Pasteur [1] and George Sternberg [2] were able to recover bacteria that were most likely pneumococci from the bloodstream of the animals. Their accounts are regarded as the first documentations of laboratory isolation of this pathogen. During the years 1882-1886, the role of the bacterium as the major etiology of pneumonia was gradually elucidated, as summarized by Robert Austrian [3, 4]. Briefly, Carl Friedländer was able to observe bacteria in preparation of lung tissue from deceased patients with lobar pneumonia, but mistakenly assumed that the first organism he successfully isolated was the predominant cause of the disease [5, 6]. This was however probably *Klebsiella pneumoniae*, a.k.a. *Friedländer's bacillus*, and inconsistencies between his description of the bacterium and those that were isolated from pneumonic lungs by Albert Fraenkel shortly after sparked a polemic between the two [7]. Thanks to the novel staining technique developed by Friedländer's laboratory colleague Hans Christian Gram [8], that could distinguish the two bacterial species, and additional work by Anton Weichselbaum [9], it was although established that the pneumococcus was the most important pathogen responsible for the disease. During the same decade, research on pneumococcal disease progressed rapidly with descriptions of different manifestations of invasive pneumococcal disease (IPD) such as bacteraemia, meningitis, arthritis, and endocarditis [4]. The bacterium initially received the scientific name *Diplococcus pneumoniae* but was renamed to the name of today, *i.e.*, *Streptococcus pneumoniae* in 1974.

Key observations that laid the foundation for the development of anti-pneumococcal serum therapy and, later, pneumococcal vaccines followed. In his continued studies in 1886, Fraenkel demonstrated that rabbits infected with pneumococci were immune to reinfection [10, 11]. Some years later, it was shown that this immunity was strain-dependent and that serum from the exposed animals conferred passive protection [12]. The protective potential of immune serum was

found to be mediated by facilitating phagocytosis of bacteria by leukocytes in 1904 [13]. Further experience with convalescence serum revealed a significant serologic heterogeneity among pneumococci, which lead to the definition of the first pneumococcal serotypes during the first decades of the 20th century [14-18]. Therapy with serotype-specific concentrated equine serum was used regularly during this time and reduced mortality in severe pneumococcal pneumonia. However, the treatment was limited by the need to determine the serotype of the infecting strain before administration, adverse effects such as serum sickness and anaphylaxis, and the lack of antiserum for all serotypes [18-21]. In the 1940s, the establishment of antibiotic treatment, predominantly with penicillin, as the standard remedy for pneumococcal infections revolutionized patient outcome and led to the abandonment of serum therapy [22]. However, the pursuit of a pneumococcal vaccine continued, which is described in a later section.

Early on, it was noted that the type-specific serum reaction also occurred with supernatants from pneumococcal broth cultures and work ensued to determine the nature of the "specific, soluble substance of pneumococci" leading to the conclusion that the bacterium produced a polysaccharide capsule [23-25]. The importance of the capsular polysaccharides (CPS) for pneumococcal virulence was demonstrated by the inability to infect mice with bacteria that had lost their ability to produce a capsule [25]. Likewise, administration of an enzyme capable of specifically degrading serotype 3 CPS simultaneously or before inoculating mice with this serotype protected them from infection [26].

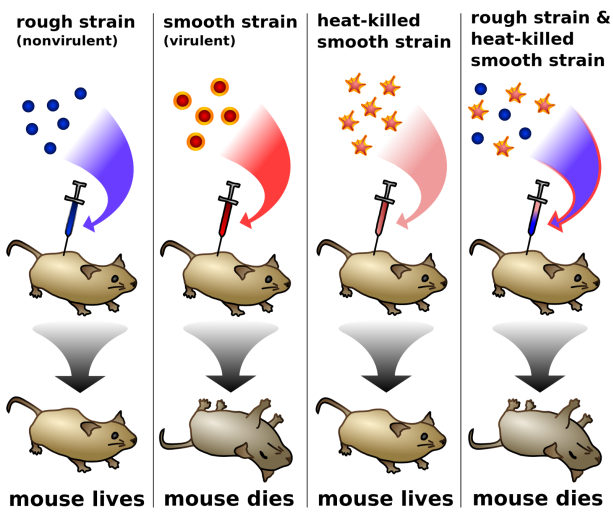


Figure 1. Griffith's experiment.
In 1928, Fred Griffith published the report from his experiments of mouse infection with different pneumococcal strains, demonsrating both the importance of the polysaccharide capsule for virulence, and genetic transformation although its mechanism was not understood at the time [25]. Figure reproduced from https://en.wikipedia.org/wiki/Griffith%27s_experiment, "Griffith's experiment discovering the "transforming principle" in pneumococcus bacteria" by Madprime licenced under CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0/).

Interestingly, experiments by Fred Griffith on capsulated and non-encapsulated pneumococci revealed a mechanism what was later perceived to be genetic transformation [25]. Upon inoculating mice with avirulent non-encapsulated pneumococci derived from a serotype 2 strain simultaneously with a suspension of heat-killed serotype 3 bacteria, the animals succumbed, and he was able to recover viable serotype 3 bacteria (*Figure 1*). The formerly serotype 2 pneumococci had apparently acquired the ability to produce a serotype 3 capsule. These observations triggered additional studies to define the "transforming principle", culminating in the report by Oswald Avery *et al*. in 1944 [27], providing the first description of deoxyribonucleic acid (DNA). The role of DNA was thereafter further elucidated in famous papers on bacteriophages by Alfred Hershey and Martha Chase [28], and on its double-helix structure by James Watson and Francis Crick [29], the latter report based partly on results from experiments by Rosalind Franklin who was although not properly attributed [30].

Taken together, *S. pneumoniae* played a central role in the early decades of microbiological research. With contribution of the mentioned studies, several key immunological and bacteriological mechanisms were clarified, important microbiological methods were developed, and DNA was discovered. In the modern era, the capsular serotypes remain a central aspect in studies of pneumococcal immunity and epidemiology such as those outlined in this thesis, due to the importance of CPSs in host-pathogen interactions and their incorporation in pneumococcal vaccines.

The bacterium

Microbiological characteristics

The pneumococcus is a Gram-positive, catalase-negative, facultative anaerobic bacterium belonging to the mitis-group of the *Streptococcus* genus. Like all streptococci, it is a fastidious organism that relies on fermentation metabolism, and increased CO_2 levels (5%) are required for optimal growth. Under the microscope, it is most often observed as diplococci, hence the historical name, but also as single cells or short chains. On blood agar, the bacterium exhibits alfa-haemolysis in aerobic conditions and beta-haemolysis in anaerobic conditions, and a characteristic colony morphology with a central depression (*Figure 2*) [31, 32]. However non-encapsulated strains give rise to small pinpoint colonies, and the extensively encapsulated serotypes 3 and 37 grow as large mucoid colonies [18, 33].

The complete genome of *S. pneumoniae*, from the strain TIGR4, was first described in 2001 [34]. Contained in a single circular chromosome, it varies in size between approximately 2.0-2.2 million bases. Natural competence and a high degree of horizontal gene transfer are hallmarks of the species. This trait is upregulated

during colonization of the host, providing a setting for genomic recombination events that may lead to the spread of antibiotic resistance and capsular switching [35, 36].

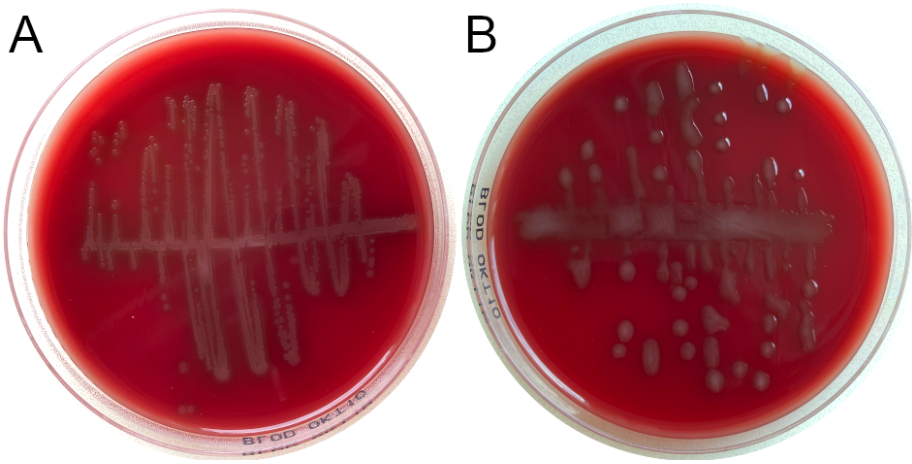


Figure 2. *Streptococcus pneumoniae* colonies.
Pneumococci may exhibit different colony morphologies on blood agar. In the figure, serotype 21 (A) grow as normal "smooth" colonies, while serotype 3 (B) has a mucous appearance due to excessive capsule production.

The bacterial cell membrane and the overlying Gram-positive peptidoglycan cell wall is elongated at a single equatorial growth zone that subsequently invaginates to form the septum for cell division. A unique property of the pneumococcal cell wall is the incorporation of the phosphorylcholine (ChoP) in its teichoic and lipoteichoic acid, also referred to as pneumococcal cell wall polysaccharide or C-polysaccharide [37, 38]. Choline is thus a nutritional requirement for the bacterium and the ChoP residues of the cell wall serves as binding motif for the pneumococcal choline binding proteins (CBPs) but also interact directly with the host [38, 39]. Additional groups of surface proteins include lipoproteins, sortase-recognized LPxTG-motif proteins, and non-classical surface protein that lack known secretion and anchoring motifs [40, 41]. Approximately 30-40% of clinical pneumococcal strains express pili which increase virulence [37, 42].

The polysaccharide capsule and its serotypes

The pneumococcal polysaccharide capsule covers the cell wall and is widely regarded as its most important virulence factor (*Figure 3*). It is the varying chemical structure of the capsule that gives rise to the serological differences defining its serotypes. The serotypes are sorted into serogroups based on serological similarity. To the author's best knowledge, 101 serotypes have been described to date (*Table 1*) [18, 43, 44].

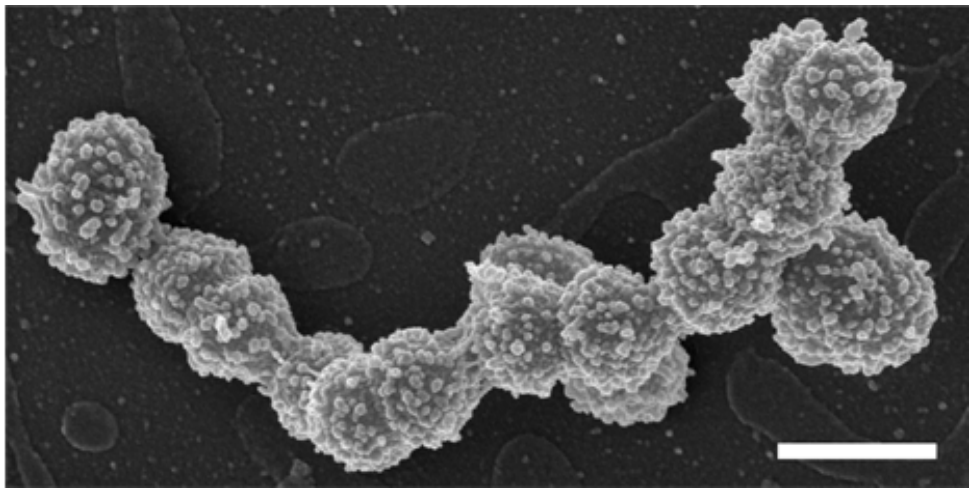


Figure 3. *S. pneumoniae* with capsule.
Scanning electron micrograph of serotye 3 strain A66 pneumococci. The capsular material is visible on the bacterial surface as irregular round protruding structures. Figure reproduced from Hammerschmidt *et al*. 2005 [45] with permission from American Society for Microbiology. Image kindly provided by Sven Hammerschmidt and Manfred Rohde.

Table 1. The 101 known pneumococcal serotypes.

Serogroup	Serotypes	Serogroup (continued)	Serotypes
-	1	24	24F, 24A, 24B, 24C
-	2	25	25F, 25A
-	3	-	27
-	4	28	28F, 28A
-	5	-	29
6	6A, 6B, 6C, 6D, 6E, 6F, 6G, 6H	-	31
7	7F, 7A,7B, 7C, 7D	32	32F, 32A
-	8	33	33F, 33A, 33B, 33C, 33D
9	9A, 9V, 9N, 9L	-	34
10	10F, 10A, 10B, 10C, 10D	35	35F, 35A, 35B, 35C, 35D
11	11F, 11A, 11B, 11C, 11D, 11E	-	36
12	12F, 12A	-	37
-	13	-	38
-	14	-	39
15	15F, 15A, 15B, 15C	-	40
16	16F, 16A	41	41F, 41A
17	17F, 17A	-	42
18	18F, 18A, 18B, 18C	-	43
19	19F, 19A, 19B, 19C	-	44
20	20A, 20B	-	45
-	21	-	46
22	22F, 22A	47	47F, 47A
23	23F, 23A, 23B	-	48

The capsule consists of chains of oligosaccharide repeat units that are synthesized intracellularly and then polymerized on the cell surface. In generally, the CPSs are negatively charged, but this particular characteristic varies between serotypes. The enzymes that are involved in the synthesis, extracellular transportation, and polymerization of the CPS are encoded by the *cps* locus, located between genes *dexB* and *aliA*, in all serotypes except serotype 37, which relies on a single gene (*tts*) for the biosynthesis of its purely glucose-containing capsule [33, 46]. Serotype 3 CPS is also unique as it consists of a simple disaccharide repeat unit (glucose-glucuronic acid) that is produced and translocated by a single synthase, while the CPS of remaining serotypes comprise more complex oligosaccharide repeat units and rely on the polymerase (*wzy*)-dependent pathway [18, 46, 47]. Additionally, serotype 3 produces large amounts of CPS which is not linked to the cell wall with covalent bonds as in other serotypes, and consequently sheds capsule material [47]. Unencapsulated strains, denoted non-typeable (NT) pneumococci, are common in carriage and conjunctivitis, but are less virulent than the capsulated variants and very seldom cause IPD [18, 48].

The CPS layer contributes to the pathogenesis of pneumococcal disease through immune evasion via several mechanisms that vary vastly in efficacy between serotypes and are further discussed below, but most importantly the capsule interferes with phagocytosis by host immune cells in the absence of specific antibodies. Consequently, the induction of a humoral immune response against the CPSs is important for preventing and clearing pneumococcal infections why they are used as antigens in pneumococcal polysaccharide and conjugate vaccines (PPV and PCV, respectively) [18, 46].

Species identification and serotyping

In the clinical laboratory, *S. pneumoniae* is generally distinguished from other mitis-group streptococci based on optochin susceptibility and bile solubility [31, 32]. However, the presence of optochin-resistant pneumococci, optochin-susceptible *S. pseudopneumoniae* and other mitis-group streptococci, and the interpretability of the bile solubility test are obstacles for its identification [49]. Serological methods, Matrix-Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS), and polymerase chain reaction (PCR) for detection of pneumococcus-specific genes may be used to improve the diagnostics [49, 50]. A urine antigen detection test, reacting with pneumococcal teichoic acid, aimed at diagnosing pneumococcal etiology in pneumonia has been available since the early 2000s [51]. More recently, serotype-specific urine antigen detection tests have been developed, which are able to detect 13 and 24 different serotypes, respectively, including those covered by the currently available 13-valent PCV (PCV13) [52, 53]. Interestingly, false positive results with these tests occur in young children that carry the bacterium in the nasopharynx, indicating that CPSs and other cell wall components are systemically absorbed during asymptomatic colonization.

Even though Neufeld described the visible reaction between the pneumococcal capsule and the corresponding antiserum known as the Quellung reaction already in 1902 [54], it was not established as the standard method for serotyping until the 1930s [55, 56]. This test, also called the Neufeld method, is currently considered the gold standard method for determining pneumococcal serotypes. With this method, the serogroup and/or serotype is determined by the combination of positive reactions with different pool, group, type, and factor antisera [57]. Under the microscope, it is characterized by increased visibility and swelling of the capsule, and in many cases agglutination of the bacteria (*Figure 4*).

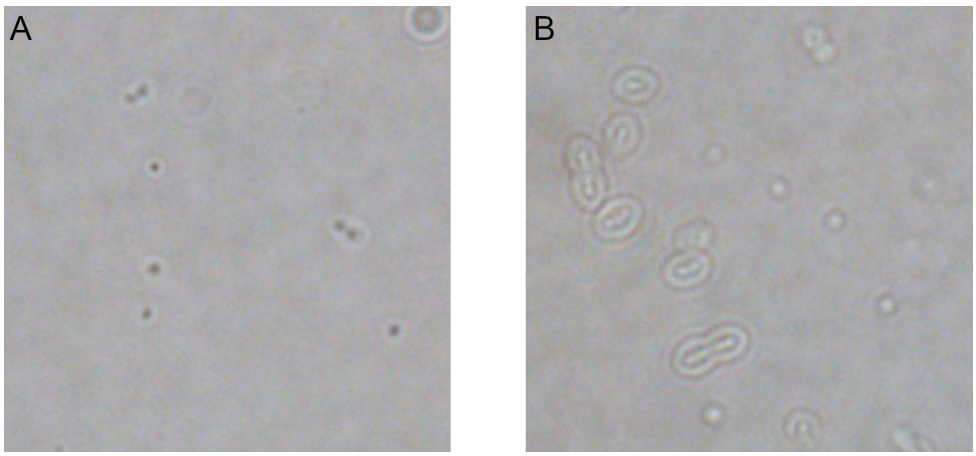


Figure 4. The Quellung reaction.
Micrographs of serotype 19F pneumococci suspended in saline with Neufeld antisera. No reaction occurs with factor serum 19c which is specific for serotype 19A (A), while a positive Quellung reaction is observed with factor serum 19b (B).

Due to the time-consuming nature of Neufeld typing other strategies for serotyping have been developed. A latex agglutination kit, based on latex beads coated with antibodies from Neufeld antisera, was developed at Statens Seruminstitut (SSI), Copenhagen in 2004 (*Figure 5*) [58]. Molecular detection of serotype-specific genes in isolated pneumococcal strain or directly in sample material with conventional or real-time PCR have also become widely adopted serotyping methods [59-61]. With genomic data from whole genome sequencing (WGS), both species and the capsular genotype may be rapidly confirmed through different bioinformatic methods, including multi-locus sequence analysis of housekeeping genes, detection of pneumococcus-specific genes or sequence characteristics, and automated pipelines for *cps* locus analysis [62-65].

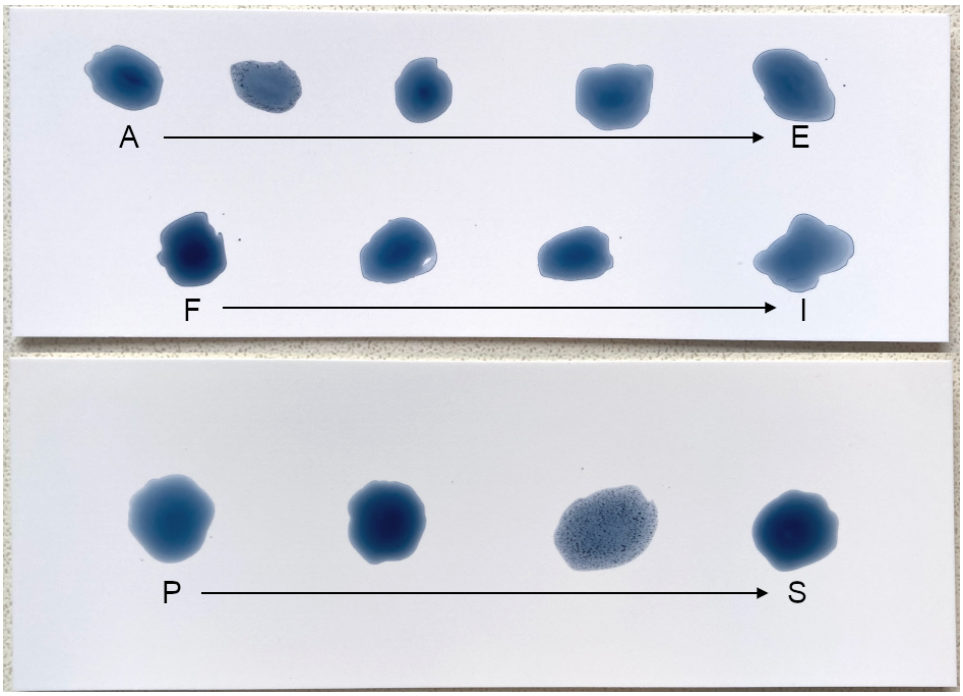


Figure 5. The latex aggluttination serotyping test.
Serotyping of a pneumococcal isolate with a latex aggluttination test. The visible agglutination represents a positive reaction with pool sera B and R, indicating that the tested isolate exhibits serotype 3.

Molecular typing

In addition to serotype, a trait that correlates only to a limited degree with clonality, determination of genetic lineages is of importance for studying the epidemiology of the pneumococcus. Multi-Locus Sequence Typing (MLST) is used to assign sequence type (ST) based on the allelic profile of 7 housekeeping genes (*i.e.*, *aroE*, *gdh*, *gki*, *recP*, *spi*, *xpt*, and *ddl*), and STs that are single locus variants of each other may be grouped in clonal complexes (CC) [66]. An online database of STs and corresponding clinical isolates is available at the PubMLST website [67]. The use of MLST has been important for tracking antibiotic resistant lineages, investigating capsule switching event, and the changing pneumococcal population structure after vaccine introduction. For example, the expansion of multidrug resistant serotype 19A ST320, which is a double locus variant of the Pneumococcus Molecular Epidemiology Network (PMEN) clone Taiwan^{19F}-14 ST236, is significant [68].

With the improved availability of WGS, the molecular epidemiology of pneumococci is currently studied with higher resolution than is possible with MLST to an increasing extent. The classification of Global Pneumococcal Sequence Clusters (GPSCs) is a recently developed WGS based tool for defining genetic

lineages and is used in studies of serotype epidemiology, antibiotic resistance, and invasiveness [69]. An online platform (Pathogenwatch) developed by the Centre for Genomic Pathogen Surveillance, University of Oxford can be used to assign bacterial species, GPSCs, STs, and various antimicrobial resistance genes with genomic data [70]. Furthermore, the Global Pneumococcal Sequencing Project website provides a database of all GPSCs and corresponding clinical isolates [71].

Nasopharyngeal carriage of *S. pneumoniae* and friends

Despite the acute, severe, and sometimes fulminant courses of many pneumococcal infections, the bacterium mostly resides as a commensal in the human nasopharynx. This is its natural habitat where it in most cases persists asymptomatically or with mild catarrhal symptoms for a few weeks or months before being cleared by the host [72-74]. However, nasopharyngeal acquisition is the first step to, and a prerequisite for, subsequent development of clinically significant pneumococcal infection [75].

Although depending on geographical setting and risk factors for transmission such as crowding, pneumococcal carriage rates among children <5 years of age are often around or over 50% at any given time [76-78]. Frequencies among adults are lower than those among children but have probably been underestimated during recent decades as efficient detection of pneumococci in the adult nasopharynx is dependent on the use of molecular methods as well as collection of multiple samples due to a lower bacterial density [77, 79, 80]. A recent meta-analysis of pneumococcal carriage in adults >60 years of age detected an overall rate of 9%, with individual study results ranging between 0-39% [81]. Similar frequencies are reported in studies of younger adults [77]. Even if toddlers are regarded as the main reservoir from which pneumococci spread to cause disease in other age groups, carriage in older children and adults, with a partly different serotype distribution than among the youngest, may also be an important source of disease-causing strains [81, 82].

The pneumococcus shares the nasopharyngeal niche with many other bacteria, and its relationships with *Haemophilus influenzae* and *Moraxella catarrhalis* are the most well studied [83-85]. Like the pneumococcus, encapsulated strains of *H. influenzae*, first and foremost type b (Hib), are important causes of pneumonia, bacteraemia and meningitis in infants and the elderly, but widespread use of the Hib conjugate vaccine has successfully reduced these infections [86]. Non-encapsulated strains, non-typeable *H. influenzae* (NTHi), may also cause invasive infections, but are predominantly of importance, similarly with *M. catarrhalis*, in exacerbations of chronic obstructive pulmonary disease (COPD) and acute otitis media (AOM) [87-89]. *Staphylococcus aureus* may also be present in the nasopharynx, and colonization with this species is negatively correlated with pneumococcal carriage [83].

Pneumococcal infection

The pathogenesis of pneumococcal disease is dependent on several circumstances including the pathogen and immune status of the host in addition to ecological factors that facilitate evasion of host immune defences, tissue adhesion, dissemination, and invasion. Successful establishment of nasopharyngeal colonization after transmission is a prerequisite for subsequent development of clinical infection [75]. Several major pneumococcal virulence factors are listed in *Table 2*, exemplifying different functions that aid the bacterium at different stages of pathogenesis.

Transmission and colonization

Spread of the pneumococcus from a colonized individual to a new host typically occurs through direct close contact or with respiratory droplets, but inoculation by contaminated objects is also possible, and aerosol transmission has been demonstrated in an animal model [90]. Infection with Influenza A or other respiratory viruses greatly increase transmission due to local inflammation with increased secretions [72, 90, 91]. Similarly, secretion of the exotoxin pneumolysin (Ply) also stimulates local inflammation and drives transmission [92]. Increased bacterial shedding is also associated with serotypes with CPSs that more efficiently inhibit mucus entrapment and with expression of a thicker capsule [93].

Whether the colonization process is successful or not depends on avoidance of the host defence, out-competition of other bacteria, and binding to extracellular matrix proteins and epithelial cells [72, 91, 94]. The different steps are also aided by the mechanism of phase variation. Briefly, this is the switching between different bacterial phenotypes characterized by either high or low expression of CPS and different levels of various surface proteins. While the heavily encapsulated phenotype more efficiently inhibits clearance by mucus and immunoglobulins (Igs) to penetrate the mucus layer, and can survive during systemic invasion, the phenotype with less capsule adheres better to the epithelium and is important for sustained colonization [95]. Furthermore, pneumococci form biofilms in the nasopharynx that hinders clearance [96]. Tissue damage, which is utilized for adhesion and invasion by the bacterium, is caused by the host inflammatory response stimulated by several pneumococcal antigens and directly by Ply and H_2O_2 [72, 73, 94].

Table 2. The most important and studied pneumococcal virulence factors and their roles in pathogenesis [72, 73, 91, 94].

Virulence factor	Description	Host interactions	Role in pathogenesis
CPS capsule	Covers outer surface and determines serotype; Effectivity of functions vary between serotypes	Repels mucus; Inhibits depositon and interactions with phagocytes of complement and Ig; Prevents entrapment in neutrophil extracellular traps (NETs)	Hampers mucociliary clearance; Immune evasion; Survival in the bloodstream
Peptidoglycan	Cell wall component	TLR2-ligand	Stimulates inflammatory response; Inhibits formation of membrane attack complex by complement
Teichoic acid and lipoteichoic acid	Cell wall component	TLR2-ligand	Stimulates inflammatory response
ChoP	Binding motif for CBPs on (lipo)teichoic acid	Binds to platelet activating factor receptor on host epi-/endothelium	Adhesion and translocation to the blood stream and the CNS
Pneumolysin (Ply)	Cytotoxin; Released upon bacterial lysis	TLR4-ligand; Lyses host cells through pore formation; Activates classical complement pathway; Ipairs ciliary function	Induces tissue damage and a strong inflammatory response; Bacterial escape from lysosome; Depletes complement; Hampers mucociliary clearance
LytA	CBP; Autolysin	Degrades the pneumococcal cell wall leading to CPS shedding and Ply release	Stimulates inflammatory response; Immune evasion
SpxB	Pyruvate oxidase	Produces H_2O_2	Induces tissue damage
PspA	Surface exposed CBP	Inhibits complement and CRP deposition; Binds and inactivats lactoferrin	Immune evasion
PspC/CbpA	Surface exposed CBP	Inhibits complement depostion and activation through binding of factor H; Binds to polymeric Ig receptor on host epi-/endothelium	Immune evasion; Adhesion and translocation to the bloodstream and CNS
CbpE	Surface exposed CBP	Degrades platelet activating factort; Binds plasminogen	Reduces neutrophil recruitment; Promotes tissue damage
Eno	Surface protein; Enolase	Binds plasminogen	Promotes tissue dagamge and invasion
NanA	Neuraminidase	Degrades siliac acid	Access to the epithlium; Provides nutrients
ZmpA	Zink metalloprotease/IgA1 protease	Degrades IgA1	Immune evasion
ZmpC	Zink metalloprotease	Degrades extracellular matrix components	Promotes invasion
PavA	Surface protein	Binds fibronectin	Promotes adhesion
RrgA	Pilus subunit	Binds to epithelium	Promotes adhesion

Local and systemic dissemination

The step from colonization to local or disseminated clinical infection often occurs shortly after the acquisition of a new pneumococcal strain but may also be caused by disruption of the host-pathogen equilibrium with an already present strain by a viral infection. Eustachian tube dysfunction caused by viral infection leading to AOM and the well-studied association between Influenza A virus and pneumococcal pneumonia are examples that illustrate this [96-98]. Influenza, and other viral lung infections such as respiratory syncytial virus, increases the risk for developing bacterial pneumonia and, correspondingly, *S. pneumoniae* is the most frequently detected bacteria in these cases. Pneumococcal pathogenesis in the lung is facilitated through several mechanisms, including by exposing targets for bacterial adhesion in the damaged epithelium, impeding mechanical clearance of bacteria from the lower respiratory tract, and depleting alveolar macrophages [97, 99]. Simultaneous viral and pneumococcal pneumonia promotes a strong inflammatory response which may lead to a severe clinical course, and the incidence of bacteraemic pneumococcal CAP follow that of influenza temporally [100]. It is generally thought that secondary bacterial infection, predominantly with *S. pneumoniae*, caused a majority of fatal cases during the 1918 influenza pandemic, illustrating the unfortunate result of coinfection [101, 102].

The bacterium reaches the lung through (micro)aspiration and is either cleared by alveolar macrophages or causes pneumonia depending on the balance between bacterial virulence factors and host immune status. From the lung, or directly from the nasopharynx, it may translocate to the bloodstream through transcytosis mediated by interactions of ChoP and PspC with the platelet activating factor receptor and the polymeric Ig receptor, respectively, or paracellular invasion through tissue damage. Partly, the same mechanisms are responsible for crossing the blood brain barrier to invade the central nervous system. The capsule is the single most important virulence factor for bacterial survival in the bloodstream [72, 73, 94]. Recent studies have demonstrated that the pneumococcus in the setting of bacteraemia may replicate intracellularly in splenic macrophages and reseed into the bloodstream, and act as intracellular pathogens in cardiomyocytes, causing cardiac damage in IPD [103, 104].

Clinical manifestations

Pneumococcal disease occurs in all ages and varies in severity across a broad spectrum, ranging from mucosal infections such as AOM, conjunctivitis, and sinusitis to community-acquired pneumonia (CAP), sepsis and meningitis. Infections leading to bacterial invasion of normally sterile sites such as the pleural space, the bloodstream, cerebrospinal fluid, or joints constitute IPD (*Figure 6*) [72, 105].

Lobar pneumonia is the classical disease manifestation that is associated with *S. pneumoniae* and has given it its name. Even if definitive aetiology is not detected in approximately 50% of CAP cases, the species is still considered the major bacterial cause of the infection. The etiological fraction of pneumococci varies vastly between studies, but throughout a lower proportion is detected in the United States (5-15%) compared with Europe (approximately 20-40%) [105-107]. The archetypical clinical presentation of pneumococcal CAP is characterized by an acute onset and includes fever, chills, productive cough, and dyspnea but the symptomatology may be more unspecific in elderly or immunocompromised individuals [108]. Even if pneumococcal CAP is not accompanied by bacterial spread to the bloodstream in the absolute majority of cases, bacteraemic CAP is the most common form of IPD, followed by bacteraemia with unknown focus, and meningitis [105]. Endocarditis and septic arthritis are additional manifestations that have although become very rare since effective antibiotic treatment became widely available [109, 110]. As in CAP, the pneumococcus is one of the most common bacteria causing AOM, especially the first episode of the condition and cases with severe symptoms [96, 111]. In some cases, pneumococcal disease may cause hemolytic uremic syndrome [112, 113].

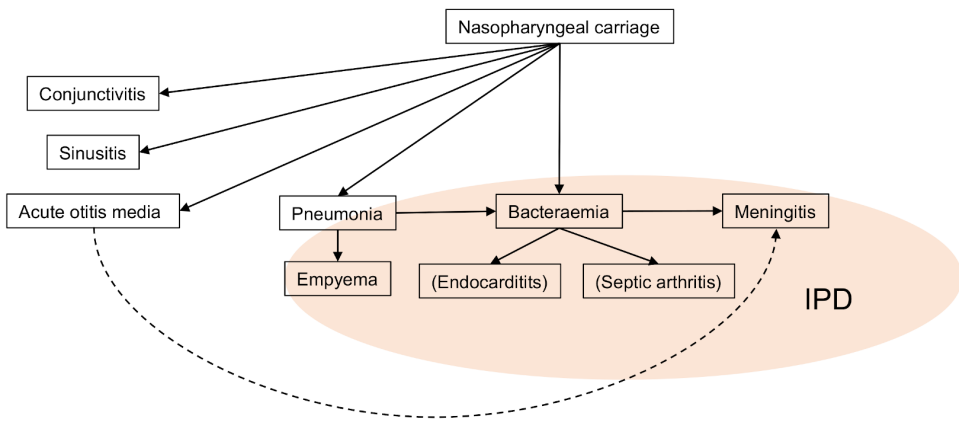


Figure 6. Diseaes manifestations casued by pneumococci.
Clinical infection with *S. pneumoniae* is preceeded by nasopharyngeal acquisition and carriage. From the nasopharynx the bacterium may spread locally to cause mucosal infections or reach the lungs through aspiration. Direct invasion from the nasopharynx to the bloodstream also occurs. Dissemination to normally sterile sites is defined as invasive pneumococcal disease (IPD; indicated by the beige area in the figure). Pneumonia is non-invasive in the vast majority of cases but is considered IPD when accompanied by bacteraemia or empyema. Endocarditis, septic arthritis, and local spread from the middle ear to the central nervous system are rare complications of pneumococcal infection.

Clinical significance of the capsule

As mentioned above, the serotype-defining capsule is important for pneumococcal pathogenesis through several functions that contribute to inhibiting

opsonophagocytosis, mucociliary clearance, and bacterial lysis. These functions vary considerably between serotypes which results in differences in their association with carriage or specific disease manifestations, clinical importance, and prevalence [18, 46, 114]. The exact mechanisms that confer these differences are not fully clarified, but divergent inhibition of complement binding, varying amounts of CPS produced, and differences in electronegativity and chemical composition are factors that probably are important [47, 115, 116].

An inverse relationship between the occurrence of serotypes in carriage and IPD is generally seen, and these observations are used to define their invasive potential by calculating either carriage to IPD ratios or rates of IPD cases per colonization events. Serotype-specific trends are generally conserved between studies, but discrepancies are seen over time and between settings. Serotypes that are often reported to have a higher invasive potential include serotypes 1, 5, 7F, 8, 12F, 14, 18C, 19A, 22F, 24F, and 33F while examples of strains with lower invasiveness are serogroups 6, 11, 15, 23, 35, and serotype 19F [117-119]. The serotypes with the absolute highest invasive potential, such as serotypes 1 and 7F, more often infect young and otherwise healthy individuals, and are associated with high survival rates. Conversely, low-invasive serotypes are associated with infections in individuals with risk factors and confer worse prognosis [120-122]. Results vary regarding the invasiveness of serotype 3 and it predominantly causes IPD in adults and is associated with severe clinical presentation, while it is seldom detected in carriage among children [117, 118, 123, 124].

Even if there are clear associations between specific serotypes and genetic lineages, most GPSCs encompass several serotypes and, conversely, most serotypes occur in many different lineages. The relative proportion of different serotypes within lineages fluctuate over time and is affected by vaccine use, and novel capsule switching events within lineages may occur. Virulence may therefore vary among strains expressing the same serotype as genetic lineages differ in their expression of non-capsule virulence genes [69, 120]. Furthermore, strong associations between serotypes and antimicrobial resistance patterns are generally seen, which is also explained by the mutual association to specific genetic lineages [69, 125].

Risk-factors for pneumococcal disease

Children <5 years of age and the elderly are at high risk for pneumococcal infection, as reflected by the massive disease burden in these groups [126]. For the youngest children (<2 years) this is partly explained by the inability to produce antibody directed against the CPS, a function that then gradually improves. Importantly, natural exposure to pneumococci also gradually builds up serotype-independent immunity during the first years of life, contributing to the declining incidence of pneumococcal infections [18, 127]. In the elderly, impaired function of various anti-pneumococcal immunological mechanisms may contribute [128].

As described above, viral respiratory tract infection predisposes for pneumococcal infections [96, 97]. Factors that interfere with epiglottis function or cough reflex such as certain neurological disorders or alcohol abuse alters the risk for pneumonia by increasing aspirations [129, 130]. Several concomitant conditions with systemic consequences increase the risk for pneumococcal infections. Individuals with compromised immune status due to Ig- or complement-deficiencies, asplenia, human immunodeficiency virus (HIV)-infection, haematological malignancies, and immunosuppressive treatment are at considerably higher risk. Likewise, chronic organ failure of the heart, lungs, kidney, and liver confers a higher incidence of pneumococcal infections [131-133]. Additionally, patients with cochlear implants or cerebrospinal fluid leakage are disproportionally affected by meningitis [134]. Other factors that predispose for pneumococcal disease and have a paramount impact on the global disease burden are childhood wasting syndrome and indoor air pollution from cooking with solid fuels [126]. Naturally, the likelihood of acquiring nasopharyngeal carriage of the bacterium is higher for children that have siblings or attend day-care, and for parents with children who are often colonized [135-137].

Antibiotic treatment and antimicrobial resistance

Penicillin and other beta-lactams constitute the traditional treatment for pneumococcal infections, but several antibiotic groups are frequently used to treat the bacterium (*Table 3*). Beta-lactam antibiotics confer their bactericidal effect through disrupting cell wall formation by binding to various penicillin binding proteins (PBPs), a group of proteins that are responsible for important stages of peptidoglycan polymerization. However, penicillin non-susceptible pneumococci (PNSP), exhibiting a minimum inhibitory concentration (MIC) greater than the epidemiological cut-off 0.06 mg/L, were first detected in 1967 [138]. During the subsequent decades, high-grade penicillin resistance as well as resistance to tetracyclines, macrolides, trimethoprim-sulfamethoxazole, and chloramphenicol emerged, and antimicrobial resistance among pneumococci became an increasing clinical problem [139, 140]. Multi-drug resistant (MDR) and extensively drug resistant (XDR) pneumococci are commonly defined as isolates exhibiting reduced susceptibility to ≥3 or ≥5 relevant antibiotic classes, respectively, but the use of increased exposure (I) or resistant (R) breakpoints as cut-off for this classification vary between studies [141, 142].

Reduced susceptibility to beta-lactams is caused by alterations of the transpeptidase domains of PBP1a, PBP2b, and PBP2x, and the penicillin MIC can be inferred from the amino acid sequence of these domains [143, 144]. Selection pressure from beta-lactam use in the population proportionally contribute to an increased prevalence of PNSP strains and a gradual drift towards higher MIC values in the bacterial population through promoting survival of non-susceptible lineages,

recombination of resistance-conferring PBPs between lineages or species, and *de novo* PBP mutations [144-147]. Non-susceptibility to different antibiotics is also associated with certain serotypes because both traits may be common in the same genetic lineage, why the use of PCVs have an impact on antimicrobial resistance [125]. Interestingly, the *cps* locus is flanked by the *pbp1a* and *pbp2x* genes why recombination events may occur that result in simultaneous capsule switching and acquisition of beta-lactam non-susceptibility, further strengthening the association between certain serotypes and PNSP phenotype [148, 149].

Table 3. Important anti-pneumococcal antibiotics, mechanisms of action, and resistance mechanisms [139].

Antibiotic group	Commonly used drugs	Mechanism of action	Resistance mechanism
Beta-lactams	Penicillin Amoxicillin Cefotaxime	Binds to PBPs, inhibits cell wall synthesis	Alterations of PBPs
Macrolides	Erythromycin Azithromycin	Binds to ribosomal 50S subunit, inhibits protein synthesis	Methylation of binding site (*ermB*); efflux pump (*mefA*)
Lincosamides	Clindamycin	Binds to ribosomal 50S subunit, inhibits protein synthesis	Methylation of binding site (*ermB*); efflux pump (*mefA*)
Tetracyclines	Tetracycline Doxycycline	Binds to ribosomal 30S subunit, inhibits protein synthesis	Modification of binding site (*Tet* genes)
Fluoroquinolones	Levofloxacin	Binds to DNA topoisomerase, inhibits DNA replication	Accumulating mutations in *gyrA*/*parC*
-	Trimethoprim-sulfamethoxazole	Inhibits folate metabolism	Insertion/point mutations in *folP*/*folA*
-	Chloramphenicol (topical treatment)	Inhibits protein synthesis	Enzymatic inactivation of the antibiotic (*cat*)

Resistance rates differ greatly between geographical regions due to natural variation of the pneumococcal epidemiology in addition to local differences in antibiotic and vaccine PCV use [125]. A recent meta-analysis by Andrejko *et al*. [150], and a surveillance programme including several sites on different continents [151], provide current global estimates. These studies demonstrated that PNSP were least common in north-western Europe, with frequencies around 10-15%, compared with 20-40% in most other settings. According to the European Centre for Disease Control and Prevention, PNSP (*Figure 7*) and erythromycin resistance rates in Sweden were 8.5% and 6.6% in 2020, respectively, which was lower than the European Union population-weighted averages (15.6% and 16.9%, respectively) [152]. In sub-Saharan Africa, PNSP (38%), and resistance to trimethoprim-sulfamethoxazole (69%) and tetracyclines (33%) is common, while erythromycin resistance is less frequent (9%) [150]. Overall, the use of PCV has resulted in reductions of non-susceptibility and resistance to most antibiotic groups, but non-PCV serotypes that are associated with antimicrobial resistance are an increasing concern. In Sweden, peroral or parenteral penicillin is the first line treatment for non-severe, uncomplicated pneumococcal infections, including empirical treatment for AOM and CAP, based on the low frequency of PNSP and positive observed

outcomes [153]. Internationally, amoxicillin is instead considered the first-hand peroral drug for treating the bacterium [150].

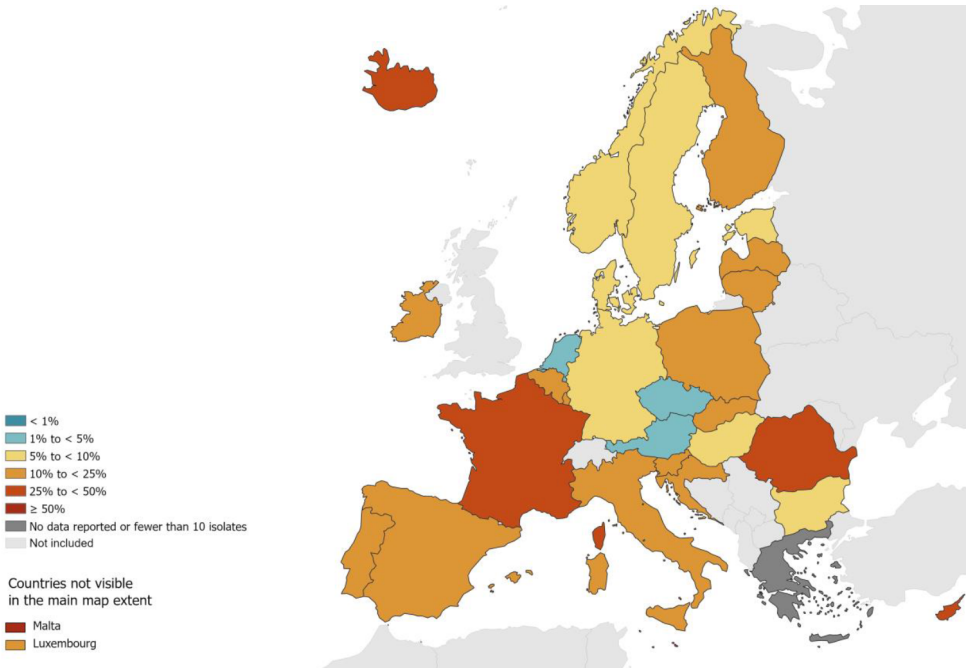


Figure 7. PNSP rates in the European Union in 2020.
Frequencies of PNSP among pneumococcal isolates causing IPD in the European Union. Figure reproduced from the European Centre for Disease Control and Prevention [152].

The immune response to pneumococcal exposure

The human mechanisms of protection against infection comprise a multitude of anatomical features, physiological process, and unspecific as well as specific cellular and chemical reactions. Innate immunity encompasses the non-specific barriers and early responses that contribute to protection directly at, or very soon after, first contact with a pathogen. This includes the external and internal surface epithelia, the chemical barriers comprised of antimicrobial proteins and complement, the early inflammatory response stimulated by pattern recognition receptors (PRRs), and non-specific effector functions of innate immune cells. In a delicate interplay with the early inflammatory response, phagocytic antigen presenting cells constitute a bridge between innate and adaptive immunity through their role in activating antigen-specific T-lymphocytes in the secondary lymphoid tissues such as lymph nodes, mucosal lymphoid tissues, and the spleen. In contrast to innate immunity, the adaptive response comes into play approximately one week

after the exposure to a novel pathogen and is characterized by T-cells that both have effector functions and orchestrate the antigen-specific response, and antibody-producing B-cells. The adaptive immune response includes the establishing of immunological memory which leads to improved initial protection and a faster as well as stronger secondary response upon re-exposure to the same antigen through circulating and secreted antibodies, and memory T- and B-cells. Importantly, the innate and adaptive immune responses to infection are not two parallel independent systems, but intermutually enhance their different effector functions to achieve clearing of an invading pathogen [154]. Below, the most important protective mechanisms for preventing and clearing pneumococcal colonization and infection are described. Additionally, the bacterium has a great number of virulence factors that contribute to avoiding the host defences, and the balance between pathogen and host factors will determine whether colonization and/or clinical infection occur and the clinical course.

Interactions with innate immunity

In the respiratory tract, the first line of defence against pneumococci is the mucosal epithelium. Epithelial cells are joined by tight junctions, making it non-permeable, and goblet cells secrete the respiratory mucus that covers the surface. The bacterium and other foreign particles are trapped in the mucus and transported by the ciliated respiratory epithelium of the nasal cavity and lower respiratory tract to the pharynx (*i.e.*, mucociliary clearance), where they are eliminated by swallowing or coughing [155]. The presence of antimicrobial proteins secreted by epithelial cells, such as lactoferrin and lysozyme, contributes directly to lysis of *S. pneumoniae* cells [156]. The pneumococcal capsule prevents mucus entrapment as the negative charges of CPSs repel the equally negative charges of sialic acid on mucins. This function varies between serotypes and the stages of phase variations due to differing chemical structures, total negative charges, and capsule thickness [46, 95]. Furthermore, NanA cleaves sialic acid from mucins which counteracts entrapment. Both Ply and H_2O_2 decrease mucociliary clearance by slowing ciliary beating [155]. The abundant virulence factor PspA interacts with lactoferrin to inhibit its lytic effect, and the bacterium expresses enzymes that modify peptidoglycans to prevent their degradation by lysozyme [72, 156].

If the bacterium overcomes the first line of defence and reaches the epithelial cellular surface or invades tissues, several PRRs on epithelial cells, mucosal macrophages, and dendritic cells are activated by pathogen associated molecular patterns (PAMPs), leading to a local inflammatory response and cytokine signalling. Cell wall components peptidoglycan, teichoic acid, and lipoproteins are recognized by Toll-like receptor (TLR) 2, Ply by TLR4, and bacterial DNA by TLR9 in phagosomes. Additionally, nucleotide-binding oligomerization domain-like receptor 2 is activated by cytosolic cell wall components [73, 156]. Consequently, neutrophils and macrophages are recruited to the site of infection and may clear

bacteria through phagocytosis. Pneumococci are however resilient to this process in the absence of specific antibodies thanks to their capsule, and if phagocytized, the bacterium may escape the phagosome via release of pore-forming Ply. Neutrophils may also kill or entrap bacteria through the secretion of antimicrobial defensins and NETs [72, 73].

As illustrated by the susceptibility of individuals with congenital complement deficiencies, the complement system plays an important role in the defence against pneumococcal infections, both in the innate response and by contributing as effector molecules in the adaptive response. The complement cascade is activated on the pneumococcal cellular surface by the classical, alternative, as well as lectin pathways. In addition to being triggered by anti-capsular and non-capsular antibodies, the classical pathway is initiated by C-reactive protein (CRP) binding to ChoP [116]. All three pathways induce cleavage of C3 on the bacterial surface resulting on deposition of the strong opsonin C3b and release of C3a which further stimulates the inflammatory response. In contrast, the formation of membrane attack complexes initiated by C5 contributes little to the killing of pneumococci [154]. The CPS layer reduces the effects of the complement system by covering surface antigens that bind antibodies, acute-phase proteins, or directly trigger complement activation. It also reduces the interaction between bound C3b and complement receptors expressed by phagocytic cells. The inhibitory effects of the capsule vary substantially between serotypes [115, 116]. The bacterium also evades complement with aid of several surface proteins such as PspA, which inhibits both the classical and alternative pathways, and PspC, which inhibits the formation of C3 convertase through binding the negative regulator factor H [116, 157]. Certain allelic variants of PspC also bind the complement inhibitor C4b binding protein [158]. In addition, excreted Ply activates C3 and thereby depletes available complement and diverts the reactions from the bacterial surface [116].

Adaptive anti-pneumococcal immunity

> "From the point of view of the adaptive immune system, each serotype of *S. pneumoniae* represents a distinct organism"
>
> Charles Janeway Jr. [18]

As demonstrated by the findings in early studies of convalescence serum and the protective effect of CPS-based vaccines, the adaptive immune response to the pneumococcal capsule results in antibodies that may prevent and resolve pneumococcal infection. It has generally been presumed that anti-CPS antibodies are the principal mechanism for naturally acquired immunity against the pneumococcus [154]. However, exposure to the bacterium also elicits antibodies and cellular responses to serotype-independent protein antigens and growing evidence indicate that these also have major roles, why the above quote by Charles

Janeway Jr. may have to be partially reconsidered [127]. Experimental bacterial challenge studies and longitudinal studies of nasopharyngeal carriage suggest that naturally acquired anti-pneumococcal immunity is established and maintained in humans by repeated colonization events in the childhood and through the lifetime [78, 128, 159, 160]. Consistently, sera from healthy adults generally contain antibodies to both capsular antigens and many surface proteins [127, 161]. How the different aspects of the immune response are affected by episodes of clinically significant infection has not been studied to the same extent. However, inadequate responses after pneumococcal infection characterized by low antibody concentrations and immune tolerance to the corresponding CPS have been described [162-167].

Initiation of the adaptive immune response occurs in the secondary lymphoid organs, where naïve B- and T-lymphocytes are activated by antigens arriving by lymphatic drainage or carried by antigen presenting cells, respectively. While protein antigens induce T-cell and T-dependent B-cell activation, polysaccharides such as the pneumococcal CPS activate T-independent marginal zone B-cells and possibly B-1 B-cells. Briefly, the T-cell mediated anti-protein response against *S. pneumoniae* gives rise to $CD4^+$ helper T-cells and germinal center formation resulting in affinity matured, class-switched IgG-producing plasma cells and memory B-cells. Production of IgA is also induced, which secreted on the mucosal membranes. On the other hand, T-independent B-cells are activated rapidly to predominantly produce IgM antibodies [154]. However, class switching and induction of both IgM^+ and IgG^+ memory B-cells as well as long-lived Ig-secreting clones also occur in these lineages, but the mechanisms are poorly understood [168, 169].

The relative importance of the different arms of adaptive anti-pneumococcal responses depends on the anatomical local where the pathogen is encountered. In mice, a mucosal IL-17 producing helper T-cells (Th17) response, strongly stimulating macrophage and neutrophil functions, has been shown to be of great importance for clearing and preventing nasopharyngeal carriage. Data from human challenge studies support this mechanism but indicate that anti-protein and anti-CPS Ig also contribute. Specifically, secreted IgA antibodies are of importance for mucosal immunity [127, 156]. In the setting of pneumonia, the Th17 response and antibodies to capsular and non-capsular antigens contribute to prevent infection, but it is unknown which are most important [127, 170]. In contrast, protection against IPD is clearly antibody-mediated, and recent findings interestingly indicated that anti-protein may be a more important factor than anti-CPS Ig in naturally acquired immunity [161].

The most important effector functions of anti-CPS and anti-protein Ig against pneumococci are agglutination, which facilitates mucociliary clearance in the airways, and opsonization for subsequent phagocytosis, further stimulated by activation of the classical complement pathway [127, 128]. The pneumococcus counteracts these functions through degradation of secreted IgA1 with the IgA1-

protease ZmpA on mucosal surfaces. Furthermore, the capsule reduce binding of anti-protein antibodies by covering these epitopes [156]. Excessive shedding of CPS by serotype 3 may divert anti-CPS antibodies from the bacterium and has been proposed as the explanation why higher concentrations of capsular Ig are needed to confer protection against this serotype compared to most other [47].

Measuring anti-pneumococcal immune status

Adaptive immune status against *S. pneumoniae* may be assessed through measurements of both antibody titres and function. Most standardized methods have been developed for the evaluation of pneumococcal vaccines but have also been applied in studies of naturally acquired immunity [171]. Concentrations of antibodies against CPS and proteins are determined with enzyme-linked immunosorbent assays (ELISA). Briefly, a plastic surface is coated by the antigen of interest to which the tested serum is then added. The bound Ig is then detected by a secondary antibody which is coupled to an enzyme. When the enzyme substrate is subsequently added a measurable visual signal is produced and its intensity can be compared to a reference to determine the concentration of the detected antibody [154]. A standardized World Health Organization (WHO) protocol for quantification of anti-CPS IgG is widely used [172, 173].

Antibody-function may be studied with different approaches. The avidity of antibodies (*i.e.*, the relative strength with which they bind an antigen) may be determined by ELISA with addition of a binding inhibitory substance at different concentrations [174]. More physiologically appealing methods that are frequently applied are various opsonophagocytic assays (OPA) that measure the opsonic activity of sera. OPAs exist in the form of killing assays that determine the ratio of bacteria surviving after opsonization with serum and exposure to phagocytes, and assays that utilizes flow cytometry to measure uptake of opsonized bacteria in phagocytes [171]. A standardized WHO multiplexed OPA is systematically used for evaluation of pneumococcal vaccines [175]. Other approaches that are used in experimental studies include the use of serum in mouse infection models and examination of different B-cell pools [159, 176, 177].

For PVC-induced responses in children, defined cut-offs that are regarded to indicate protective immunity exist for anti-CPS IgG concentrations and OPA titres and are used in vaccine immunogenicity trials. However, it is unclear which cut-offs are optimal in adults. Furthermore, the two methods do not always correlate. In immunogenicity trials, the fold-increase of OPA titres of after immunization as compared to baseline is therefore often used as an important measure of vaccine responsiveness in addition to absolute values [171].

Pneumococcal immunity through life

As previously discussed, children under 1-2 years of age are unable to mount an adequate humoral immune response to polysaccharide antigens and are therefore susceptible to the pneumococcus, and this function improves gradually thereafter. The emergence of a functional T-independent response coincides with rapidly decreasing risk for severe infections. Furthermore, gradually improved serotype-independent immunity during the first years of life, stimulated by frequent colonization, does also contribute to declining frequencies of carriage and infection [127, 128]. Resilience towards the pathogen is sustained in adults, as reflected by the small number of infections in this group, until a change occurs at approximately 60-70 years of age. Thereafter the risk for severe pneumococcal disease increases gradually to high levels. This is thought to be due to declined function of several immune mechanisms that. Both concentration of anti-CPS Ig (specifically IgM) and, to an even higher degree, OPA titres decrease in the elderly. Reduced levels of IgM^+ memory B-cells and B-1 cells have been observed. Additionally, elderly individuals are less frequently colonized by pneumococci and the events that occur are not as immunogenic as in younger adult [128, 178].

Pneumococcal vaccines

Development of pneumococcal vaccines

In the beginning of the 20th century, the first trials with a pneumococcal vaccine, in the form of a heat-killed whole cell vaccine, were conducted among gold mine workers in South Africa. The researchers were not aware of the importance of the serotype-specificity of the response but short-lived positive effects on pneumonia incidence rates and mortality were noted. Further studies in South Africa and the United States using mixtures containing the most common serotypes in the respective settings showed greater reductions, although all studies on the whole cell vaccine included significant design flaws [179]. In light of the discovery of the CPSs, continued research focused on their use as vaccine antigens. It was clearly proven that this was a feasible strategy for preventing pneumococcal infections by successful interruption of an ongoing serotype 1 pneumonia outbreak at a psychiatric hospital in 1937 by immunizing patients with the corresponding CPS [180], and by trials performed in military camps some years later with polyvalent formulations [181]. Neither the whole cell nor CPS-based vaccines did, however, reach any wide impact at the time, and in face of the breakthrough of effective antibiotic therapy interest in pneumococcal vaccines dwindled. Consequently, the hexavalent CPS formulation that was available in 1946 was withdrawn from the market in 1954 [182].

Despite the routine use of antibiotics, a considerable mortality rate due to pneumococcal infections remained, especially among the elderly and individuals with complicating illnesses [22]. This fact, and the reports of antibiotic resistant pneumococci spurred renewed interest in vaccine development. Government-sponsored research initiatives subsequently lead to the licensure of a 14-valent PPV in 1977, which was replaced by the currently used 23-valent formulation (PPV23) in 1983. Alike the situation today, the PPVs were from start recommended to individuals >2 years with a high risk of contracting pneumococcal disease. Because of the absence of an effective adaptive immune response to PPV in children <2 years of age, a vaccine was however still lacking for this group that was highly affected by pneumococcal disease on a global level. This situation changed in the year 2000 with the introduction of the first protein-conjugated CPS vaccine, PCV7 [182]. Since then, several higher valency PCVs have been developed, and additional vaccines are in the pipeline (*Table 4* and *Figure 8*).

Pneumococcal polysaccharide vaccine

In many industrialized countries, including Sweden, PPV23 is currently recommended for all individuals older than 65 years and individuals >2 years of age with increased risk (*i.e.*, chronic organ dysfunction, diabetes mellitus, alcohol, or drug use etc.) for pneumococcal infections [183, 184]. In similar with other polysaccharide vaccines, PPV23 elicits a T-cell independent antibody response that relies on marginal zone B-cells, which are not yet developed in children <2 years of age, explaining its poor immunogenicity in this group. Furthermore, the response induced by polysaccharide vaccines seems to exhaust the memory B-cell pool, which is thought to explain hyporesponsiveness (further discussed below) which has been observed after repeated immunization with PPV23 [177, 185]. The efficacy and effectiveness of PPV23 against IPD and pneumonia, and its role in vaccination strategies have been extensively debated. A significant risk reduction for IPD is confirmed but results from the three most recent systematic reviews performed diverge regarding the vaccine effectiveness against all-cause and pneumococcal CAP [184, 186, 187].

Pneumococcal conjugate vaccines

Since PCV7 became available at the turn of the millennium, followed by PCV10 and 13 in 2010, these vaccines have been gradually introduced in paediatric national immunization programmes (NIPs) worldwide which is recommended by the WHO [188]. Unlike pure polysaccharide vaccines, PCVs induce a T-cell dependent response in children as well as adults characterized by germinal center formation and development of memory B-cells [177].

Table 4. Currently available and in-development pneumococcal vaccines.

Vaccine (trade/development name)	Carrier proteins	Current status
PPV23 (Pneumovax)	None	Recommended for indidivuals >65 years, and risk groups >2 years
PCV7 (Prevenar)	CRM197[a]	Discontinued
PCV10 (Synflorix)	NTHi protein D, tetanus and dipheteria toxoids	Used in childhood vaccination programmes
PCV10 (Pneumosil)	CRM197[a]	Used in childhood vaccination programmes
PCV13 (Prevenar 13)	CRM197[a]	Used in childhood vaccination programmes; recommended for adult risk groups
PCV15 (Vaxneuvance)	CRM197[a]	Approved for use in adults; recommended for adult risk groups [189]
PCV20 (Apexxnar)	CRM197[a]	Approved for use in adults; recommended for adult risk groups [189, 190]
PCV24 (ASP3772/AFX3772)	*S. pneumoniae* surface proteins	Phase 1/2 study completed in adults [191]
PCV24 (VAX-24)	CRM197[a]	Preclinical studies completed [192], phase 1/2 study innitiated
PCV25 (IVT-PCV-25)	Not published	Preclinical development

[a] Non-toxic mutant diphteria toxin.

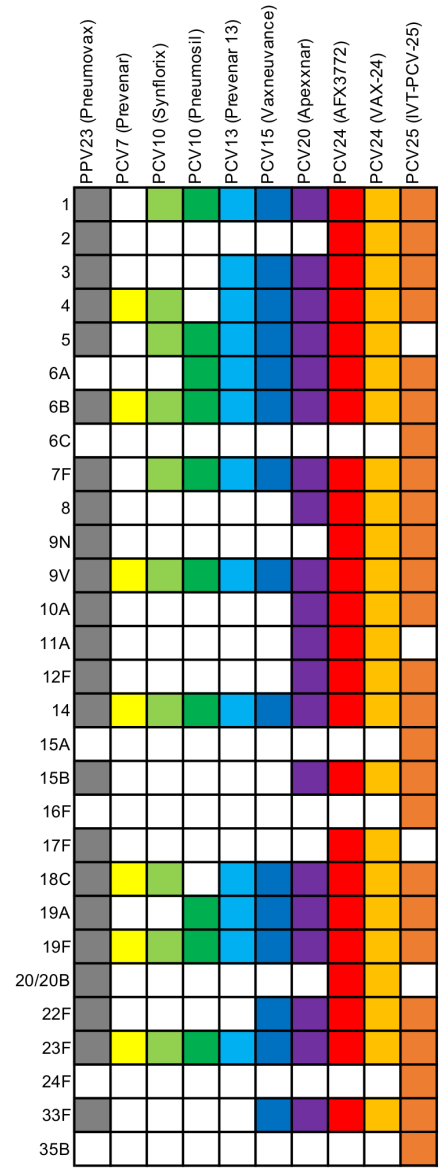


Figure 8. Included serotypes in currently available and future pneumococcal vaccines.
Serotype covrage of the pneumococcal vaccines that are listed in Table 4.

Infant immunization with PCVs effectively reduces the risk for IPD and CAP caused by the included serotypes, resulting in a considerable impact on overall incidence reductions among children. The incidence of IPD caused by any serotype has decreased by approximately 60-70% in young children in several countries [193-198]. The vaccines also provide protection against pneumococcal AOM, but their effectiveness for lowering the overall AOM incidence is uncertain [194, 199]. In adults aged 65 years or older, vaccination with PCV13 substantially reduced the risk for IPD and CAP caused by the included serotypes with a moderate effect on overall IPD and pneumococcal CAP [200].

In addition to preventing clinical pneumococcal infection, PCVs reduce nasopharyngeal colonization by the included serotypes [201]. Because young children constitute the main reservoir of pneumococci in the population, immunization of this group has achieved herd effects with fewer infections caused by the included serotypes also in nonvaccinated age groups. The indirect effect on all-cause IPD incidences that have been observed in adults, approximately in the range 10-30%, were although lesser among those aged ≥65 years which is the most heavily affected group in absolute numbers [196, 197]. Furthermore, the generally slight reductions among older adults have been more pronounced and long lasting in the United States compared to Europe. The reason that no greater effects are reached in these groups is the increased incidence of infections caused by non-PCV serotypes, a phenomenon termed serotype replacement [195-197, 202].

The PCVs may be administered according to schedules including 2 or 3 primary doses during the first 6 months of life, followed by a booster dose at least 6 months later (2+1 and 3+1, respectively), or including only 3 primary doses without a booster (3+0) [188]. No clear benefits have been noted in comparisons of the different schedules, but it has been proposed that a booster dose may provide a more long-lived response and lead to greater herd effects [203].

Two novel PCVs, PCV15 and PCV20, have recently been approved for use in adults in the United States and the European Union. In updated guidelines from 2022, PCV15 followed by PPV23, or a single dose of PCV20 was recommended to all adults ≥65 years and all adult risk groups in the United States [189], and PCV20 was recommended to adults with very high risk for pneumococcal infection (*i.e.*, asplenia, immunosuppression, cerebrospinal fluid leakage etc.) in Sweden [190]. For individuals with hypo- or asplenia specifically, PCV20 vaccination should be followed by a single dose of PPV23 according to the Swedish guidelines. Additional high-valency PCVs, including two 24-valent and one 25-valent formulation, are currently in different stages of development (*Table 4* and *Figure 8*). Interestingly, a PCV24 candidate, which have recently been assessed in a phase 1/2 study in adults, contains CPSs linked to a fusion protein comprising segments from two pneumococcal surface proteins that are linked to virulence in serotype 3. Immunogenicity analyses indicated that vaccination induced positive antibody and cellular Th17 responses towards the fusion protein, although the clinical importance of the anti-protein response for this vaccine is yet unknown [191].

Serotype replacement

The use of PCV effectively reduces the risk for nasopharyngeal colonization by the included serotypes in children. However, the overall carriage rate of pneumococci is not affected, which is due to increased prevalence of non-PCV serotypes [201]. Gradually, this effect leads to a shift of the serotype distribution carried by children and many PCV serotypes that were previously common have virtually been eradicated. Because nasopharyngeal colonization is a prerequisite for clinical infection and because this group constitutes the main reservoir of the bacterium, the spectrum of serotypes causing disease in both children and adults also changes as a result. This process, termed serotype replacement, have important consequences for disease incidences in different age groups and antimicrobial resistance rates. Which serotypes emerge as the most clinically important replacement strain vary considerably between settings and age groups even if certain serotypes are recurrent, such as serotype 3 in adults and serotypes 12F and 22F in young children [204].

Serotype replacement has been extensively studied through carriage studies in children and IPD in all age groups. Among children, the increasing incidences of non-PCV serotype IPD only somewhat offset the positive impact of the vaccines on all-cause disease thanks to the low invasiveness of most replacement serotypes in children [195-198]. More invasive serotypes may although emerge, as was demonstrated by the increase of PCV13 serotype 19A IPD in the United States after PCV7 introduction and, more recently, in Belgium after reversion to PCV10 from PCV13 in the paediatric NIP [198, 204]. Non-PCV13 replacement serotypes with moderate to high invasive potential in children include serotypes 8, 12F, 22F, 24F, and 33F [117, 119, 205]. In the elderly and individuals with predisposing illnesses more non-PCV serotypes however cause IPD to a higher degree, resulting in the observed limited PCV herd effects in these groups [121, 195, 197, 202].

Overall, antimicrobial resistance to several drugs has declined slightly in pneumococci as a result of PCV use [150]. Several replacement serotypes that exhibit high frequencies of PNSP and MDR have although emerged. In some cases, these strains belong to the same GPSCs or CCs as well-known resistant vaccine-type clones and have been able to expand when an ecological niche is made available by PCV use [125].

Evaluation of vaccination programmes

Because the spectrum of circulating serotypes differs between settings prior to the introduction of PCVs in paediatric NIPs and the relative proportions of specific replacement strains vary, local monitoring of vaccine effects is required to evaluate the effects on pneumococcal epidemiology [204]. The WHO recommends that this is done through active surveillance of IPD and with nasopharyngeal carriage studies [188]. While national surveillance systems for IPD exist in most high incomes settings, this is not the case in many low- and middle-income countries (LMICs)

where only carriage studies may instead be used [206]. However, carriage studies only in young children is not optimal for predicting the most clinically relevant serotypes for older adults in the population [82].

Hyporesponsiveness after immunization

In addition to the poor immune response elicited by PPV in children <2 years of age, insufficient responses after pneumococcal vaccination have been noted in several other situations. Hyporesponsiveness is defined as the inability to raise a greater, or at least equal, antibody response against a vaccine antigen after booster immunization compared to the primary dose, reflecting development of immune tolerance. However, the term is also used regarding absent responses upon primary immunization [207]. For pneumococci, this reaction is serotype-specific has been detected with both quantitative measurement of IgG and functional methods such as OPA. Well-studied examples of hyporesponsiveness may occur after repeated vaccination with PPV or after a PCV administration in individuals primed with PPV, however the immunotolerant state seems to resolve over time [208-210]. Other clinical situations associated with an absent or diminished response against the corresponding serotype are immunization after or during colonization, or following IPD [211-216]. Interestingly, in a recent study of infants receiving PCV7 or PCV13, a lower immune response was observed towards serotypes 6A, 6B, 18C, and 19F when the serotype was simultaneously carried, compared with other serotypes, indicating serotype-specific differences [214]. Hyporesponsiveness towards a Hib polysaccharide vaccine has also been observed in children following infection with the bacterium [217], and towards *Neisseria meningitidis* serogroup C upon repeated immunization with meningococcal polysaccharide vaccines [218].

In this context, *S. pneumoniae* serotype 3 is of particular concern. Clinical trials with PCV13 [219], and the preliminary PCV11 formulation that later resulted in the licenced PCV10 after exclusion of serotype 3 [220], demonstrated hyporesponsiveness against this serotype after revaccination. These findings probably explain the fact that immunization with PCV13 does not reduce the risk for colonization with this specific serotype, possibly contributing to reduced herd effects [201, 221]. A recent meta-analysis of surveillance data although indicated that a degree of herd protection against serotype 3 is actually established in regions where PCV13 is used [222]. Additionally, the anti-CPS IgG concentration estimated to correlate with protection against serotype 3 is substantially higher than other serotypes [47]. Both positive and nonsignificant vaccine efficacies of PCV13 for serotype 3 IPD in children have been reported in randomized controlled trials, but the baseline incidences for this serotype have been low in many cases and observational studies indicate a protective direct effect [194, 223]. Furthermore, observations of vaccine breakthrough infections are very scarce, indicating that infants vaccinated with PCV13 are although protected against the serotype [123, 224].

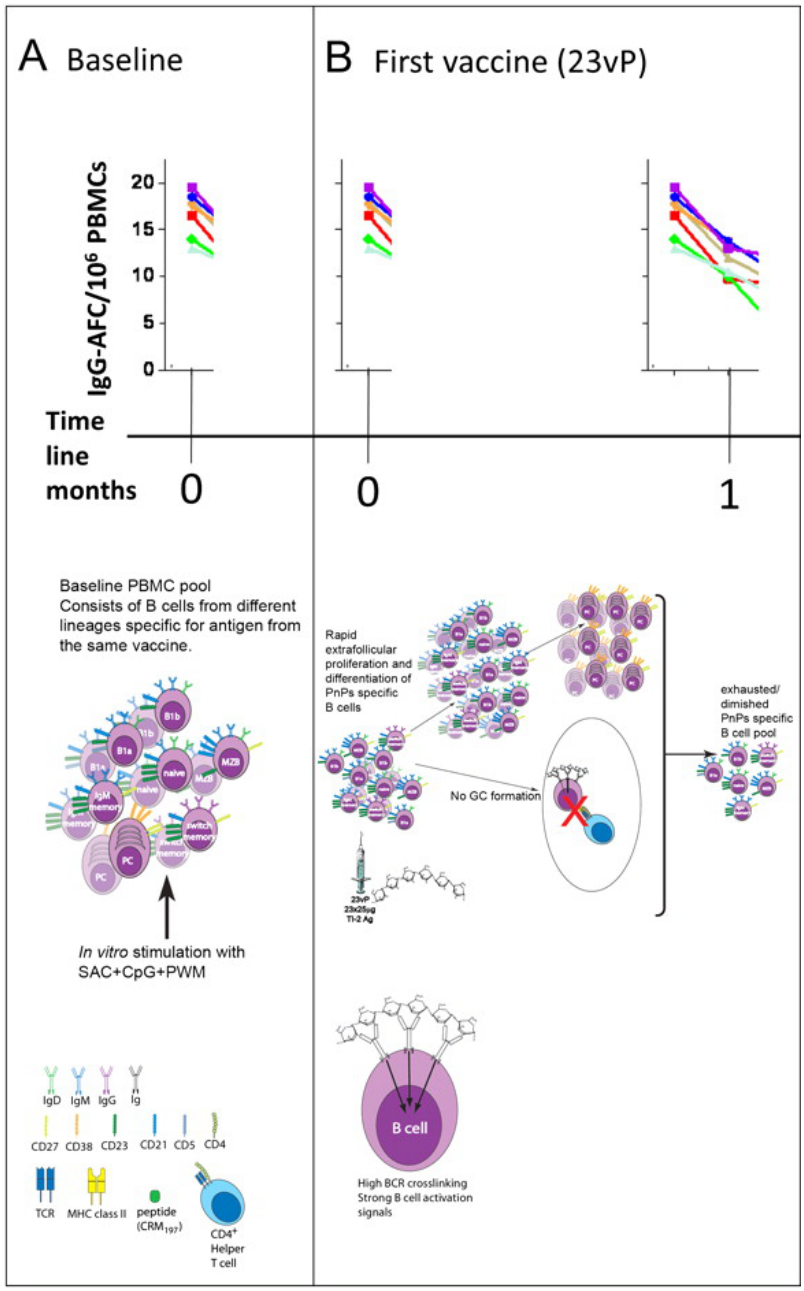
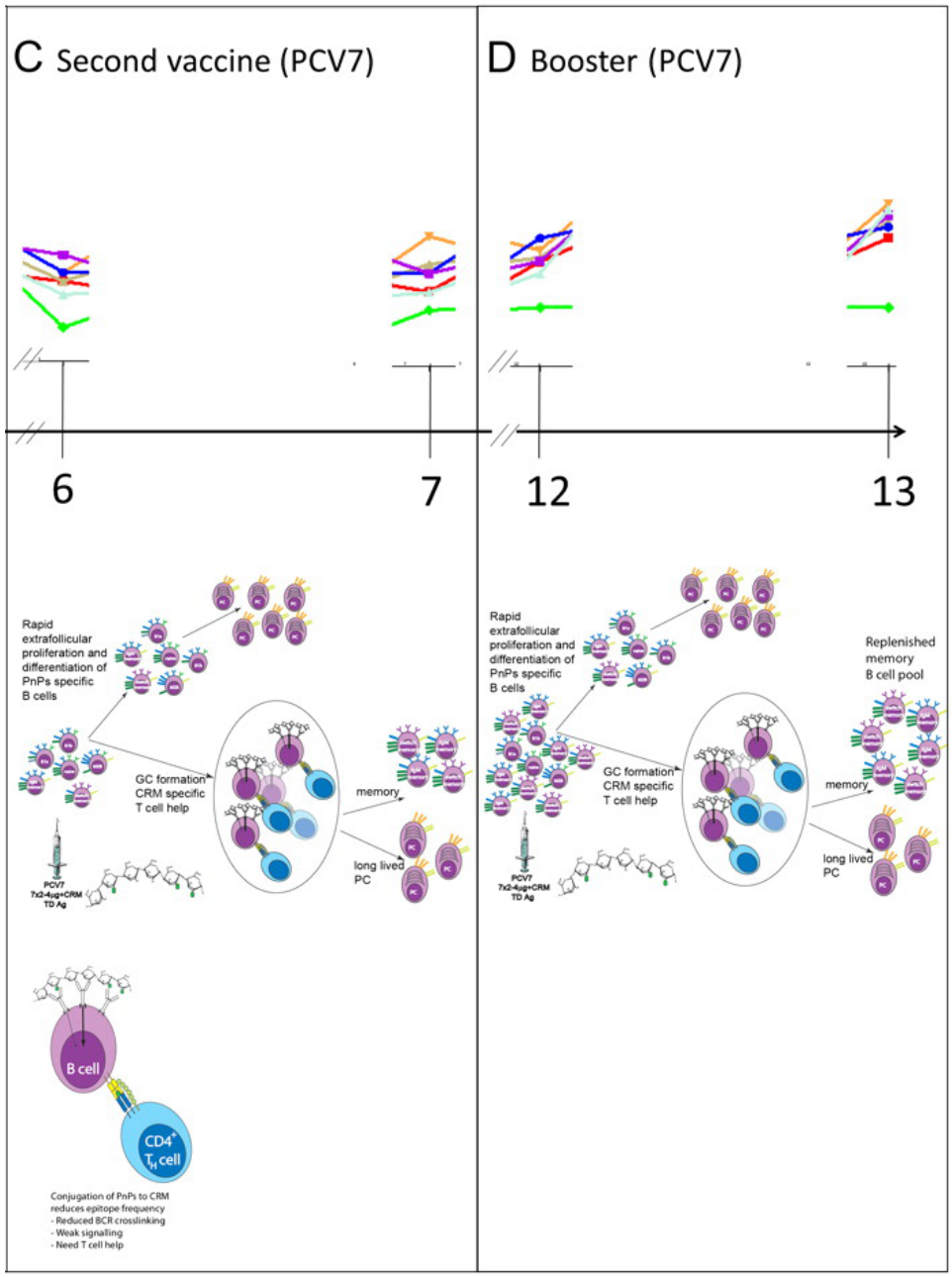


Figure 9. A model of the mechanism explaining PPV23-induced hyporesponsiveness towards PCV.

Before immunization with pneumococcal vaccine (A) a naïve B-cell pool detected in peripheral blood encompass cells specific for different epitopes of the vaccine antigens. Upon immunization with pneumococcal polysaccharide vaccine (B) rapid T-cell independent terminal differentiation of B-cells to antibody secreting cells occur without formation germinal centers or memory B-cell induction. As a result, the pool of B-cells that are responsive to pneumococcal CPSs diminish (represented by the line graph in the top panel). When the conjugate vaccine is administered 6 months after



the polysaccharide vaccine (C), memory B-cells are formed by the T-dependent repsonse but the responsive B-cell pool is lower than baseline. An additional booster of the conjugate vaccine (D) however leads to a replenished pool of responsive cells consisting of memory B-cells. PCV7, 7-valent pneumococcal conjugate vaccine; PBMC, peripheral blood mononuclear cell; 23vP, 23-valent pneumococcal polysaccharide vaccine; Figure reproduced from Clutterbuck *et al.* 2012 [177], with permission of the Infectious Diseases Society of America.

The underlying immunological mechanisms for hyporesponsiveness in different situations are not fully understood. For PPV induced hyporesponsiveness, depletion of the B-cell pool by the T-independent response caused by polysaccharide antigens is probably involved, leading to a lesser response upon a following immunization (*Figure 9*). Specifically, reductions of circulating B1- and marginal zone B-cells, and IgM^+ as well as switched memory B-cell populations appear to be involved [177, 185]. It is hypothesized that the systemic load of CPS during IPD, or systemically absorbed capsular antigens from colonizing strains, may similarly induce immune tolerance by exhausting the B-cell pool that would otherwise be activated by subsequent PCV immunization [214]. Lastly, the clinical importance of vaccine hyporesponsiveness to different serotypes are not known. Due to the polyvalent nature of the vaccines, the serotype-specificity of hyporesponsiveness, and herd effects in the population, a possibly sustained susceptibility to infection by a specific serotype may comprise only a minimal overall risk increase for the individual.

Serotype-independent vaccines

To counter the problem with serotype replacement, a serotype-independent vaccine providing protection against all strains of *S. pneumoniae* would be optimal. Different formulations have been proposed through the years based on various pneumococcal proteins such as PspA, PspC/CbpA, Ply, PhtD, in addition to pilus subunits. Whole-cell vaccines have also been explored. Several Phase 1 studies have been conducted and protein combination vaccines including Ply and PhtD, and a whole cell vaccine have proceeded to Phase 2 trials. The candidates have generally proven safe and immunogenic, but no evident advantages over PCVs have been demonstrated to date [225]. Regarding PspA, concerns have been raised about possible induction of autoantibodies due to the homology of certain regions of the molecule to human cardiac myosin [226].

Epidemiology in the conjugate vaccine era

Global disease burden

Infections caused by *S. pneumoniae* remain a major health concern globally, causing extensive morbidity and mortality predominantly among children <5 years of age and the elderly, even though the widespread adoption of PCVs in paediatric NIPs has led to substantial reductions. The distribution of severe infections and deaths is skewed, with higher rates in LMICs compared to high income settings, and this difference is most pronounced in children (*Figure 10*) [107, 227].

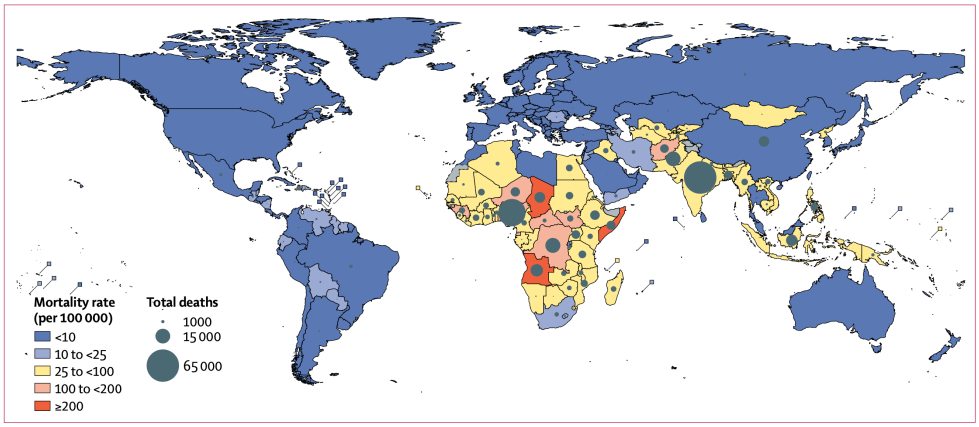


Figure 10. Global mortality rates and total numbers of deaths caused by pneumoccocal infections in children <5 years of age in 2015.
In 2015, a total of 318,000 deaths among children aged <5 years were estimate to be caused by *S. pneumoniae*. Most fatal cases and the highest deaths rates occurred in LMICs. Figure reproduced from Wahl *et al*. 2018 [107], licenced under CC BY 4.0 (https://creativecommons.org/licenses/by/4.0/).

According to the Global Burden of Disease Study (GBD) 2019, lower respiratory tract infections (*i.e.*, pneumonia and bronchiolitis) caused 2.5 million deaths in 2019, representing the second most common cause of death among children up to 9 years of age, surpassed only by neonatal disorders, and the sixth most common among elderly adults. The total number of fatal cases were 672,000 and 1,280,000 in children <5 years and adults ≥70 years, respectively [227, 228]. Different etiologies were not presented in this report, but in an analysis for the GBD 2016 *S. pneumoniae* was estimated to cause 46-52% of lower respiratory tract infection deaths in all age groups, corresponding to 341,000 cases in children <5 years and 494,000 in individuals >70 years, which is consistent with the numbers for 2019 [126]. Similar numbers were reported from another estimation of the global pneumococcal CAP and IPD disease burden among children aged 1-59 months in 2015. This study attributed a total of 318,000 deaths to the bacterium in this age group, corresponding to a mortality rate of 45/100,000 children, ranging between extremes from 8-9/100,000 in the Americas and Europe to 92/100,000 in Africa (*Figure 10*) [107]. Even greater differences were found regarding lower respiratory tract infection deaths in the GBD 2019, with approximately 3/100,000 deaths among children in high income settings compared with 220/100,000 in sub-Saharan Africa [227].

During the last three decades, the mortality rates caused by pneumococci, and lower respiratory tract infections overall, in young children have approximately been halved. In contrast, the rate has only slightly declined in elderly adults, and the total number and fatal cases in this group has increased due to ageing populations [107, 227]. On a global scale, reductions of childhood wasting and household air pollution have had the largest impact on pneumococcal mortality rates in young

children during these years, followed by PCV use. However, the attributable fractions differed greatly between settings and PCV vaccinations were the most important factor in high-income countries. As of 2019, childhood wasting remains the single most important risk factor responsible for respiratory tract infection deaths [126, 227]. Further increasing PCV coverage is also of great importance as only 48% of eligible children were estimated to have received all three doses of the vaccine in 2019 [229].

In addition to the extensive disease burden caused by pneumococcal CAP and IPD, the species is a major etiology of AOM, one of the most common infections affecting children worldwide. Although mortality due to AOM is uncommon, the diagnosis contributes to considerable suffering, antibiotic consumption, and health care costs [230]. In 2005, the global AOM incidence was estimated at 10/100 individuals. Half of cases occurring in children <5 years and the incidence in this age group ranged from 25-30/100 in parts of Europe and the Americas to 100-150/100 in sub-Saharan Africa (*i.e.*, more than one episode/child per year) [231]. In countries where PCVs are used in the paediatric NIP, the proportion of AOM caused by pneumococci has decreased and observational studies have indicated a reduction of the total AOM incidence [232-234].

Pneumococcal disease in Sweden

In 2009, PCV7 was introduced in the paediatric NIP of Sweden, although some counties begun using the vaccine a few years earlier. In 2010, this vaccine was replaced by either PCV10 or PCV13 depending on county until 2019 when PCV10 was adopted nationally. Since 2004, all cases of IPD must be reported to the relevant authorities, currently the Public Health Agency of Sweden, and the corresponding pneumococcal isolates are referred for serotyping. For this reason, it is possible to evaluate vaccine effects on IPD thoroughly in the country.

A study comparing counties using either PCV10 or PCV13 report a total IPD incidence of 15.9/100,000 in 2007, which decreased by 18% to 13.0/100,000 in 2013-2016 [197]. The most pronounced decrease occurred in children <5 years of age, from 13.9/100,000 to 5.1/100,000, with the highest numbers and sharpest declines among those <2 years. Conversely, herd effects have only had little impact on the incidence among adults ≥65 years. No significant differences were detected regarding overall IPD incidences in any age group between counties using PCV10 or PCV13. The age group-specific incidences reported by the Public Health Agency during the years around PCV introduction are shown in *Figure 11A* [235]. Interestingly, the incidence of IPD among adults was reduced by approximately half in 2020 and 2021 due to containment policies related to the Covid-19 pandemic, a pattern that was seen in many countries (*Figure 11B*) [236]. The corresponding reduction in 2020 among children was not as substantial, and the 2021 incidence among those <2 years was virtually equal to that in 2019 [235].

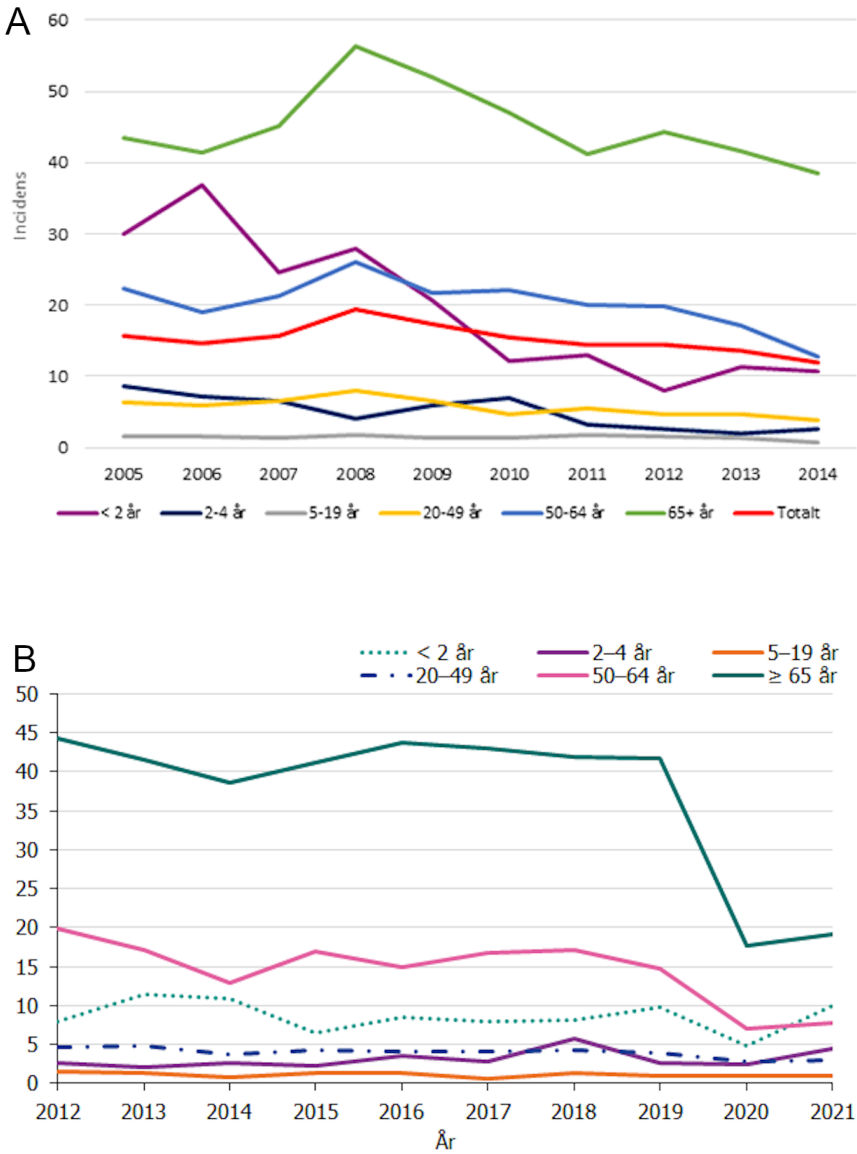


Figure 11. Age group-specific incidences of IPD in Sweden 2005-2014.
Incidence rates (cases/100,000 individuals) of IPD cases reported to the Public Health Agency of Sweden in different age groups and in total during 2005-2014 (A) and 2012-2021 (B). PCV7 was introduced successively during 2007-2009 and replaced by PCV10 and PCV13, depending on county, in 2010. After vaccine introduction, the incidence declined in children <5 years of age. In 2020, the first year of the Covid-19 pandemic, IPD incidences declined sharply. Graphs reproduced from the Public Health Agency of Sweden [235].

The serotype distribution causing IPD in Sweden has shifted considerably since the introduction of the different PCVs. Presently, all serotypes covered by PCV10, plus serotype 6A, are uncommon causes in all age groups [235]. In the previously mentioned study based on national data, serotype 3 was infrequently detected in children during the whole time-period but increased among older adults after commencement of vaccinations. Likewise, serotype 19A has become increasingly important in the older age groups, while the increase of the serotype that occurred among children <5 years in counties using PCV10 did not result in a significant difference compared to those using PCV13 [197]. Emerging serotypes that have become frequent causes of IPD in Sweden are 6C, 8, 9N 15A, and 22F [235]. Importantly, many of the replacement non-vaccine serotypes that have increased in IPD are associated with infections in individuals with chronic medical or immunocompromising conditions [121, 122].

In contrast to IPD, no exact data on pneumococcal CAP or AOM is available as the etiology of these infections is most often unknown. The results of a population-wide register study based on ICD10 diagnosis codes and an observational study on hospital admissions of children although indicate that the incidence of pneumococcal CAP has decreased among children, while no clear herd effects were seen. Among adults with comorbidities the incidence even increased [237, 238]. Hospitalizations for sinusitis and AOM-related diagnoses in children also decreased after PCV introduction [233, 234, 238]. Serotype replacement in carriage has been studied in Stockholm County [135, 239].

Pneumococcal disease in Angola

In similar with many other countries in sub-Saharan Africa, Angola is affected by a high pneumococcal disease burden, but no comprehensive monitoring system for pneumococcal disease is yet established. Although considerable improvements have occurred during the last 20 years, the country had a high overall under 5 mortality rate at 71/1,000 live births in 2020 [240]. PCV13 was introduced in the national paediatric NIP in 2013.

In 2015, the mortality rate caused by pneumococcal infections in children under 5 years of age was estimated at 216/100,000 children (*Figure 10*; p. 42) [107]. However, the mortality rate caused by lower respiratory tract infections in this age group in Angola decreased by approximately one third between 2015 and 2019 according to the GBD 2019 [241]. Furthermore, lower respiratory tract infections represented approximately 9% of all deaths among those aged >70 years in 2019, corresponding to a rate of 705/100,000 individuals [241]. Studies of bacterial meningitis in the Angolan capital Luanda demonstrated that *S. pneumoniae* has become most common etiology in children with the disease since vaccination against Hib commenced in 2006 [242], and that PCV13 serotypes caused approximately 50% of pneumococcal meningitis in children aged 3 months-15 years in 2016-2017 [243]. Before PCV introduction, serogroups 6, 23, and 19, and

serotype 1 were the most frequently detected strains, based on a very limited number of pneumococcal isolates from meningitis (*n*=45) [242]. To the author's best knowledge, no additional previous reports on the serotype distribution of pneumococci detected in carriage or disease in Angola exist. Lastly, Angola is one of the most heavily affected countries by AOM worldwide [231].

Chronic suppurative otitis media (CSOM)

Despite not being caused directly by *S. pneumoniae*, development of CSOM is usually initiated by an episode of AOM and is thus often a complication of pneumococcal disease. The condition is defined as a chronic state of middle ear inflammation, perforation of the tympanic membrane, and otorrhoea (*Figure 12*). According to the WHO definition, 2 weeks symptom duration is enough to diagnose CSOM, but many consider persistence for at least 6 weeks as a requirement. Symptoms may resolve spontaneously but frequently recur, resulting in "active" and "inactive" phases of the disease. Even when the disease resolves completely, chronic hearing impairment may often persist due to imperfect healing of the tympanic membrane and damage to the auditory organs. Debut often occurs before 5 years of age and, in addition to causing discomfort and psychosocial suffering, the high rate of hearing impairment has a serious detrimental effect on childhood development. Although rare, serious complications of CSOM include brain abscess and bacterial meningitis which are the main contributors to fatal cases of CSOM [244-246].

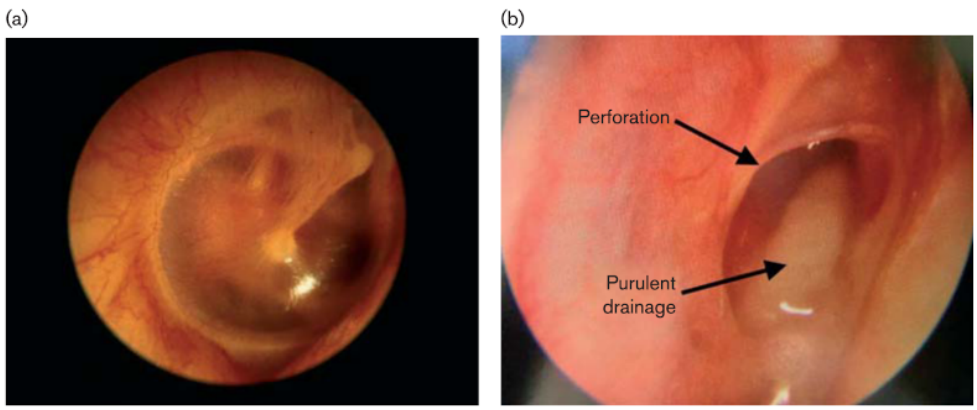


Figure 12. Otoscopic appearance of CSOM.
Pictures from otoscopy of a healthy tympanic membrane (a) and of a CSOM affected ear (b) with visible tympanic membrane perforation and otorrhoea. Figure reproduced Mittal *et al*. 2015 [246] with permission conveyed through Copyright Clearance Center, Inc.

Pathogenesis

Chronic suppurative otitis media is most often developed as a result of inadequately treated AOM with spontaneous rupture of the tympanic membrane. It may however also occur in patients with a tympanostomy tube or after traumatic perforation. The process starts with invasion of bacteria through the auditory canal that generally replace the pathogen that caused the acute infection of the middle ear [245, 246]. Reflux of pathogens from the nasopharynx due to perforation-induced Eustachian tube dysfunction has also been proposed [247]. Colonization with biofilm formation is established and often comprises multiple bacterial species, sustaining chronic inflammation and causing tissue damage [246, 248, 249]. Higher levels of pro-inflammatory cytokines but lower expression of TLRs in the middle ear of CSOM patients compared to those with other forms of OM was detected in one study [250]. This finding may reflect an inefficient host response, explaining the inability to clear the infection.

Microbiology

In contrast to AOM, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* are seldomly detected in cultures from the CSOM affected middle ear. Instead, *S. aureus* and *Pseudomonas aeruginosa* are often reported as the two most common species, but Gram-negative rods of the *Proteus* and *Klebsiella* genera are also frequent findings [246]. In sub-Saharan Africa, including reports from Angola, *Proteus* spp. are often found to be the most or second most frequently detected bacteria, and *S. aureus* is less common compared studies performed in the middle east and Asia [251]. Additionally, anaerobic species such as *Peptostreptococcus*, *Fusobacterium*, *Prevotella*, and *Porphyromonas* spp. are often isolated in studies that apply sufficient methods [252]. Fungi may also be involved, and are often found during ongoing antibacterial treatment [253].

Treatment

The different treatment strategies have been insufficiently studied in clinical trials and are mostly based upon clinical experience. Aural toileting (*i.e.*, dry mopping through the auditory canal), topical antibiotic treatment with chloramphenicol, colistin, quinolones or aminoglycosides, and use of topical antiseptics such as boric acid, iodine, aluminium acetate, or acetic acid are the most used treatments. Topical treatment is often prescribed in combination with aural toileting [254, 255]. Relapse is common due to antimicrobial resistance and persisting biofilms that promote bacterial survival [256]. Furthermore, potential ototoxicity of topical aminoglycosides and antiseptics may be an issue [246, 254]. A recent Cochrane review concluded that topical treatment with quinolones is probably more efficient than boric acid, but the data was insufficient for additional conclusions regarding other substances [257]. Systemic antibiotic treatment is considered a second line treatment, but it is unclear if sufficient tissue concentrations are achieved in the

middle ear and there is no good evidence to support its use [258]. In high income settings, surgery is performed in a high number of cases, resulting in lower recurrence rates than topical antibiotics [259].

Disease burden

Being one of the most prevalent infectious diseases globally, CSOM affects a vast number of individuals. Monasta *et al*. [231] estimated that a total of 31 million new cases occurred in 2005, with the highest incidence rates in Oceania (9.4/1,000 individuals) and central sub-Saharan Africa (7.6/1,000) individuals. Almost one in 4 cases were children younger than 5 years. Specific groups that are affected by the highest CSOM disease burden are the Inuit populations of North America and Greenland, and Australian Aborigines [260-262]. Socioeconomic factors, limited access to healthcare, and genetic predisposition are thought to contribute to these observations [261]. A recent literature review of CSOM among children <16 years in sub-Saharan Africa reported prevalence estimates between 1.5-7% in HIV-noninfected children [251]. In Luanda, 27% of HIV-positive children aged 0-15 years compared with 4% of aged-matched controls were diagnosed with CSOM in a study performed in 2008 [263].

Aims of the present investigation

The general aims of this thesis are to improve the understanding of the adaptive immune response to pneumococcal infection, to explore the epidemiology of *S. pneumoniae* in Skåne County, Sweden in relation to the use of PCV, and to characterize respiratory tract pathogens in addition to bacteria associated with CSOM in Angola.

Specific objectives addressed in the included papers are:

- To assess the serotype-specific opsonic antibody response that is induced by pneumococcal CAP and IPD in relation to various background factors (Paper I and II).
- To evaluate the association between anti-capsular antibody concentrations and opsonic activity in the setting of clinical pneumococcal infection (Paper I and II).
- To describe the serotype distribution, antimicrobial resistance rates, and invasiveness of *S. pneumoniae* detected in clinical respiratory tract samples after introduction of PCV13 in Skåne County (Paper III).
- To examine the molecular epidemiology, resistance mechanisms and virulence factors of XDR pneumococci detected in Skåne County (Paper IV).
- To determine nasopharyngeal carriage rate, serotypes, and antimicrobial susceptibility patterns of *S. pneumoniae* among children in Angola in the early PCV era, and to investigate the molecular epidemiology and resistance mechanisms of isolated MDR pneumococci. Finally, to characterize co-colonizing strains of *H. influenzae*, and *M. catarrhalis* (Paper V).
- To describe bacterial species and antimicrobial resistance patterns associated with CSOM in Angola (Paper VI).

Materials and methods

Definitions

Sepsis

In Paper I, the Sepsis-2 definitions for sepsis, severe sepsis, and septic shock, based on systemic inflammatory response criteria, were used in the data analysis. Conversely, the Sepsis-3 definition that is based on sequential organ failure assessment scores were applied in Paper II [264].

Pneumococcal CAP

In Paper I and II, pneumococcal CAP was defined as an acute onset of illness with radiologic signs of pulmonic consolidation; growth of *S. pneumoniae* in a culture sample from the nasopharynx, sputum, or blood; and ≥2 of the following criteria: fever of 38°C, dyspnea, cough, pleuritic chest pain, or abnormal lung auscultation.

Serotype invasiveness

A meta-analysis by Brueggeman *et al*. [265] was used to classify pneumococcal serotypes as having low (3, 19F, and 23F) or high (1, 4, 7F, and 14) invasive potential in the analyses of Paper I, based on the pooled estimate ORs comparing each serotype's frequency in carriage and IPD, respectively.

Multi-drug resistant pneumococci

Pneumococci were defined as putatively MDR or XDR if screening with disk diffusion tests indicated non-susceptibility to ≥3 or ≥5 of the following 6 antibiotic classes, respectively: beta-lactams, macrolides, lincosamides, tetracyclines, fluoroquinolones, and folate metabolism inhibitors (*i.e.*, trimethoprim and sulfamethoxazole) [142]. Definitive MDR or XDR phenotypes were confirmed after MIC determination with broth microdilution. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints were applied [266].

Study outline, populations, and data collection

Paper I and II

The rationale of these investigations was to assess the adaptive anti-pneumococcal immune response by examining changes in opsonic activity against the infecting serotype that occurred after an episode of pneumococcal infection. Paired acute-phase/pre-infection and convalescence sera were examined with an OPA, measuring the capacity of the present antibodies to induce phagocytosis of pneumococci. The observed change between the two time points was classified as either a functional or non-functional response. Associations of these outcomes with anti-capsular antibody concentrations and background factors were analysed.

A total of 80 patients with pneumococcal CAP and/or IPD were enrolled retrospectively in two, partially overlapping, studies. One assessing the immune response of IPD patients (n=40) with regard to different infecting serotypes (Paper I), and the other concerning patients (n=54) with invasive or non-invasive pneumococcal CAP (Paper II). The 54 participants that are described in Paper II were adult patients with radiologically confirmed pneumococcal CAP from a previously described cohort at Örebro University Hospital in 1999-2002 [166, 267]. The remaining 26 individuals, only included in Paper I, were patients with IPD admitted to Skåne University Hospital in 2006-2017.

Clinical and demographical data was collected from medical records. From 60/80 patients, including all 54 patients from the Örebro University Hospital cohort, acute-phase serum samples were collected within 2 days from hospital admission and convalescence samples approximately 1-3 months thereafter (range 20-82 days). From the remaining 20 IPD patients, pre-infection sera were collected 2-24 months before admission and convalescence samples 6-60 months after the infectious episode. OPA was performed with all sera to determine OPA titres, and serotype-specific anti-CPS IgG concentrations were measured with an ELISA in Paper I. In Paper II, data from a previous study regarding serotype-specific anti-CPS Ig (not specifically IgG) concentrations was used. Furthermore, data on the concentration of pneumococcal DNA in plasma were available for 25 of the patients [166]. All acute-phase/pre-infection sera were screened for total IgG and IgG2 deficiencies.

Paper III and IV

A description of pneumococci associated with respiratory tract infections was provided by characterizing strains detected in clinical respiratory tract cultures 2-4 years after introduction of PCV13 in Skåne County. The serotype distribution was assessed in relation to the use of PCV10 and PCV13 before and during the study period, respectively, by comparing the results with a previously studied period (2011-2013) [268]. Additionally, antimicrobial susceptibility patterns of different serotypes; clinical factors associated with PCV serotypes; and molecular

epidemiology, resistance mechanisms, and virulence factors of XDR pneumococci were investigated.

Skåne County is the southernmost and third most populous county of Sweden with 1,349,000 inhabitants at the end of the study period [269]. Infants were immunized with PCV10 in the county from May 2010 until May 2014, when it was replaced by PCV13. The uptake of PCV has been approximately 97-98% among eligible children born in 2009 and later in Skåne County [270]. Pneumococci isolated between October 2016 and March 2018 from upper and lower respiratory tract samples, including the middle ear and conjunctiva, at the single clinical microbiology laboratory (Laboratory Medicine Skåne, Lund) that processes all microbiological samples from healthcare providers in the study area were included (*n*=2,131). Results from antimicrobial susceptibility testing conducted at the clinical laboratory, and background data was collected, including patient age and sex, sample type, sample date and referring department.

Available pneumococcal isolates (*n*=1,858) corresponding to the included samples were obtained and stored at the research laboratory. Serotyping was performed with PCR, latex agglutination and the Quellung reaction. Age-stratified serotype data on all IPD cases in Skåne County during 2016-2018 was collected from the Public Health Agency of Sweden to enable comparisons between the serotypes' relative frequency among the studied respiratory tract samples and samples from IPD (Paper III).

As described in Paper IV, a minority of isolates (*n*=25), reported as XDR based on the screening at the clinical laboratory, were subjected to additional susceptibility testing with broth microdilution and WGS. Genetic lineages, and the presence of resistance mechanisms and virulence factors were determined. For comparison in the analyses of virulence genes and for construction of phylogenetic trees, additional genomes of pneumococci belonging to GPSC1 (*n*=79), GPSC9 (*n*=50) and GPSC10 (*n*=52) were acquired from the European Nucleotide Archive. These were reference strains previously described by Gladstone *et al*. [69], selected to represent different STs within the GPSCs.

Paper V

To characterize *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* present among children in Angola, a cross-sectional nasopharyngeal carriage study was performed. Describing the detected pneumococcal strains was considered the focus of the study. In addition to carriage rates and antimicrobial resistance patterns, that were determined for all three species, frequencies of *H. influenzae* capsule types and pneumococcal serotypes were assessed. Furthermore, all isolated MDR pneumococci were subjected to WGS to investigate the molecular epidemiology and resistance mechanisms.

Enrolment of children aged 4-12 years (*n*=940) was carried out in the community of the Angolan capital Luanda (*n*=654), at outpatient departments at Hospital Josina Machel and Hospital Pediátrico David Bernardino in Luanda (*n*=126), and in

villages near the province capital Saurimo of Lunda Sul (*n*=160) in November and December 2017. PCV13 was introduced in the paediatric NIP in 2013 and the Hib vaccine in 2006, both administered according to a 3+0 schedule at 2, 4, and 6 months of age. According to official data from the Angolan Ministry of Health regarding 2017 (personal communication), the 3rd dose-coverage among eligible children was 67% for PCV13 and 65% for the Hib vaccine in Luanda, compared with 84% and 83%, respectively, in Lunda Sul. The corresponding national numbers reported by the WHO were, however, lower (52% for both vaccines) [271].

Children accompanied by their parents or guardians were approached by personnel from the otorhinolaryngology department of Hospital Josina Machel and asked to participate in the study at sampling stations set up outdoors or at outpatient departments. Oral consent to participate were given by the parents or guardians. Nasopharyngeal swabs were collected and transported in skimmed milk-tryptone-glucose-glycerine (STGG) medium [79] on ice before freezing at -80 °C at the National Institute for Health Research, Luanda. The collected samples were then transported in cooler-bags with ice blocks to Malmö, Sweden where culturing, species identification and further microbiological analyses were performed. Background information was gathered regarding demographics, medical background, and living conditions according to a pre-printed form.

Paper VI

To investigate the spectrum of bacteria associated with CSOM in Angola and corresponding antimicrobial susceptibility patterns, a cross-sectional study was performed during January to December 2016. To thoroughly assess which pathogens were present in the infected middle ear and in the nasopharynx, samples were analysed to identify the species of all different colonies that could be discerned after culturing.

Patients of all ages (*n*=152) with anamnesis of otorrhoea lasting more than 2 weeks, attending the otorhinolaryngology department of Hospital Josina Machel, Luanda, or healthcare centres in the provinces Lunda Sul, Namibe and Zaire were offered to be enrolled in the study. Examination with otoscopy was subsequently performed and patients were included if tympanic membrane perforation was confirmed. Patients, or their parents or guardians, provided informed consent orally. Examination with otoscopy and microbiological sampling from middle ear discharge and the nasopharynx was performed by otorhinolaryngology specialists using standard techniques. Flocked swabs were used to collect samples of middle ear discharge, after cleaning of the ear canal with 70% ethanol. Samples were collected in STGG medium [79] and were subsequently stored at -80 °C at the National Institute for Health Research, Luanda, before transportation to Malmö, where culturing, species identification and further microbiological analyses were performed. Patient interviews in Portuguese regarding demographical and clinical information according to a questionnaire were conducted by study doctors at the different study sites.

Laboratory methods

Opsonophagocytic assay

To assess the opsonic antibody activity of sera a single-serotype OPA was performed. The method was based on a Centers for Disease Control and Prevention protocol developed by Romero-Steiner *et al*. [272], available from the WHO Bacterial Respiratory Tract Reference Laboratory at the University of Alabama, Birmingham [273]. Some modifications of the method were done according to a more recent multiplexed OPA protocol from the reference laboratory [274].

Briefly, a serial dilution of heat-inactivated serum was added in duplicates to a microtiter plate together a fixed amount of pneumococci of the corresponding infecting serotype (*Figure 13*). After incubation, human phagocytic cells and complement were added, followed by additional incubation to facilitate phagocytosis. Finally, the contents of each well were cultured overnight on blood agar plates to assess bacterial survival. Negative control reactions, without addition of serum were included on each microtiter plate, as well as a serial dilution of a positive control serum. The OPA titre of a serum was defined as the inverse of the lowest serum dilution that caused >50% killing of bacteria compared to the negative controls. The OPA titres of the paired sera from each patient were then compared to determine if the opsonic antibody activity was either increased, unchanged or decreased after the infectious episode.

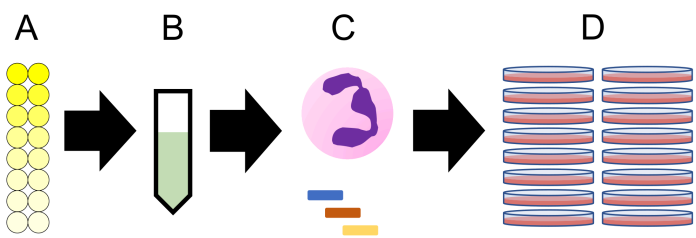


Figure 13. Outline of opsonophagocytic assay (OPA).
A single serotype OPA was performed with paired sera from patients with pneumococcal infections. Initially, heat-inactivated serum was added to a microtiter plate in a dilution series in duplicates (A). A fixed amount of target strain pneumococci of the infecting serotype was then added to each well, followed by incubation to allow opsonization to occur (B). Thereafter, activated phagocytes and complement was added, and samples were incubated to facilitate phagocytosis of bacteria (C). Finally, equal volumes of the reaction mix in each well were transferred to blood agar plates, bacteria were grown overnight and colony forming units were counted (D). OPA titres were determined by comparing bacterial killing induced by patient sera as compared to a negative control with no serum added to the reaction.

Residual antibiotic activity in patient sera, which could theoretically generate a false positive result in OPA, was excluded by agar susceptibility testing based on a previously described method [275]. Briefly, acute-phase serum samples were added to 2 mm holes in blood agar plates inoculated with the *S. pneumoniae* target strain of the corresponding infecting serotype which were then cultured overnight. The

complete absence of a zone of growth inhibition excluded significant antimicrobial activity in the tested serum.

ELISA for determination of Ig concentrations in serum

In Paper I, a serotype-specific ELISA was used to determine absolute concentrations of anti-capsular IgG based on a WHO reference laboratory protocol [276]. The WHO international standard anti-pneumococcal capsule serum 007sp was used as reference serum. The serotype-specific anti-capsular Ig concentrations that were used in the analyses in Paper II were measured in a previous study [166]. This was done according to a previous WHO protocol measuring the antibody concentration in arbitrary units (AU) compared to a reference serum [277, 278]. To exclude total IgG or IgG2 deficiencies as confounding factors, a sandwich ELISA was performed with all studied acute-phase/pre-infection sera and calibration sera with known concentrations for human IgG and IgG2, respectively.

PCR for determination of pneumococcal DNA in plasma

Concentrations of pneumococcal DNA in acute-phase plasma samples were measured with a real-time PCR in a previous study and were available for 25 of the patients included in Paper II [166]. DNA was extracted from samples with an automatic NucliSENS easyMAG instrument (bioMérieux. Marcy-l'Étoile, France) and *Spn9802* DNA was measured according to a previously described method [279].

Identification of bacterial species

Clinical isolates of *S. pneumoniae* described in Paper III and IV were identified at the clinical laboratory based on typical colony morphology with α-hemolysis and optochin sensitivity, positive latex agglutination test or, in case of optochin resistance, positive PCR for *LytA*. In paper V and VI, only colony morphology with α-hemolysis and optochin sensitivity was used as criteria to confirm pneumococcal species. Identification of other bacterial species was done by MALDI-TOF MS using a microflex MALDI-TOF instrument with flexControl software (Bruker Daltonics, Bremen, Germany) set to default settings. Species identification was thereafter done using the Bruker Daltonic MALDI Biotyper 4.1 software with Bruker taxonomy library (n=6,903).

Serotyping of pneumococci

Pneumococci were serotyped with PCR, latex agglutination testing and the Quellung reaction with Neufeld antisera. All isolates were primarily analysed with a sequential multiplexed PCR assay with 6 reactions, each including primers pairs specific for 5-6 different serotypes or groups of serotypes. Latex agglutination testing and the Quellung reaction were used to further specify the PCR results and to serotype samples run through all PCRs twice without a positive reaction (*Figure 14*). Latex agglutination and the Neufeld method were also used if uncertain results

were yielded with PCR. If a serotyping result from the Quellung reaction for an isolate differed from that in PCR, the serotype assigned with the former method was used in the final results. As indicated in *Figure 14*, certain serogroups were not analysed further to determine the specific type.

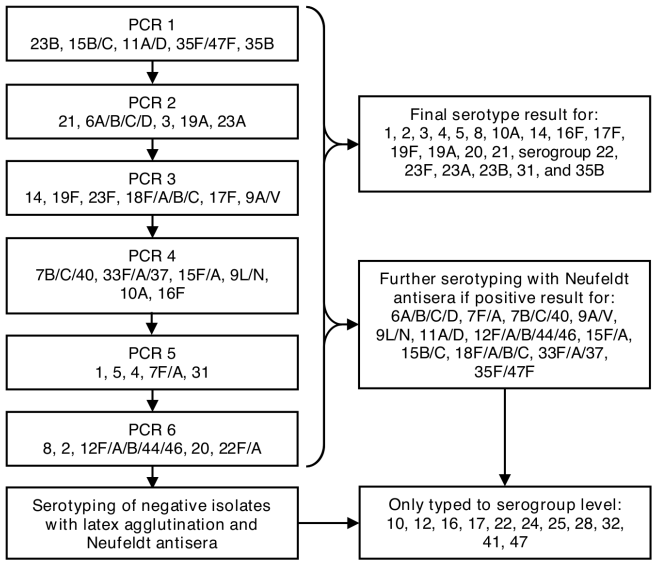


Figure 14. Serotyping scheme for pneumococci.
All available pneumococcal isolates were serotyped according to a multiplexed PCR scheme comprising 6 separate reactions, each with 5-6 primer pairs reacting with capsular gene segments specifc for certain serotypes or groups of serotypes. Serotyping with Neufeld antisera (the Quellung reaction) was performed to specify the PCR results. Isolates that did not react positively with serotype-specific primers in any of the sequential PCRs twice, were serotyped using a latex aggluttination kit and, if needed, Neufeld antisera.

The protocol used for PCR was based on the method previously described by da Gloria Carvalho *et al.* [59] with some modifications. Sequences of the oligonucleotide primers used were available from the Centers for Disease Control and Prevention Streptococcus Laboratory [280]. Primer pairs were selected for the sequential PCRs to attain the highest representation of serotypes previously shown to be common in Sweden in early reactions [135, 239, 268]. For each sequential PCR, isolates representing the included serotypes, previously confirmed with latex agglutination testing or the Quellung reaction, were included in all runs as positive controls (*Figure 15*). Additionally, a primer pair detecting the *S. pneumoniae* capsule locus gene *cpsA* was always included to indicate the presence of DNA from an encapsulated pneumococcus. Isolates that were negative for *cpsA* twice, after repeated DNA isolation, were considered NT pneumococci.

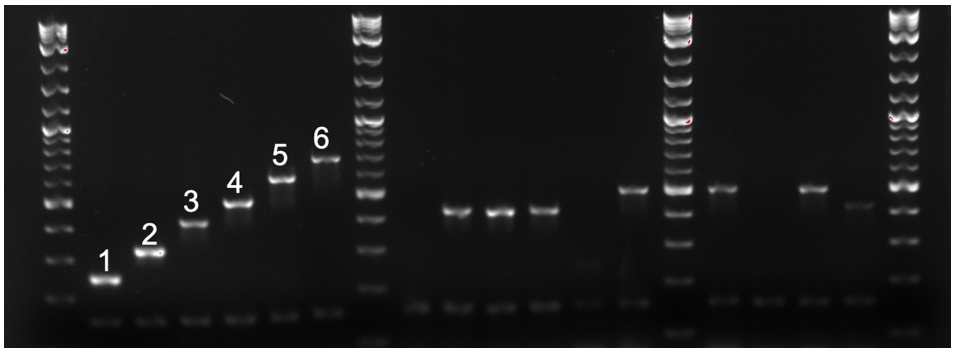


Figure 15. Multiplexed polymerase chain reaction (PCR) for serotyping pneumococci.
An example of serotyping results from the 4th sequential PCR in the serotyping scheme including positive control samples for the six included primer pairs representing serotypes 7B/C/40 (1), 33F/A/37 (2), 15F/A (3), 9L/N (4), 10A (5), and 16F (6), and several positive results. At the bottom, bands representing the positive control capsule locus gene *cpsA* are seen.

Serotyping with latex agglutination was performed with the ImmuLex Pneumotest kit (SSI Diagnostica, Copenhagen, DK) according to the manufacturer's instructions. Isolates that did not produce any positive reaction after testing twice were considered NT. Additional serotyping with the Quellung reaction using Neufeldt antisera (SSI Diagnostica) was done to determine serotypes that could not be distinguished using either the multiplex PCR scheme or latex agglutination.

Capsule typing of H. influenzae

Isolates of *H. influenzae* that were identified in the study described in Paper V were capsule typed with MALDI-TOF MS as previously described by Månsson *et al*. [281, 282] and the results were confirmed with conventional typing with PCR [283-285].

Antimicrobial susceptibility testing

Disk diffusion tests (Thermo Fisher Scientific) were used to screen for antimicrobial non-susceptibility and Etests (BioMérieux) were used to determine MIC values of betalactams. Additional MIC determination with broth microdilution (Sensititre Streptococcus STP6F AST Plate; Thermo Fisher Scientific) was performed with pneumococci analysed in Paper IV and V that were reported as XDR or MDR upon screening, respectively. Additionally, broth microdilution was used for determining colistin MICs for isolates included in Paper VI. All results were interpreted according to the most recent EUCAST clinical breakpoints available at the time of testing [266].

Whole genome sequencing

Pneumococci were sequenced as previously described by Kavalari *et al*. [286]. Genomic DNA extraction was done using the DNeasy Blood & Tissue Kit

(QIAGEN, Hilden, Germany) whereafter fragment libraries were constructed using the Nextera XT Kit (Illumina, Little Chesterford, UK). Finally, 250-bp paired-end sequencing was performed with a MiSeq system (Illumina) according to the manufacturer's instructions.

Bioinformatic analyses

Genome assembly of the paired-end Illumina data was performed using the SKESA assembler [287]. Uploading of genome data to Pathogenwatch [70] was done to detect specific antimicrobial resistance genes and mutations, determine PBP profiles, and assign STs and GPSCs [69, 125, 143]. Clonal complexes of the detected sequence types were acquired using a 6/7 allele threshold from the Global Pneumococcal Sequencing Project database [71] and PubMLST [67]. Virulence genes were detected by using the Basic Local Alignment Search Tool in NCBI Genome Workbench version 3.6.0. Phylogenetic trees were constructed with isolates belonging to GPSC1, GPSC9 and GPSC10 using MinTyper 1.0 [288]. Finally, the PneumoCaT tool was used to determine capsular genotypes [64].

Statistical methods

Collected data was presented with descriptive statistics. To compare differences in scale variables between any two or three groups the Mann-Whitney U and Kruskal-Wallis *H* tests were used, respectively. Categorical variables were compared with the Chi-squared test or, if the expected count of any cell was <5, Fisher's exact test. Uni- and multivariate logistic regression analyses were used to determine the association of background variables with different outcomes. Calculated ORs were presented with 95% confidence intervals (95%CI). The above analyses were performed in SPSS Statistics v. 22, 24 or 26 (IBM, Armonk, NY), or Prism Graphpad v. 7 (GraphPad Software, San Diego, CA).

Anti-CPS Ig ratios were calculated in Paper I and II by dividing the concentration measured in convalescence serum with the concentration in the corresponding acute-phase or pre-infection serum. In Paper III, ORs were calculated to compare frequencies of individual pneumococcal serotypes among IPD and respiratory tract samples, 95%CIs were determined for these ratios as described by Kirkwood and Sterne [289].

Results

Paper I

In total, 40 patients with IPD were included in the study of the anti-pneumococcal antibody response regarding different pneumococcal serotypes. Half of the patients were infected with serotypes with low invasive potential (serotype 3, *n*=12; 19F, *n*=4; 23F, *n*=4) according to Brueggemann *et al*. [265], while the remaining infections were caused by medium or high invasive serotypes (serotype 7F, *n*=11; 14, *n*=5; 1, *n*=2; 4, *n*=2).

Overall, convalescence sera from 40% (*n*=16) of patients yielded a higher OPA titre, or an identical titre >8, than that of corresponding pre-infection or acute-phase sera. These were considered functional anti-pneumococcal opsonic antibody responses. Sera from the remaining patients exhibited either a decreased convalescence OPA titre (45%; *n*=18) or an unchanged OPA titre <8 (15%; *n*=6), patterns that were regarded as non-functional. Infection caused by serotypes with low invasive potential was more common among patients with a decreased (50%) or unchanged OPA titre <8 (68%), compared with patients exhibiting a functional response (25%) (*p*=0.029). A multivariate logistic regression analysis showed an association between low-invasive serotypes and a non-functional opsonic response, adjusted for age and the presence of severe sepsis or septic shock (OR 0.13, 95%CI 0.024-0.67).

Furthermore, the calculation of anti-CPS IgG concentration ratios revealed a correlation between the quantitative antibody response and a functional one measured by OPA. In patients with a functional OPA response the median anti-CPS IgG ratio was 2.3 (interquartile range [IQR] 1.8-5.5), compared with 1.8 (IQR 0.6-2.6) and 0.5 (IQR 0.1-0.8) in those with an unchanged OPA titre <8 or a decreased convalescence OPA titre, respectively (*p*=0.041).

Paper II

In Paper II, the anti-pneumococcal antibody responses of 54 patients with pneumococcal CAP were assessed The infecting serotypes were 3 (*n*=11), 14 (*n*=10), 7F (*n*=9), 23F (*n*=6), 9V (*n*=4), 18C (*n*=3), 19A (*n*=3), 19F (*n*=3), 1 (*n*=2),

4 (*n*=2), and 6B (*n*=1). Thirty percent of patients (*n*=16) were bacteremic, while the remaining patients had non-invasive CAP.

Analysis of corresponding acute-phase and convalescence sera with OPA demonstrated that almost half of patients (*n*=24; 44%) produced a functional antibody response, with increased convalescence OPA titres (*Figure 16*). Conversely, 35% (*n*=19) exhibited a non-functional response, characterized by a decreased convalescence OPA titre or undetectable opsonic activity at both time points. A third group, comprising 20% (*n*=11) of patients, exhibited identical acute-phase and convalescence OPA titres at both time points. In contrast with Paper I, no cut-off was used to classify OPA responses characterized by equal acute-phase and convalescence OPA titres as functional or non-functional.

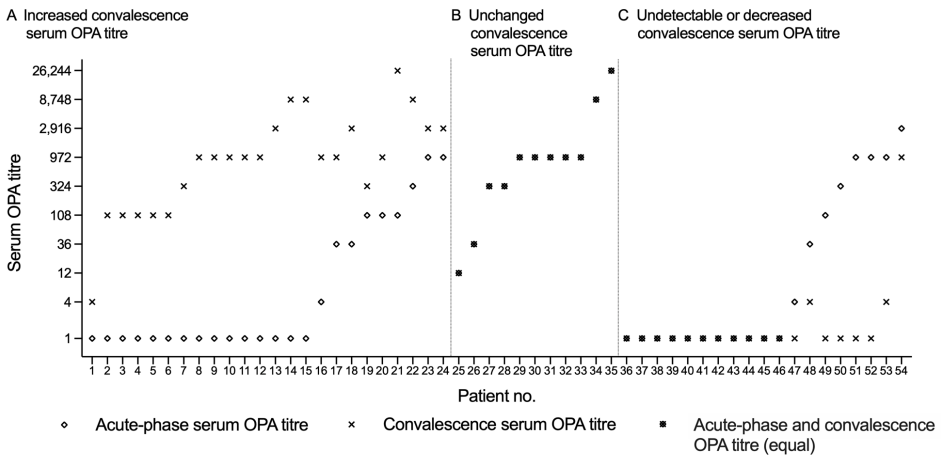


Figure 16. Opsonic antibody responses of patients with pneumococcal CAP.
Patient serum OPA titres during the acute-phase of infection and during convalescence. Cases were classified as producing either a functional repsonse, with an increased convalescent titre (A; *n*=24), or a non-functional response, characterized by a decreased or undetectable convalescent titre (C; *n*=19). One group exhibited equal, but detectable OPA titres at both time points (B; *n*=11). A serum OPA titre of 1 in the figure indicates that no opsonic activity was detected with the sample.

Acute-phase sera with detectable opsonic activity (OPA titre >1) generally were from older patients (median age 75 years) compared to acute-phase sera that did not induce any bacterial killing in the OPA (median 60 years) (*p*=0.032). However, patients with sera exhibiting a functional convalescent antibody response were younger (median 61 years) than those with unchanged or decreased/undetectable convalescent OPA titres (median 78 and 69 years, respectively) (*p*=0.028). Interestingly, more than half (*n*=10; 53%) of patients with non-functional antibody responses were bacteremic, compared with 21% (*n*=5) and 9% (*n*=1) of patients showing increased or unchanged convalescent OPA titres, respectively (*p*=0.019).

No difference was detected in anti-CPS Ig concentrations between acute-phase sera that yielded a detectable OPA titre or not. In contrast, the median Ig

concentration in convalescence sera with OPA titres >1 (105 AU, IQR 58-337) was higher than that in sera with no detectable opsonic activity (28 AU, IQR 12-109) (p=0.003). Likewise, the highest median convalescence/acute-phase Ig ratio (3.2, IQR 1.4-13.7) was found with sera from patients with a functional OPA response, compared with ratios of 1.1 (IQR 0.9-2.6) and 1.3 (IQR 0.9-2.1) among those with either an unchanged or decreased titre, respectively (p=0.002).

Multivariate analysis of factors affecting the opsonic response (unpublished data)

Infection with a low-invasive serotype was more common among patients with a non-functional OPA response also in Paper II (50% vs. 23%; p=0.041). To further elucidate the relationships between OPA outcome and background factors uni- and multivariate logistic regression analyses were performed (*Table 5*). These analyses confirmed the independent associations of a non-functional opsonic response with low-invasive serotypes and IPD.

Table 5. Logistic regression analysis of factors associated with a non-functional OPA response.

Variable	Univariate OR (95%CI)	*p*	Adjusted OR (95%CI)	*p*
Age[a]	1.01 (0.98-1.04)	0.60		
Age >65 years	0.94 (0.31-2.87)	0.91		
Female sex	2.06 (0.66-6.41)	0.21		
Current smoking	1.69 (0.51-5.60)	0.40		
Comorbidity[b]	0.69 (0.22-2.12)	0.51		
CRB-65 >1	1.01 (0.30-3.35)	0.99		
Bacteraemia	5.37 (1.53-18.90)	**0.009**	6.71 (1.68-26.78)	**0.007**
Sepsis[c]	1.44 (0.44-4.70)	0.54		
Symptom duration[a]	1.03 (0.93-1.13)	0.60		
CRP maximum[a]	1.00 (1.00-1.01	0.22		
Days between samples[a]	0.99 (0.95-1.03)	0.57		
Low-invasive serotype[d]	3.29 (1.03-10.53)	**0.045**	4.24 (1.13-15.91)	**0.032**

[a] Continuous variable.
[b] COPD, heart disease, diabetes mellitus, liver disease, renal insufficiency, neoplasm, or immunosuppression.
[c] Fulfilment of the Sepsis-3 definition.
[d] Serotypes 3, 6B, 19A, 19F, and 23F.

Paper III

In total, 2,131 respiratory tract samples collected in Skåne county between October 2016 and March 2018 positive for growth of *S. pneumoniae* were included in the study. A large proportion of samples were collected from children <5 years of age (26%) or individuals ≥60 years of age (36%) and most were referred from outpatient departments (69%). The majority of samples were nasopharyngeal swabs (63%) or sputum samples (23%) (*Figure 17*).

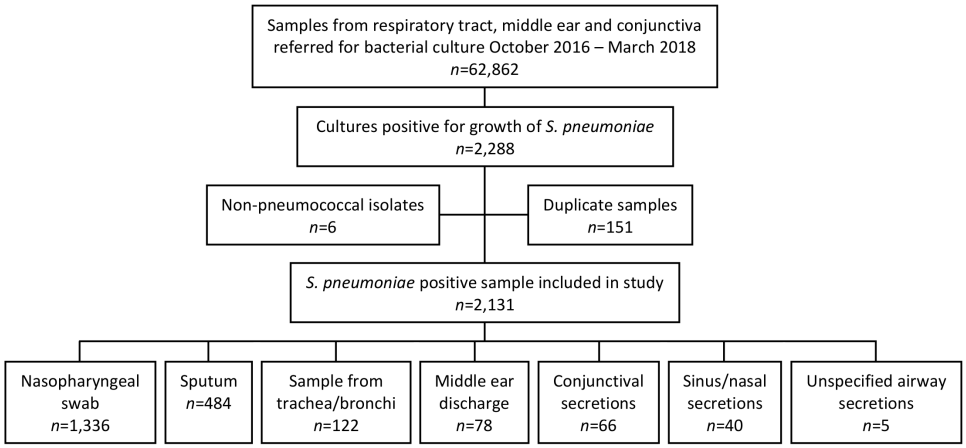


Figure 17. Pneumococcal samples detected in Skåne county in October 2016-March 2018.
An flowchart depicting the the inclusion of pneumococcal isoaltes from respiratory tract samlpes and the distribution of individual sample types. The 6 excluded non-pneumococcal isolates were revealed to be other species during further analyses after collection, it is unknown if species assignment at the clinical laboratory was incorrect or if the discrepancy was due to contamination or mix up of isolates after species determination.

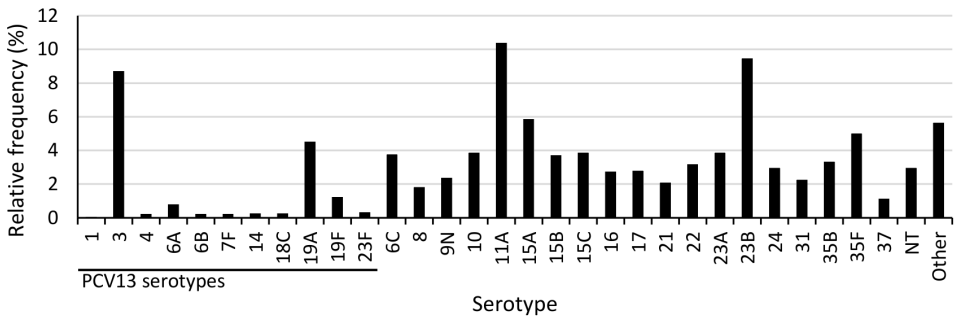


Figure 18. Serotype distribution of pneumococci detected in clinical respiratory tract samples.
The realtive frequency of detected serotypes among pneumoccoci isolated from clincial respiratory tract samples referred for culturing in Skåne county between October 2016 and March 2018. PCV13 serotypes are indicated by the bar benteath the x-axis. The serotype coverage of PCV13 was 17% in total. Non-vaccine serotypes that individually represent <1% of isolates are grouped in the figure as “Other”. NT, non-typeable.

Pneumococcal isolates corresponding to 87% (*n*=1,858) of included samples were available for serotyping. The most common serotypes were 11A (10.4%), 23B (9.5%), 3 (8.7%), and 15A (5.9%) (*Figure 18*). Seventeen percent of isolates exhibited serotypes covered by PCV13, representing predominantly serotype 3 (8.7%) and 19A (4.5%), while PCV10 coverage was 2.8%. A multivariate logistic regression analysis showed that PCV13 serotypes were less frequently detected in samples from children born after the introduction of PCV13 in the paediatric NIP

(OR 0.46, 95%CI 0.32-0.67). Furthermore, PCV13 serotypes were more common in samples referred from hospital settings (OR 1.43, IQR 1.11-1.85), while their association with lower respiratory tract samples became non-significant after adjusting for the two aforementioned factors (OR 1.31, IQR 1.00-1.71).

During the study period, 519 pneumococcal isolates from patients with IPD in Skåne county were serotyped by the Public Health Agency of Sweden, mostly from individuals ≥60 years of age (76%). The calculated IPD vs. respiratory tract ORs indicated that PCV13 serotypes (1.8, 95%CI 1.5-2.1) were more invasive than most non-vaccine serotypes (NVTs) that were frequent in respiratory tract samples, exhibiting ORs near 1 or <1. The NVTs with the highest ORs were serogroup 12 (OR 9.8, 95%CI 3.1-30.8), serotype 8 (OR 6.8, 95%CI 4.6-10.2), and serogroup 22 (OR 6.8, 95%CI 4.6-10.2).

Non-susceptibility against any routinely tested antibiotics or MDR phenotypes were more frequent among several PCV13 serotypes compared with the overall rates (*Figure 19*). However, this was also detected in certain NVTs such as serotypes 15A (MDR), 23B, and 35B (PNSP). In addition, NT pneumococci were often MDR.

Results of serotyping with different methods (unpublished data)

No formal analysis was performed to compare the serotyping scheme in this study (*i.e.*, PCR + serological methods) with the Quellung reaction only. In total, 1,493 (80%) of isolates were either positive for a PCR-included serotype (*n*=1,452) or *cpsA* negative (*n*=41). The serotypes were further specified with Quellung typing for 663 PCR-positive isolates. Conversely, 300 isolates (16%) were *cpsA* positive but negative for all serotypes included in the PCR and were thus serotyped with latex agglutination and the Neufeld method. Of these, almost half exhibited serotypes (*n*=140) that were included in the PCR (*i.e.*, false negatives), while 160 were other serotypes or NT. The remaining 65 isolates were also serotyped by latex agglutination and/or the Neufeld method due to uncertain PCR results or conflicting results between PCR and antisera.

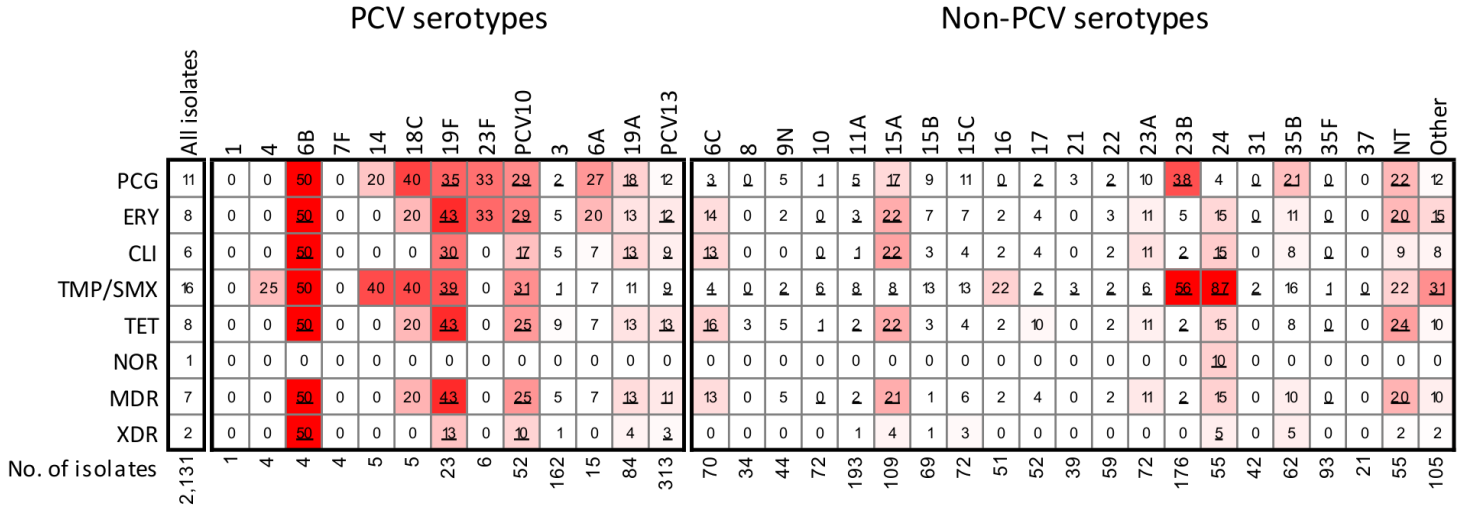


Figure 19. Antimicrobial non-susceptibility rates among detected pneumococcal serotypes.

Percentages of non-susceptibility to commonly used antibiotics among serotypes detected in Skåne county between October 2016 and March 2018. Several PCV13 serotypes exhibited a high degree of resistance while these phenotypes were restricted to a smaller number of NVTs. Red colouring of cells indicate increasingly higher non-susceptibility rates to the corresponding antibiotics compared to the overall detected frequency. Underlined numbers indicate a statistically significant difference between that serotype and isolates of all other serotypes combined. NT, non-typeable; PCG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TMP/SMX, trimethoprim-sulfamethoxazole; TET, tetracycline; NOR, norfloxacin; MDR, multi drug resistance (*i.e.*, non-susceptibitlity to ≥3 antibiotics); XDR, extesively drug resistance (*i.e.*, non-susceptibitlity to ≥5 antibiotics).

Paper IV

The 26 available pneumococcal isolates classified as XDR (non-susceptible to ≥5 antibiotic classes) during antimicrobial susceptibility screening were selected for WGS and MIC determination. One of the isolates were subsequently excluded due to repeated failure to acquire sequencing data of sufficient quality, bringing the total to 25 studied isolates. Serotypes of the studied isolates and the STs, CCs, and GPSCs assigned by uploading genomic data to Pathogenwatch are outlined in *Table 6*. Most isolates belonged to GPSC1 (*n*=7), GPSC9 (*n*=5) or GPSC10 (*n*=5). These GPSCs, and two additional detected GPSCs (GPSC16; *n*=1 and GPSC47; *n*=1), are classified as dominant global MDR lineages [69].

Confirmation of the antimicrobial resistance patterns by MIC determination with broth microdilution revealed that 10/25 isolates exhibited MDR rather than XDR phenotypes, with resistance against 3-4 antibiotic classes. The overall correlation between phenotypic resistance and the presence of resistance mechanisms was although high (*Table 6*). The ten reclassified isolates were susceptible to trimethoprim-sulfamethoxazole according to current EUCAST clinical breakpoints, however, 9 of these isolates harboured a *folP* amino acid insertion indicating the presence of a resistance mechanism. This is explained by the fact EUCAST cut-off value for disk diffusion testing with trimethoprim-sulfamethoxazole was increased in 2019, between isolate detection at the clinical laboratory and the analyses performed for Paper IV [266]. The only other discrepancies were one tetracycline susceptible isolate carrying the *tetM* gene, and one clindamycin susceptible isolate carrying *ermB*.

All studied isolates carried the pneumococcal virulence genes *ply*, *lytA*, *lytC*, *cbpE*, *pavA*, *hysA*, *eno*, *piuA*, *psaA*, *cppA*, *htrA*, and *tig/ropA*. The *zmpC* gene was exclusively detected in GPSC9 isolates, while PI-1 and PI-2 were present in all GPSC1 isolates and, only PI-1, in two isolates belonging to GPSC47 and GPSC115, respectively. Phylogenetic trees were constructed for GPSC1 (*n*=86), GPSC9 (*n*=55), and GPSC10 (*n*=57) using the studied isolates and additional genomes acquired from the European Nucleotide Archive. The expression of serotypes, resistance genes, and virulence genes was generally similar for closely related isolates.

Table 6. Molecular epidemiology, resistance mechanismsms, and MICs of MDR and XDR pneumococci determined with broth microdilution.

Isolate ID	Serotype	GPSC[a]	ST	CC	PBP Profile (1a-2b-2x)	Inferred PCG MIC	PCG MIC	*ermB*/ *mefA*	ERY MIC	CLI MIC	*tetM*	TET MIC	*folA*/ *folP*	TMP/ SMX MIC
1	19F	**1**	236[b]	-	13-16-47	2	1 (I)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
2	3	**1**	271[b]	320	17-16-47	2	1 (I)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)
3	19A	**1**	320	320	13-11-16	4	4 (R)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
4	19A	**1**	320	320	13-11-16	4	4 (R)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)
5	19F	**1**	2920[b]	-	13-16-New	4	4 (R)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)
6	19A	**1**	4768	320	13-11-16	4	4 (R)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
7	19F	**1**	8359	-	13-14-20	4	4 (R)	+/+	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)
8	15A	**9**	63[c]	63	New-7-138	0.5	0.5 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	1 (S)
9	15A	**9**	63[c]	63	24-27-28	0.25	0.12 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/-	≤0.5 (S)
10	15C	**9**	782[c]	63	17-53-36	2	0.5 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	4 (R)
11	15A	**9**	3816[c]	63	24-27-13	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	1 (S)
12	15A	**9**	3816[c]	63	24-27-13	0.25	0.12 (I)	+/-	>2 (R)	>1 (R)	+	≤1 (S)	-/+	1 (S)
13	24	**10**	230[d]	230	New-15-22	0.5	1 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	≤0.5 (S)
14	15B	**10**	4253[d]	230	New-15-367	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	1 (S)
15	24	**10**	6227[d]	230	17-15-22	0.5	0.5 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	≤0.5 (S)
16	7B	**10**	New1	-	17-144-8	2	2 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	1 (S)
17	7B	**10**	New1	-	17-144-8	2	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	-/+	1 (S)
18	15C	**16**	83[e]	81	15-12-18	2	4 (R)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)
19	11A	43	8605	-	7-12-135	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
20	6B	**47**	2040[f]	315	New-53-35	0.5	0.25 (I)	+/-	>2 (R)	0.25 (S)	+	>8 (R)	-/+	1 (S)
21	NT	81	4149	-	25-7-56	2	2 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
22	35B	91	373	-	7-1-New	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	2 (I)
23	35B	91	373	-	7-1-242	0.25	0.5 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
24	35B	91	373	-	7-1-242	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	4 (R)
25	6B	115	135	1348	8-67-103	0.25	0.25 (I)	+/-	>2 (R)	>1 (R)	+	>8 (R)	+/+	>4 (R)

[a] Bold font indicates dominant global MDR GPSCs [69].

[b] Identical or single locus variant of PMEN clone Taiwan19F-14 ST235.

[c] Identical or single locus variant of PMEN clone Sweden15A-25 ST63.

[d] Identical or single locus variant of PMEN clone Denmark14-32 ST230.

[e] Single locus variant of PMEN clone Spain23F-1 ST81.

[f] Single locus variant of PMEN clone Poland6B-20 ST315.

Abbreviations: PCG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; TMP/SMX, trimethoprim/sulfamethoxazol.

Paper V

Nasopharyngeal swabs were collected from 940 children aged 4-12 years (median 8, IQR 7-12). Overall carriage rates of *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis* were 35% (*n*=332), 13% (*n*=121), and 15% (*n*=139), respectively. A multivariate logistic regression analysis showed that pneumococcal carriage was positively associated with simultaneous carriage with *H. influenzae* (OR 2.37, 95%CI 1.51-3.72) and negatively associated with antibiotic treatment within the last month (OR 0.27, 95%CI 0.09-0.83).

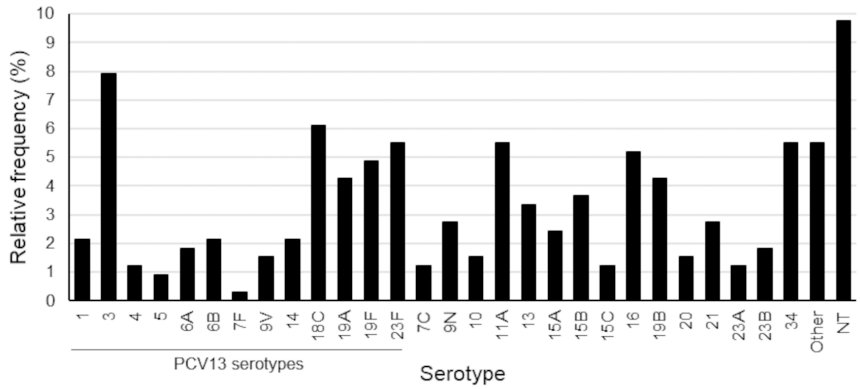


Figure 20. Serotype distribution of pneumococci detected in carriage among children in Angola.
The relative frequencies of individual serotypes isolates from nasopharyngeal samples in children aged 4-12 years in 2017. Serotypes included in PCV13 (41%), which was introduced in the country in 2013, are indicated by the bar beneath the x-axis. Serotypes representing <1% of all isolates are pooled as "Other". NT, non-typeable.

All but 4 isolates (*n*=328), that were lost due to contamination, were serotyped, and 41% were covered by PCR13, including serotypes 3 (8%), 18C (6%), and 23F (6%) (*Figure 20*). The most prevalent NVTs were 11A (6%), 34 (6%), and serogroup 16 (5%). The majority of *H. influenzae* isolates were NTHi (90%) while 12 encapsulated strains were detected, representing Hif (*n*=7), Hie (*n*=2), Hib, Hic, and Hid (all *n*=1).

A substantial number of pneumococci were PNSP (40%), although all exhibited benzylpenicillin MICs in the I interval. Serotypes covered by PCV13 were PNSP more frequently than NVTs (50% vs 33%; *p*=0.003), due to high rates among serotype 6A, 6B, 14, 18C, 19F, and 23F. However, several NVTs also exhibited PNSP phenotypes frequently (*i.e.*, serotypes 11A, 15A, 23A, 23B, and NT isolates). Very high frequencies of trimethoprim-sulfamethoxazole resistance were detected among all three studied species (71-84%) and tetracycline resistance was moderate (13-17%) in pneumococci and *H. influenzae*. Twenty-five (7%) MDR pneumococci

were detected during AST screening. Determination of MICs with broth microdilution confirmed MDR phenotype in all but one of these isolates which was sensitive to trimethoprim-sulfamethoxazole, and WGS revealed that GPSC94 (*n*=7) and GPSC10 (*n*=5) were the most common MDR lineages, mostly comprising serotypes 23F and 3, respectively.

Results of serotyping with different methods (unpublished data)

The serotyping of pneumococcal isolates included in Paper V yielded less false negative results in the PCR (*n*=8; 2%) and less conflicting serotype results between PCR and the serological methods (*n*=9; 3%) compared to the analyses presented in Paper III.

Paper VI

A total of 184 ear discharge samples and 151 nasopharyngeal swabs were collected from 152 individuals with CSOM aged 0-77 years (median 13 years, IQR 5-22 years). Cultures yielded 533 individual isolates from ear discharge samples and 289 from nasopharyngeal swabs. In individuals with bilateral disease, 27% of the detected species were present in both ears, while correspondence of species isolated from ear discharge and the nasopharynx was lower (9%).

Eighty-seven different species were identified in samples from the middle ear, mostly representing aerobic bacteria, but a small number of fungal isolates (*n*=16) were also found. On average, 3 different species were found per sample. The most frequently detected species in ear discharge samples were *Proteus* spp. (15%), *P. aeruginosa* (13%), and *Enterococcus* spp. (9%). Of the 34 pneumococcal isolates detected in nasopharyngeal samples, 12 (35%) were covered by PCV13.

Antimicrobial susceptibility testing was performed on the species that are generally regarded as relevant pathogens in CSOM and AOM (i.e., Enterobacterales, *P. aeruginosa*, *S. aureus*, *Streptococcus pyogenes*, *S. pneumoniae*, *H. influenzae*, and *M. catarrhalis*). Susceptibility rates to fluoroquinolones and aminoglycosides were generally moderate to high among tested bacteria (69-100%), with exception of *S. aureus* which was susceptible to tobramycin in only 30% of cases. Conversely, only 57% of non-*Proteus* Enterobacterales were susceptible to chloramphenicol. Additionally, 54% of *S. aureus* were methicillin resistant, 53% of pneumococci were PNSP, and resistance rates against trimethoprim-sulfamethoxazole were high among all bacteria (44-78%).

Discussion

A high systemic antigen load may hamper the anti-pneumococcal antibody response

The analyses of paired sera with OPA in Paper I and II revealed that many patients with IPD and/or CAP developed a functional opsonic antibody response during the convalescence period. Conversely, a large proportion exhibited decreased or unmeasurable convalescent antibody function, patterns that were associated with bacteraemia and with low invasive pneumococcal serotypes which generally produce thick polysaccharide capsules [166, 290].

It is important to point out that the definition of a functional response differed between the two studies regarding cases with identical OPA titres at both timepoints. In Paper I, a cut-off at a titre of ≥8 was used to classify responses characterized by equal titres at both time points as functional or non-functional. In Paper II, paired acute-phase and convalescence sera with equal titres were considered a separate group. The more conservative approach used in Paper II may be more appropriate because the cut-off at an OPA titre of 8 is an arbitrary limit which is used to indicate protective immunity in young children in studies of PCV and does not *per se* indicate if the immunological response is appropriate or not [171]. With this approach, the percentage of patients with a functional response in Paper I would be 35% rather than 40%. Furthermore, the studies should be regarded as exploratory and hypothesis-generating due to their retrospective and partially *post hoc* nature, the limited sample size, as well as the great variability in the temporal relation between sample collection and the infectious episode in Paper I.

How an episode of clinically significant pneumococcal infection affects naturally acquired anti-pneumococcal immunity has been scarcely studied but impaired production of anti-CPS Ig after infection has been reported in other studies applying only quantitative methods [162, 164-167]. In only one earlier study opsonic activity was measured at different time points for 14 patients which, in line with the present results, revealed considerably lesser increases and overall lower titres of convalescence sera from bacteraemic patients compared to those with non-invasive CAP [291]. The current investigations confirm these results with a larger sample size and contribute with the observation that a negative result seem to be associated with certain serotypes.

There are parallels between the current findings and vaccine hyporesponsiveness to pneumococcal vaccines, including the association with IPD, which leads to the question if the biological mechanisms are shared. It is often proposed that hyporesponsiveness is caused by depletion of serotype-specific memory B-cell pools by previous exposure to unconjugated CPS, through PPV immunization, carriage, or IPD, leading to a lower response upon subsequent vaccination [176, 177, 207, 214]. Additionally, apoptosis of memory B-cells, which led to fewer Ig-secreting activated B-cells, was observed after repeated vaccination with a meningococcal polysaccharide vaccine in mice [292]. It has also been hypothesized that high levels of polysaccharide antigens may persist in the human body for long periods of time and continually downregulate corresponding B-cell clones and neutralize specific Igs [163, 177, 207, 293]. Possibly, this is because T-independent antigens with high epitope density such as CPS may not activate naïve marginal zone B-cells, but instead render them unresponsive, if adequate cytokine co-stimulation is not present [154]. It seems plausible that these mechanisms could contribute to explain the current findings, and the associations with both bacteraemia and serotypes producing large amounts of CPS indicate that a high systemic level of polysaccharide antigens may be a key factor. Another mechanism that has been proposed to be of importance for the attenuated immune response to pneumococcal CPS are immunosenescence in the elderly [294]. However, a negative effect of high age on the opsonic response was not confirmed in the current studies even if it was indicated. To put the present results in context, it is possible that the observed absent antibody responses to infection correspond to what has occurred in patients described in previous reports that do not respond to vaccination after IPD.

Opsonic activity and anti-capsular Ig concentration correlate poorly in naturally acquired immunity

In the current studies, the concentrations of anti-CPS antibodies and outcomes in the OPA correlated to some degree. However, high OPA titres were also detected with sera that exhibited low serotype-specific Ig concentrations, and vice versa. Furthermore, the acute-phase to convalescence changes of the two measures were in some cases completely divergent. In the previous study of the patients recruited from a historical cohort, a negative quantitative Ig-response was associated with low-invasive serotypes, which is confirmed with the current results, but the association with bacteraemia was only detected with OPA [166]. These discrepancies highlight the importance of applying both quantitative and functional methods in immune response studies.

Divergence between OPA and anti-CPS Ig concentrations is commonly reported and may have several explanations [216, 291, 295, 296]. Firstly, anti-CPS Ig with

low avidity may be detected with ELISA but not affect the outcome in OPA [174, 291, 296]. Secondly, the presence of IgM against the pneumococcal capsule is of importance for opsonophagocytosis, but these antibodies are not measured in IgG-specific assays, as the one applied in Paper I [297]. Both these factors are age dependant and may contribute to lower opsonic function in elderly individuals [128]. As discussed above, neutralization of antibodies by persisting circulating CPSs may occur, which possibly affects Ig with high avidity to a higher extent [177, 207, 293]. Moreover, opsonic antibodies against various protein antigens, which are of importance for naturally acquired adaptive immunity, could affect the OPA results [161].

Although these observations indicate that the OPA is a better surrogate marker for protection than anti-CPS Ig concentrations, factors that are not reflected in this assay also contribute to anti-pneumococcal immunity. This is highlighted by the fact that many of the currently studied individuals developed pneumococcal infections, and in many cases IPD, despite exhibiting high OPA titres before or shortly after the onset of symptoms. One such variable is the functionality of the cell-mediated response, which has been shown to be important for preventing pneumococcal lung infection after colonization in mice [170]. Interestingly, a non-opsonic anti-CPS antibody induced by pneumococcal vaccination was shown to interfere with serotype 3 colonization and systemic dissemination in mice, demonstrating yet another aspect of anti-pneumococcal immunity that may be important also for naturally acquired immunity [298]. Predisposing factors that were not investigated in the currently studied patients, such as complement deficiencies, may also have contributed to infection despite high OPA titres.

Finally, the clinical significance of the findings presented in Paper I and II is not fully clear. On one hand, the importance of opsonic antibodies for protection against IPD is undisputed why it is reasonable to believe that the individuals with a non-functional opsonic antibody response after IPD are more susceptible to reinfection by the same serotype than those with high convalescence titres. An analysis of national IPD data in Australia determined that the incidence of recurrent disease after a previous episode of IPD was 247/100,000 person-years compared to the overall incidence of 8/100,000, and approximately half of those that experienced reinfection (243/512) did not have any known predisposing risk factors [299]. It is although unknown to what extent these episodes were caused by the same serotype. In an earlier report from the United States, 25% of 80 cases of recurrent IPD was with the same serotype [300]. These observations may be explained by inadequate immune responses such as those described in the present reports. On the other hand, it is possible that the impaired antibody function detected with OPA may be compensated by other aspects of anti-pneumococcal immunity or resolve naturally after some time before any significant cumulative risk for recurrent infection has been reached.

Serotypes 3 and 19A circulated in Skåne county during 2016-2018 despite use of PCV13 in infants

As all pneumococci detected in respiratory tract samples in the county were included in the analyses presented in Paper III and IV, it is presumed that they represent a cross-section of the strains that were present in the population during the study period. Because the isolates were from clinical specimens, it may also be assumed that many of the corresponding patients suffered from significant infection by the detected bacterium, but an overlap with asymptomatic carriage probably exists in the material studied. A previous study found differences in the serotype distribution detected in carriage and mucosal infections in children, respectively, which implies that surveys of clinical samples may provide better information regarding which serotypes are important strains causing disease [301].

Compared with 2011-2013, PCV10 serotypes (12% vs. 3%), and of the PCV13 non-PCV10 serotypes 6A (3% vs. 1%) and 19A (10% vs. 5%) declined. In contrast, serotype 3 constituted approximately 9% of isolates in both studies [268]. Several NVTs were common in both time periods, but it is worth noting that serotypes 15A and 23B have sharply increased to become two of the most prevalent serotypes. The spectrum of emerging serotypes was similar to that previously reported in carriage among children in Stockholm County and other European countries after PCV introduction, even there are differences regarding individual serotypes between studies [135, 205, 239, 301-303]. Both the fact that individuals of all ages were included and that clinical samples were studied in the present study may contribute to such differences [82, 301].

Serotype 3 has attracted special attention due to the limited herd effects achieved for this serotype with PCV13 [222]. In the currently studied setting and in other reports, carriage of serotype 3 was rare among children both before and after PCV13 introduction, and the serotype has caused low rates of childhood IPD in Sweden since 2007 despite being a common etiology among the elderly [135, 197, 224, 239]. Taken together, serotype 3 is predominantly of clinical importance in this age group in Sweden and the presented data do not indicate an established herd effect against this serotype. A previously proposed explanation is that serotype 3 may be predominantly carried by older children and adults, representing the reservoir for this serotype in the population [82, 224, 239].

Continued circulation of serotypes 19A and, to a lesser extent, 19F in the population after PCV13 introduction has also been noted in other settings [123, 135, 239]. The prevalence of serotype 19A in Skåne county during the studied years is of concern because the PCV formulation used in the Swedish NIP was changed to PCV10 in September 2019. This may lead to an increased overall paediatric IPD incidence, caused by serotype 19A infections, which was observed in Belgium [198]. On the other hand, the reduction of the total IPD incidence was similar in Swedish counties using the different PCVs, irrespectively of an increased rate of

serotype 19A IPD in counties administering PCV10 [197]. Because the incidence of IPD has been heavily affected by the Covid-19 pandemic, it is not yet possible to draw any conclusion regarding recent data from the Public Health Agency of Sweden. However, it is alarming that serotype 19A represented 16% of all IPD cases in 2021 compared to approximately 10% during 2017-2020 [235]. Future changes in the epidemiology of this serotype need to be closely monitored.

As previously reported, many NVTs that were frequently detected in the current study are low invasive, such as serotypes 11A, 23B, 15B/C, and 35F [117, 135, 205]. However, these serotypes, and others which were slightly more invasive (*i.e.*, 6C, 15A, and 23A), cause a considerable number of IPD cases as they become widely spread in the population and individuals with predisposing factors are disproportionally affected by infections by these serotypes [121, 122, 304, 305]. The NVTs 8, 9N, 12F, 22F, and 33F are often reported as being among the most invasive emerging serotypes in IPD, which is in line with the current results. Conversely, lower invasiveness for serotype 3 and serogroup 24 was found compared with several previous studies [117, 119, 135, 205]. Differences like these may be partly due to the inclusion of samples from all age groups but may also be explained by various genetic backgrounds among isolates expressing the same serotype [69]. Continued surveillance of replacement serotypes is of importance for guiding future vaccinations strategies. Some of the frequently detected NVTs are included in the recently licenced PCV15 (PCV13 serotypes plus 22F and 33F) or PCV20 (PCV13 serotypes plus 8, 10A, 11A, 12F, 15B/C, 22F, and 33F) and the coverage of these vaccines were 21% and 41%, respectively [306]. How future vaccine formulations should be used is dependent on the prevalence of included serotypes in different age groups and a recurrent proposal is that a new vaccine may be given to the elderly to counter increasing IPD rates in this groups caused by replacement serotypes that do not cause high numbers of IPD in children [307].

A limitation of the study was the fact that not all non-vaccine serogroups were further analysed to determine specific serotypes. This approach was chosen upon initiation of the study as the focus lay on surveying the changing epidemiology of PCV13 serotypes. Furthermore, the applied PCR method resulted in some false negative reactions, which was, however, compensated by using classical serological methods. The smaller number of conflicting results or uncertain PCR results may have been caused by the presence of multiple serotypes in samples in some cases. However, it was also influenced by the presence of unspecific “ghost bands” in PCR gels and weak positive reactions during early use of the method that prompted additional use of the Neufeld method. The method improved with time, after replacement of reagents and minor modifications, and was more streamlined as demonstrated by the results in Paper V. Serotyping with multiplexed PCR is, however, a thoroughly validated and widely used method why no formal analysis was performed to compare it with serological typing [280].

Antimicrobial non-susceptibility is related to a limited number of serotypes and genetic lineages

Compared to the global situation, the frequencies of PNSP and non-susceptibility to other relevant antibiotics are considerably lower in Sweden, which is reflected in the current results [150]. The occurrence of these phenotypes was distinctly dependent on serotype with most cases found among some PCV13 serotypes and a limited number of NVTs. The association of PCV13 serotypes with antimicrobial resistance is well documented and the overall frequencies have been lowered thanks to vaccine use even if findings vary in different regions [150, 308-311]. Likewise, the NVTs that were often non-susceptible to different antibiotics (*i.e.*, 6C, 15A, 23B, 24, and 35B) have been described previously [141, 304, 310-312]. Luckily, the detected MICs for penicillin were in almost all cases in the I range. This fact indicates that it is predominantly treatment with oral penicillin that may not be successful against these strains. It may, however, result in a delay until effective treatment is started for individual patients. Furthermore, the detected MDR/XDR strains may be problematic to treat in patients that are allergic to beta-lactams.

The three most common GPSCs found among XDR pneumococci, GPSC1, GPSC9 and GPSC10, represent internationally spread lineages that are known to often exhibit high levels of antimicrobial resistance [69]. Of these, only GPSC1 comprised PCV13 serotypes, mostly 19A, which was still a prevalent serotype despite use of the vaccine. For several reasons, the presence of these lineages may be a concern. Firstly, the 19A CC320, which was common among GPSC1 isolates, was responsible for the increase of MDR serotype 19A IPD that occurred after PCV7/10 introduction in the United States and other countries, and has originated from the 19F ST236 lineage which was also found among the studied isolates [68, 313, 314]. Furthermore, a sub-clade of GPSC1 CC320 has caused vaccine breakthrough serotype 19A IPD cases in Ireland [315], and the serotype 3 ST271 isolate detected within the same GPSC represents a previously described capsule switch event giving rise to multi-resistant serotype 3 pneumococci [316]. GPSC9 [142, 317] and GPSC10 [318, 319] have also previously been reported to be common in IPD. Lastly, the expression of PI-1 and 2, which improve adherence to host cells [42], and ZmpC, which is associated with severe clinical outcomes [320], may contribute to the virulence of GPSC1 and GPSC9, respectively.

Because only a small number of isolates were sequenced, it is although unknown if these GPSCs were prevalent only among the putatively XDR pneumococci that were selected for the analysis, or if they were also common among other MDR strains. Since PCV10 was introduced in the Swedish paediatric NIP in 2019 all three lineages, however, mainly represent serotypes not covered by the currently used vaccine which could lead to their increased prevalence.

Any herd effect in carriage of pneumococci was not evident in Angola in the early PCV period

Apart from reports on a very limited number of pneumococcal isolates from bacterial meningitis in Luanda and from the CSOM patients presented in Paper VI, the results presented in Paper V comprise the first study of pneumococcal serotype epidemiology and antimicrobial resistance rates in the country [243, 321]. The WHO recommends that the serotype distribution present in a country is investigated prior to introduction of PCVs in paediatric NIPs to enable future evaluation and that this can be done with carriage studies [188]. With the current study no information was provided regarding the situation before PCV introduction in Angola, but the results may be used as baseline data as it included nonvaccinated children in the early PCV period when vaccine uptake was low.

The pneumococcal carriage rate and serotype distribution detected were largely comparable to similar studies from sub-Saharan Africa [76, 136, 322-326]. The high prevalence of serotypes 3, 18C, and 34 were although almost unique. Furthermore, the positive association between carriage with pneumococci, *H. influenzae*, and *M. catarrhalis* confirm previous findings [83, 327]. With an overall PCV13 coverage of 41%, the current report does not indicate that any herd effect has been established among non-vaccinated children. The high prevalence of vaccine type pneumococci is likely primarily due to the few years of vaccine use in combination with the low vaccine uptake. Continued circulation of these serotypes after PCV introduction, to a higher degree than is often observed in high income settings, has although been observed in other countries in the region where PCV has been in use for a longer time [136, 323, 325, 328]. It has been suggested that the use of immunization schedules lacking a booster dose during the second year of life may lead to waning immunity which could contribute to these observations [203]. The findings emphasize the need for reaching a higher degree coverage among children in Angola and the transition from the currently used 3+0 immunization schedule to one including a booster dose of PCV could be beneficial. Even if no evident indirect vaccine effects were seen, it is important to mention that signs of a decreased childhood meningitis incidence have been noted at a pediatric hospital in Luanda since 2013, indicating a positive direct vaccine effect [243, 329].

Compared to the presented results regarding pneumococci in southern Sweden, non-susceptibility to penicillin and, above all, trimethoprim-sulfamethoxazole was much more frequent in Angola and was detected in a wider set of serotypes. Resistance to tetracycline was also common, while almost all pneumococci were susceptible to amoxicillin, clindamycin, erythromycin, and norfloxacin. These findings were consistent with other data from the region and are probably influenced by selection pressure from antibiotic use [324, 330-332]. The high rate of PNSP, although all MICs were in the I range, was associated PCV13 serotypes and the situation may therefore be improved to some degree with increasing vaccine

coverage. Very high resistance rates to trimethoprim-sulfamethoxazole are commonly detected in sub-Saharan Africa and is thought to be due to persisting widespread use of this antibiotic as a first-line treatment for lower respiratory tract infections as well as prophylactic use in HIV-positive individuals [324, 331, 333]. While the antimicrobial resistance patterns detected were not unexpected, very little previous information exist regarding bacteria in Angola and the report thus contributes some background data for motivating antibiotic therapy practices in the country.

The genetic lineages that were detected among MDR pneumococci in Angola were more heterogenous than the XDR isolates described in Paper IV, probably partly due to the lower cut-off (*i.e.*, non-susceptible to ≥3 antibiotics) applied for inclusion in this analysis. However, GPSC10 and GPSC94 constituted approximately half of isolates and representing both PCV13 serotypes and NVTs, indicating that clonal expansion partly explains the presence of MDR pneumococci in Angola. As mentioned regarding the XDR isolates found in Skåne county, GPSC10 is an emerging clone exhibiting multiple serotypes associated with IPD and multi-resistance [318, 319]. To the author's knowledge, GPSC94 has not been described closely in the literature previously, but additional isolates belonging to this GPSC have although been detected in Senegal, the Gambia, Ghana, and Niger [71]. Likewise, several GPSCs, STs, and PBPs that were detected were not previously included in the databases used to assign their profiles, which reflects that the molecular epidemiology is less extensively investigated in this setting.

A high number of bacterial species are involved in CSOM

To provide local background data that can guide treatment and increase the understanding of CSOM, the microbiological spectrum present in affected individuals was investigated in Paper VI. In previous studies on CSOM, conventional microbiological methods have generally been applied which limits the number of species possible to identify. In similar with a recent study that could identify a wide range of bacterial species present in otitis media with effusion, the current aim was to determine the species of all culturable bacteria by using MALDI-TOF MS [334].

The finding of *Proteus* spp., *P. aeruginosa*, and enterococci as prevalent bacteria in the middle ear was in line with other studies from sub-Saharan Africa [251, 335-339]. However, many additional aerobic bacterial species were detected, including many Enterobacterales, indicating that the infection in CSOM is more multi-bacterial than previously reported. Because samples were not processed under anaerobic conditions, it is possible that the true number of species is even higher [252]. It is although important to point out that is unsure to what degree the different

species contribute to the pathogenesis in CSOM and if some (*e.g.*, coagulase-negative staphylococci and *Corynebacterium* spp.) only represent contaminants from the normal skin flora [340]. Concordant bacterial findings in the middle ear and nasopharynx of the same individual were rare, which does not provide any support for the hypothesis that the nasopharynx is an important reservoir for bacteria involved in CSOM [247].

Results from AST suggest that ciprofloxacin is the most feasible antibiotic for topical treatment of CSOM even if resistance rates towards quinolones were considerable among Enterobacterales and *S. aureus*. Even if treatment with topical gentamicin and quinolones occur, the most used antibiotic for CSOM in Angola is however chloramphenicol, which all *P. aeruginosa* and many Enterobacterales were resistant against [251]. In the paper, the potential of using topical antiseptics such as boric acid powder or aluminium acetate (Burow's solution) was discussed due to their low costs and higher availability, the presence of ciprofloxacin resistant bacteria, and promising results from preclinical and comparative clinical studies [246, 341-343]. A series of Cochrane reviews published in 2020 have although concluded that ciprofloxacin probably is a better treatment than boric acid, but all treatment options for CSOM have very limited scientific support [254, 255, 257]. Additional clinical trials assessing varying treatments are thus needed and it would be favourable if such studies were combined with thorough microbiological diagnostics to further clarify the significance of different bacterial species and antimicrobial resistance patterns.

Main conclusions and future perspectives

With the studies of the immune response triggered by severe pneumococcal infections presented in Paper I and II, some light was shed on an aspect of anti-pneumococcal immunity that is insufficiently studied. The main conclusion is that a positive opsonic antibody response against the corresponding serotype after infection does not occur as expected in many cases. Based on the association with bacteraemia and heavily encapsulates serotypes, the results provide indirect support for the hypothesis that a high systemic load of CPS may be the underlying cause through several mechanism. Additionally, functional assays such as the OPA contribute important information in studies of acquired anti-pneumococcal immunity compared to only measuring Ig concentrations. Preferably, future prospective studies should include sampling at additional points in time to further elucidate the dynamics of the response to pneumococcal infection and a broader set of methods should be applied to assess antibodies against several different antigens and to characterize B- and T-cell responses. The clinical importance of the different types of responses also needs to be evaluated.

From the results presented in Paper III and IV it can be concluded that the use of PCV13 has led to substantial rearrangement of the serotype distribution present in the population of Skåne county. However, herd effects were limited against serotype 19A and were non-existent regarding serotype 3. Certain emerging serotypes such as serotypes 15A and 23B that are not highly invasive may constitute a clinical problem in mucosal infections due to antimicrobial resistance but can also cause many IPD cases as their overall prevalence increases in the population. Continued surveillance, including investigations of molecular epidemiology on pneumococci causing invasive as well as non-invasive infections, is of importance to identify serotypes and genetic lineages that are of clinical importance to guide future preventative measures. Importantly, the epidemiology of serotype 19A must be evaluated after the implementation of PCV10 nationally in Sweden in 2019.

Paper V provides the first extensive description of pneumococci present in Angola which is of importance as support for treatment strategies and as baseline data for future evaluation of PCV immunizations in the country. In conclusion, there was no evidence for an established herd effect on the serotype distribution of pneumococci carried by unvaccinated children. A vast majority of bacteria were resistant to trimethoprim-sulfamethoxazole and non-susceptibility to penicillin and

tetracycline was common, while the empirical use of benzylpenicillin, amoxicillin, clindamycin, and macrolides may be recommended. Increased vaccine uptake among eligible children is paramount for reaching maximal impact on pneumococcal disease and the introduction of a PCV booster dose during the second year of life may be beneficial. Lastly, follow-up studies are encouraged to monitor the changing epidemiology of *S. pneumoniae* in Angola.

Additional studies of the microbiology and treatment of CSOM are urgently needed to improve care of this condition which is very common in resource limited settings. The study presented in Paper VI contributes to this with a thorough analysis of the aerobic bacterial spectrum associated with the disease. It is concluded that the infection of the middle ear is highly multi-bacterial and many of the commonly detected bacteria are resistant to frequently used antibiotics. Based on the resistance patterns, quinolones are the preferred topical antibiotic treatment of CSOM in Angola. To determine the clinical importance of different bacteria and resistance patterns detected in CSOM, high quality intervention studies including microbiological analyses are needed.

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Paper I

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Serotypes With Low Invasive Potential Are Associated With an Impaired Antibody Response in Invasive Pneumococcal Disease

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Pneumococcal polysaccharide vaccines may elicit a hyporesponse under certain conditions. There is limited knowledge, however, on the type of specific antibody response in individuals with invasive pneumococcal disease (IPD). The aim of this study was to investigate the functional antibody response in patients with IPD caused by different serotypes. Pre-immune and convalescent sera from 40 patients (age 14–91 years) with IPD caused by serotypes with low (serotype 3, 19F, and 23F) and high (1, 4, 7F, and 14) invasive potential were investigated. For each patient, the homologous serotype-specific antibody concentration was determined. The functionality of induced antibodies post-IPD was evaluated in an opsonophagocytic assay (OPA). Undetectable or decreased pneumococcal killing in OPA following IPD, i.e., a nonfunctional antibody response, was observed in 24 of 40 patients (60%). Patients with nonfunctional antibody responses had lower serotype specific IgG antibody ratios post-IPD than patients with increased OPA titres. A nonfunctional antibody response was associated with low invasive serotypes (3, 19F, and 23F, $p = 0.015$). In conclusion, a nonfunctional antibody response may follow IPD, and was in our cohort associated to serotypes with low invasive potential. These findings need to be confirmed in a larger material.

Keywords: antibody, opsonization, sepsis, serotype, *Streptococcus pneumoniae*

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INTRODUCTION

Despite widespread immunization with pneumococcal conjugate vaccines (PCVs) and effective antimicrobial therapy, *Streptococcus pneumoniae* is still a major cause of upper and lower respiratory tract infection as well as invasive pneumococcal disease (IPD). Pneumococci are generally shielded by an immunogenic polysaccharide capsule determining the specific serotype based upon chemically unique structures. Immunologic hyporesponses associated with serotypes included in PCVs have been reported in some settings; in clinical trials of an 11-valent PCV preparation in children, IgG titers for serotype 3 were lower following the booster dose compared with titres obtained after the initial dose (Poolman and Borrow, 2011). Following PCV7 immunization, a hyporesponse has been demonstrated in the context of prior pneumococcal

carriage in the nasopharynx (Dagan et al., 2010a; Väkeväinen et al., 2010; Rodenburg et al., 2011). Pneumovax® (PPV23), a polysaccharide-based vaccine advocated for immunocompromised hosts and individuals >65 years of age, has also been associated with an attenuated antibody response upon revaccination (O'Brien et al., 2007). The explanation for hyporesponses is not fully understood, but depletion of a specific B-cell pool (i.e., clone) has been proposed (Brynjolfsson et al., 2012). In vaccine trials, excessive concentrations of polysaccharide have been found to reduce the antibody response (Dagan et al., 2010b).

Serotypes differ in their ability to cause invasive disease and in prevalence of nasopharyngeal colonization. Brueggemann et al. (2004) studied the invasive disease potential of different *S. pneumoniae* serotypes in children . The authors concluded that some serotypes (including 3, 6B, 15B/C, 19F, and 23F) conveyed a lower risk for invasive disease, and were more frequently isolated as colonizing bacteria than other serotypes (including 1, 4, 7F, 14, and 18C). These results have been confirmed by other groups (Kronenberg et al., 2006; Zemlickova et al., 2010). Low invasive serotypes are associated with higher case-fatality rates and disease in immunocompromised patients, acting as "opportunistic" bacteria, whereas highly invasive serotypes more often infect healthy, immunocompetent individuals, acting as primary pathogens. In parallel, we have previously demonstrated that low invasive *S. pneumoniae* serotypes induced lower IgG titer ratios than did highly invasive serotypes following pneumococcal pneumonia in adults (Athlin et al., 2014). However, patient serum IgG titers alone do not necessarily reflect the functionality of antibodies, and therefore the IgG-dependent capacity to induce opsonization needs to be measured in functional assays.

Studies *in vivo* have revealed impaired B-cell function and antibody production in sepsis survivors, even long after the septic event has resolved (Pötschke et al., 2013; Griffith et al., 2016). However, it is currently unknown whether IPD may induce an impaired antibody response similar to the hyporesponse observed with pneumococcal vaccines. The objective of this explorative study was to investigate the antibody response following IPD in a cohort of IPD patients.

RESULTS

Three Different Types of Responses Are Observed in Opsonophagocytosis

To determine whether IPD related to certain pneumococcal serotypes induces a nonfunctional antibody response, patient sera were collected from two different counties in Sweden. Clinical features of all study patients are outlined in **Supplementary Material**. Patients were diagnosed with pneumonia (38/40 cases), meningitis or ethmoiditis (1 case each). Median age was 59.5 years (range 14–91 years) and 55% ($n = 22$) were women.

We analyzed sera from patients with IPD in a single serotype opsonophagocytic assay (OPA) comprising activated human phagocytes according to a well established protocol. In this functional assay with pre-IPD and post-IPD patient sera, variable OPA responses were observed between serotypes (**Figure 1A**). According to the antibody responses detected, the IPD patients were categorized either as having a functional antibody response as judged by titers in OPA ($n = 16$), a nonresponse ($n = 18$), or a decreased response (reduced titers in OPA; $n = 6$). The majority of patients ($n = 24$; 60%) thus had a nonfunctional antibody response.

IPD With a Low-Invasive Serotype Is Associated With a Nonfunctional Response

To examine whether the pneumococcal capsular serotype can be related to a nonfunctional antibody response in our cohort, patients that had suffered from IPD were divided into two groups according to the invasive potential (low invasive or highly invasive) of the infecting pneumococcal serotype according to Brueggemann et al. (2004). Interestingly, a significant difference was observed as outlined in **Figure 1B**. In total, only 4 of 20 cases (20%) with low invasive serotypes (3, 19F, and 23F) developed a functional antibody response as compared to sera from patients that had been infected with highly invasive pneumococcal serotypes (1, 4, 7F, and 14; $p = 0.022$). Twelve out of 20 cases (60%) developed an increased antibody response in this group.

In a multivariate logistic regression model with functional responses collapsed to a dichotomous response variable (functional antibody response vs. nonfunctional antibody response), infection by a low invasive serotype (3, 19F, and 23F) was the only predictor significantly associated with a nonfunctional antibody response, adjusted for old age and disease severity ($p = 0.015$, **Table 1**). Old age (>65 years), the Charlsons Comorbidity Index, and disease severity (sepsis or septic shock) were predictors not significantly associated with the results obtained by OPA. Time of sera collection post-IPD was also determined as it might influence the quality or quantity of antibodies, but was not associated to the results in OPA (**Tables 1**, **2**).

Serotype Specific IgG Correlates to an Efficient Phagocytic Killing

The concentration of specific anti-capsular IgGs plays an important role for an efficient opsonophagocytosis. IgG levels were therefore determined by ELISA, and the ratio between post- and pre-/acute-IPD IgG titers against capsular antigen of the infecting serotype was calculated for each patient. A higher ratio indicated a stronger increase in anti-capsular antibodies after IPD. As shown in **Table 2**, sera from patients with a functional response in our OPA had an increased median IgG ratio of 2.25 as compared to 1.78 for individuals with a nonresponse and 0.52 for a decreased antibody response ($p = 0.041$). The same trend was also found when the functional and nonfunctional antibody response (nonresponse and decreased response collapsed) was compared ($p = 0.076$) (**Figure 2**). However, no difference in IgG titer ratio was observed between patients infected by *S. pneumoniae* highly invasive serotypes (1, 4, 7F, and 14) compared to low invasive serotypes (3, 19F, and 23F, $p = 0.781$).

Five out of 40 convalescent sera, all from patients infected with serotype 3, did not have an IgG concentration considered to be

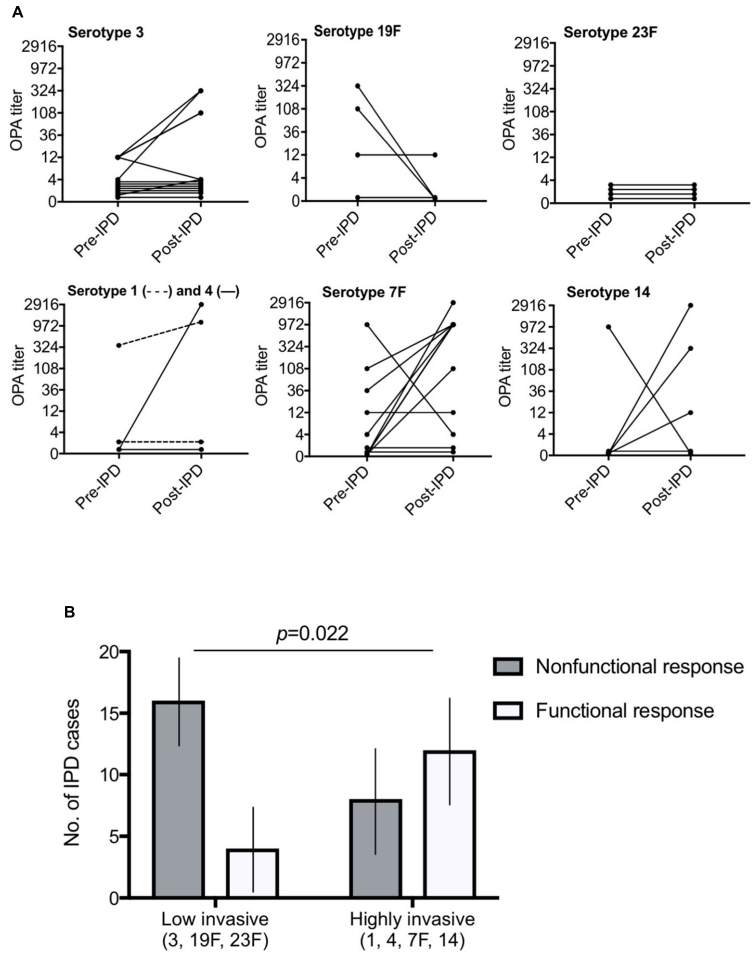

FIGURE 1 | Killing of pneumococci in an OPA revealed mixed responses. Low-invasive *S. pneumoniae* were significantly associated with a nonfunctional response compared to highly invasive serotypes. In **(A)**, 7 different pneumococcal serotypes were analyzed by OPA. The threshold for significant bacterial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

protective for invasive disease (0.35 mg/L) (Siber et al., 2007). All anti-capsular IgG titres are shown in **Supplementary Material**.

Since IgG or IgG2 deficiencies have been associated with an increased risk for IPD (Ekdahl et al., 1997a; Furst, 2009), we determined total IgG or IgG2 in pre-/acute-IPD sera by ELISA. Values according to local clinical guidelines for measurement of deficiencies in IgG (6.7–15.5 g/L) and IgG2 (1.15–5.7 g/L) were used as reference. All titers were above the lower reference limits of Ig concentrations. In conclusion, a relative increase in specific IgG titers in post-IPD sera was associated with enhanced bacterial killing. Moreover, individuals with a nonfunctional antibody response did not suffer from IgG or IgG2 subclass deficiencies.

TABLE 1 | Univariate and multivariate logistic regressions for functional (1) vs. non-functional (0) antibody response (nonresponses and decreased responses collapsed).

Predictor	Univariate odds ratio (95% CI)	*p*	Adjusted odds ratio (95% CI)	*p*
Low-invasive serotype	0.17 (0.04–0.68)	**0.013**	0.13 (0.024–0.67)	**0.015**
Age > 65 years	2.54 (0.63–10.17)	0.188	3.90 (0.70–21.08)	0.119
Sex	0.86 (0.24–3.24)	0.842		
Charlson Comorbidity Index	0.77 (0.57–1.046)	0.095		
Sepsis (severe sepsis or shock vs. sepsis)	0.26 (0.07–1.07)	0.062	0.31 (0.06–1.56)	0.157
Time post-IPD serum (months)	0.98 (0.95–1.02)	0.379		

Significant p-values (≤0.05) are indicated in bold.

TABLE 2 | Distribution of clinical predictors among individuals with different types of functional responses.

Predictor	Functional antibody response (*n* = 16)	Nonresponse (*n* = 18)	Decreased response (*n* = 6)	*p*-value
Low-invasive serotype, *n* (%)	4 (25%)	14 (68%)	3 (50%)	**0.029**[3]
Age > 65 years	3 (21%)	9 (50%)	3 (38%)	0.263[2]
Sex (% female)	63%	44%	66%	0.827[3]
Charlsons Comorbidity Index, median (IQR)[1]	1 (0–3.0)	4 (1.3–6.0)	2.5 (0.0–4.0)	0.067[2]
Severe sepsis or shock *n* (%)	6 (46%)	14 (78%)	3 (50%)	0.080[3]
Time post-IPD serum in months, median (IQR)	3.5 (1.0–20.0)	12.5 (1.0–38.5)	1.0 (1.0–14.5)	0.377[2]
IgG ratio (post-/pre-IPD), median (IQR)	2.25 (1.8–5.5)	1.78 (0.6–2.6)	0.52 (0.1–0.8)	**0.041**[2]

Significant p-values (≤0.05) are indicated in bold. [1]IQR; interquartile range, [2]Kruskal-Wallis, [3]Chi-Square test.

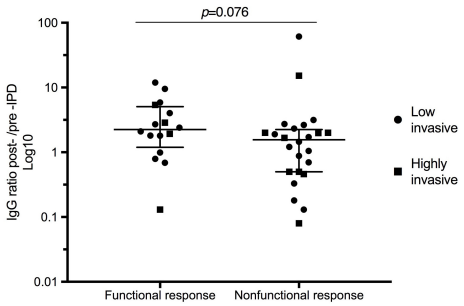

FIGURE 2 | Serotype specific IgG ratios in relation to type of functional antibody response in OPA. Low invasive serotypes are indicated by black dots and highly invasive by squares. Post-IPD IgG antibody concentrations were divided with pre-IPD IgG levels to obtain a ratio for each patient. Median values and interquartile range are indicated. The IgG levels were measured according to a WHO protocol including 007sp reference serum. Patients with a decreased response and a nonresponse were here collapsed as a nonfunctional antibody response in OPA. These patient sera had a lower median serum IgG ratio than patient sera with a functional response.

DISCUSSION

We found that a majority of patients (60%) in our cohort developed a nonfunctional antibody response post-IPD. This type of dysfunctional humoral response to IPD seems to be serotype-dependent. Low invasive serotypes (3, 19F, and 23F) were associated with a nonfunctional response in OPA, compared to highly invasive serotypes (serotypes 1, 4, 7F, and 14) that were correlated to a functional opsonophagocytosis. Prior to this study we found that low invasive serotypes were also associated with a poor IgG response in adults with pneumococcal pneumonia (Athlin et al., 2014). The results raise questions on the humoral antibody response in patients that have survived IPD. Our findings should be regarded as exploratory and descriptive (due to a low number of patients), since this study was not designed to test this hypothesis *per se*. Importantly though, in a previous study with fewer IPD cases and flow cytometry analysis as read out instead of OPA, Musher et al. (2000) also revealed that a high percentage (50%) of convalescent sera from bacteremic patients displayed poorly functional opsonization.

The thickness of the polysaccharide capsule is important for the virulence of pneumococcal serotypes. Weinberger et al. (2009) used fluorescein isothiocyanate (FITC)-dextran exclusion to reveal that serotypes 3, 19F, and 23F have thicker capsules compared to 1, 4, 7F, and 14. The nonfunctional antibody response in low invasive serotypes found in the present study may be due to this difference in capsular structure. Some support for this hypothesis can be attained in investigations on PCV:s, where excessive amounts of polysaccharide in vaccine formulas reduced the antibody response (Dagan et al., 2010b). An explanation for the impaired antibody response observed in polysaccharide vaccines was suggested by Brynjolfsson et al. (2012). In autopsies of mice immunized with meningococcal polysaccharides they found increased apoptosis of polysaccharide specific B-cells (Brynjolfsson et al., 2012). Yet another theory was put forward by Poolman and Borrow (2011). They proposed that the hyporesponse observed in relation to immunization with abundantly encapsuled serotype 3 may be due to absorption of residual antibodies upon exposure.

Other hypotheses for nonresponsiveness to pneumococcal polysaccharide capsules have also been suggested. In a study

by Simell et al. (2011) aging reduced the functionality of anti-pneumococcal antibodies and consequently killing of *S. pneumoniae* by opsonophagocytosis. In our material, age >65 years was not associated with a nonfunctional antibody response. Cases of nonfunctional antibody responses were observed in several younger patients (for example, patients aged 14–28 years as indicated in **Supplementary Material**), suggesting that other mechanisms than immunosenescence are involved.

The powerful immunological reaction observed in septic patients is followed by an immune suppressive state that might cause prolonged defects in humoral immunity, as demonstrated in mice (Pötschke et al., 2013). However, previous studies on impaired antibody responses have focused on evaluation of vaccine efficacy rather than natural immunization by IPD. Prior carriage of *S. pneumoniae* in the nasopharynx, as well as prior bacteremia has been associated with a hyporesponse to PCV and PPV23, respectively (Ekdahl et al., 1997b; Dagan et al., 2010a). Ekdahl and collaborators suggested that IgG deficiency caused the poor antibody response in patients with prior IPD when immunized with PPV23. We excluded IgG deficiency as the cause of a nonfunctional antibody response in our study.

The high rate of a nonfunctional antibody response in the present study contrasts to the low rate of recurrent IPD in epidemiological studies. However, nasopharyngeal carriage rates of pneumococci are low in the adult population, even for low invasive serotypes (Regev-Yochay et al., 2004). The risk for IPD with the same serotype at two occasions is therefore theoretically very low. King et al. (2003) suggested that host factors of immunosuppression and underlying illness were typical in patients with IPD reinfection, whereas only two serotypes (6B and 18C) were associated with reinfection . It cannot be excluded, however, that a nonfunctional antibody response induced by IPD resolves over time. In a recent vaccine trial, the hyporesponse induced in toddlers by a combined schedule of PCV7 and PPV23 was not sustained when the children were in preschool age (Licciardi et al., 2016).

In the present study, serum concentrations of serotype specific IgG were determined, and a ratio between post and pre-IPD serum was calculated (**Figure 2**). Patients with a functional antibody response had higher IgG titer ratios, which may indicate that an antibody increase is necessary for efficient pneumococcal killing. Nevertheless, a divergence in IgG ratios and results obtained by OPA was found in several cases. These findings are also supported by a Japanese study, where infants immunized with PCV7 demonstrated protective IgG titers post-IPD, but all 17 patients had suboptimal responses in OPA. Low avidity of serotype-specific antibodies was suggested as the cause (Oishi et al., 2013). We tested the hypothesis that lower avidity of anti-capsular IgG antibodies post-IPD may contribute to the discrepancy in some patients that were nonresponsive in OPA in spite of a high IgG-ratio. We did not, however, find any changed avidity that could explain the discrepancies observed with high IgG ratios and a nonresponsive opsonophagocytosis (data not shown). Another possible explanation for diverging results between the serum tested in OPA and IgG ratios may be differences in levels of anti-pneumococcal polysaccharide IgM levels. Interestingly, Park and Nahm (2011) found that low levels of IgM in older adults contributed to a poor opsonization of pneumococci.

A limitation of this study was that we only included seven of more than 90 known pneumococcal serotypes. The rationale for this was that these sera were the only specimens from patients with IPD identified in our biobanks during the study period. From a clinical point of view, however, these 7 serotypes are considered to be among the most important ones (Hausdorff et al., 2000). Furthermore, serum samples were collected with a great variation in time prior to and after the episode of IPD that has to be considered when interpreting the results of this study. Therefore, we address the importance of conducting a prospective study where the collection pre- and post-IPD sera is defined as per protocol. Finally, we did not collect any vaccination data in this study. Yet, it has to be noticed that the usage of the 23-valent pneumococcal polysaccharide vaccine, which is recommended for adults, was very low in Sweden during the study period (only 0.08 prescriptions per 1,000 adults) (The Board of Health and Welfare, 2018).

CONCLUSION

A nonfunctional antibody response may follow an episode of IPD. The risk of a poor humoral response was in our material associated to old age and infection with a pneumococcal serotype with low invasive potential.

MATERIALS AND METHODS

Patient Sera

Patient characteristics are outlined in the **Supplementary Material**. Pre/acute-IPD and post-IPD sera used in this exploratory study were convenience samples that had been collected previously. Fourteen cases were obtained from a cohort at Örebro University Hospital and were collected in 1999–2002, and 26 cases from Skåne University Hospital (Biobank Skåne, Lund) in 2006–2017. No statistically significant differences in age, Charlsons Comorbidity Index or gender distribution were found between the cohorts. Amongst the 40 patients, paired sera from 20 patients were collected in the acute phase of IPD at admission to hospital and after 5 weeks of convalescence (median 34 days; range 20–81 days). For another 20 patients, paired sera were collected from patients 2–24 months prior to (pre-IPD; median 12 months) and 6–60 months after an episode of IPD (post-IPD; median 26 months) (**Supplementary Material**). A pre-IPD or acute-IPD serum is designated as "pre-IPD" in the study.

Based upon the study by Brueggemann et al. (2004), we sorted pneumococcal serotypes into two groups according to invasive potential. Twenty paired sera (pre-IPD and post-IPD) from patients infected by low invasive serotypes were included (serotype 3, $n = 12$; 19F, $n = 4$; 23F, $n = 4$), and for comparison, 20 paired sera from patients infected by highly invasive serotypes (serotype 7F, $n = 11$; 14, $n = 5$; 1, $n = 2$; 4, $n = 2$) were also added to the analyses. The medical history of all patients was reviewed.

Disease severity of IPD was determined according to SIRS criteria (Bone et al., 1992), and comorbidities were defined according to the Charlsons Comorbidity Index (Charlson et al., 1987).

Pneumococcal Serotyping

Blood for culture was collected at admission to hospital from all patients. Bactec™ blood culturing system (Becton Dickinson, MD, United States) was used for isolation, and pneumococcal isolates were grown on 10% blood agar plates at 37° CO_2 overnight. The Quellung test was performed for serotyping with specific rabbit antisera (Statens Serum Institut [SSI], Copenhagen, Denmark) (Habib et al., 2014). *S. pneumoniae* serotype 1, 3, 4, 7F, 19F, and 23F (control isolates from BEI resources, Manassas, VA, United States) were used in the OPA (Romero-Steiner et al., 1997).

Enzyme-Linked Immunosorbent Assay (ELISA) for Determination of Capsular Antigen IgG

Homologous IgG antibody titers were quantified by ELISA according to a protocol from the World Health Organization (WHO) (Slotved et al., 2009). Serotype 22F specific capsular polysaccharide and cell-wall polysaccharide (both from SSI) were used for blocking of unspecific binding, and 007sp [kindly provided by Dr. Mustafa Akkoyunlu at U.S. Food and Drug Administration (FDA), Silver Spring, MD, United States] was used as reference serum. A ratio between pre-/acute-IPD and post-IPD was calculated for each patient.

Opsonophagocytic Assay (OPA)

The functionality of antibody titers were determined by a single serotype OPA according to a protocol available at https://www.vaccine.uab.edu/uploads/mdocs/cdc-ops3.pdf (accessed 26/06/2018). The human promyelocytic cell-line HL60 was differentiated into neutrophils by propagation with addition of 0.8% dimethylformamide (DMF) for 5–6 days before use in the OPA. To prevent bacterial clumping, the opsonization and phagocytosis steps were performed with microtitre plates on a mini orbital shaker (700 rpm) as specified in a recent multiplex OPA protocol (WHO reference laboratory at University of Alabama). Patient sera were tested in at least two separate experiments in duplicates against the homologous serotype that had caused IPD. OPA titers were defined as the serum dilution that killed 50% of bacteria compared to controls containing no patient serum but cells only. A minimum titer of 1:8 in OPA has been shown to confer protection in mice and infants, and was therefore selected as cut-off (Romero-Steiner et al., 2006). Pre-IPD and post-IPD sera were tested for all patients. An increase in pneumococcal killing or unchanged OPA titer above the 1:8 titer threshold was termed functional antibody response and a decrease in OPA titer was termed decreased antibody response. OPA titers below the threshold before and after IPD were designated as a nonresponse. To test sera and exclude a residual antibiotic activity, a previously published method was used (Driscoll et al., 2012) with some modifications. Agar susceptibility testing was performed by adding sera to holes (2 mm in diameter) on blood agar plates inoculated with respective target strain, the same ones used in the OPA. After overnight culture, absence of a zone of bacterial killing indicated no antibiotic activity.

ELISA for Determination of Total IgG and IgG2

Maxisorp™ plates (Nunc, Waltham, MA, United States) were coated with rabbit anti-human IgG (Sigma, Darmstadt, Germany) or mouse anti-human IgG2 (Sigma) overnight at +4°C. Plates were washed in wash buffer (PBS pH 7.4, 0.05% Tween20), blocked with blocking buffer (PBS, 1% skimmed milk, 0.05% Tween20) for 1 h at room temperature (RT) followed by an additional wash. Patient and calibration sera for IgG (Dako, Glostrup, Denmark) and pure IgG2 (The Binding site, San Diego, CA, United States) were diluted in a blocking buffer, titrated on plates and incubated for 1 h at RT. After one final wash, all wells were incubated for 20 min with horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG (Dako P0214). The optical density was measured at 405 nm, and absorbance between calibration sera and patient sera was compared.

Statistical Analyses

Three groups (increased antibody response, nonresponse and decreased antibody response) were compared using the Chi-squared test for categorical variables and the Kruskal-Wallis test for continuous variables. Univariate logistic regressions were performed to assess the association between the type of antibody response and clinical as well as bacterial predictors. In all regression analyses, the two types of nonfunctional responses (nonresponse or decreased response) were collapsed to create a binary outcome variable. A multivariate model was fitted using the purposeful selection algorithm. Briefly, all variables with a *p*-value below 0.2 were added to the multivariate model. The variable with the highest *p*-value was stepwise removed and the model was run again until only significant variables or variables that affected the adjusted odds ratios or *p*-values of the remaining variables remained. Comparisons of difference between the two groups in **Figure 1B** was made by Fisher's exact test and in **Figure 2** by Mann-Whitney *U* test. Statistical analyses were made in the SPSS® v.22 software or in Prism Graphpad® 7 and a level of significance was set to $p \leq 0.05$.

DATA AVAILABILTY STATEMENT

We will make materials, data, and associated protocols promptly available to readers upon request.

ETHICS STATEMENT

This study was approved by the Regional Ethics Board at Lund University Hospital (2012/86) and the Ethics committee of the Örebro County Council (868–1999). The methods were done in accordance with the Helsinki declaration and regulations of the abovementioned Universities and Biobank Skåne.

AUTHOR CONTRIBUTIONS

NL designed the study, analyzed the data, performed the experiments and drafted the manuscript. FU did the experiments and contributed to the writing. JA designed and initiated the study and revised the manuscript. FR helped in designing the study, performed the statistical analysis and revised the manuscript. H-CS contributed to the design of the study and critically revised the manuscript. SA analyzed the patient data, collected the patient sera and critically revised the manuscript. KR initiated and designed the study and critically revised the manuscript. All authors have approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02746/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary Table S1

Serotypes with low invasive potential are associated with an impaired antibody response in invasive pneumococcal disease

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Table S1. Clinical data on all patients included in the present study.

Patient ID	Serotype	Invasive potential	Sex	Age	Charltons comorbidity index	SIRS category	Period of serum collection post-IPD (months)	Anti capsular IgG pre/acute (mg/L)	Anti capsular IgG convalescent (mg/L)	Post- /pre IPD IgG titer ratio	Functional antibody response in OPA
1	1	**High**	F	61	2	Severe	1	3.10	3.26	1.05	Nonresponse
2	1	**High**	F	32	0	Severe	31	4.30	4.25	0.99	Functional response
3	3	**Low**	M	59	2	Severe	1	0.19	11.6	61.1	Nonresponse
4	3	**Low**	M	48	0	Sepsis	1	0.73	1.50	2.0	Decreased response
5	3	**Low**	M	78	7	Chock	60	0.63	0.05	0.08	Nonresponse
6	3	**Low**	F	55	1	Severe	30	0.18	0.36	2.00	Nonresponse
7	3	**Low**	F	53	1	Missing	26	0.40	0.73	1.81	Functional response
8	3	**Low**	F	61	2	Severe	35	0.18	0.30	1.67	Nonresponse
9	3	**Low**	M	70	6	Severe	38	0.09	0.05	0.50	Nonresponse
10	3	**Low**	F	46	0	Severe	25	0.75	0.1	0.13	Decreased response
11	3	**Low**	F	56	4	Chock	1	0.69	1.45	2.10	Functional response
12	3	**Low**	M	60	1	Severe	10	0.73	1.31	1.8	Functional response
13	3	**Low**	M	54	3	Chock	1	5.50	14.52	2.64	Nonresponse
14	3	**Low**	F	54	1	Severe	1	1.79	5.63	3.15	Nonresponse
15	4	**High**	M	87	7	Severe	1	0.55	0.67	1.21	Nonresponse
16	4	**High**	F	75	3	Sepsis	1	0.70	6.67	9.50	Functional response
17	7F	**High**	F	89	5	Sepsis	1	8.90	7.03	0.79	Functional response
18	7F	**High**	F	67	2	Sepsis	1	10.30	28.70	2.87	Functional response
19	7F	**High**	F	57	1	Sepsis	1	15.01	4.95	0.33	Decreased response
20	7F	**High**	M	39	3	Severe	1	3.45	8.32	2.4	Functional response
21	7F	**High**	M	31	0	Sepsis	1	2.00	11.76	5.88	Functional response
22	7F	**High**	F	28	0	Chock	39	1.10	2.1	1.90	Nonresponse
23	7F	**High**	F	67	8	Sepsis	19	0.62	1.7	2.70	Functional response
24	7F	**High**	M	55	1	Sepsis	60	0.60	9.1	15.20	Nonresponse
25	7F	**High**	F	82	6	Chock	24	0.52	1.03	1.98	Nonresponse
26	7F	**High**	F	25	0	Sepsis	23	1.03	4.15	4.03	Functional response
27	7F	**High**	M	38	0	Chock	1	0.70	8.30	11.90	Functional response
28	14	**High**	F	47	0	Sepsis	0.5	7.11	38.2	5.40	Functional response
29	14	**High**	M	67	4	Severe	25	4.75	2.2	0.46	Nonresponse
30	14	**High**	M	64	4	Sepsis	6	4.74	9.1	1.93	Functional response
31	14	**High**	M	40	1	Chock	12	37.2	4.75	0.13	Functional response
32	14	**High**	F	91	4	Sepsis	1	5.96	1.07	0.18	Decreased response
33	19F	**Low**	F	83	4	Severe	1	8.30	5.81	0.70	Decreased response
34	19F	**Low**	M	63	4	Chock	11	8.47	14.61	1.72	Decreased response
35	19F	**Low**	F	24	0	Sepsis	55	8.77	6.1	0.69	Functional response
36	19F	**Low**	M	14	6	Sepsis	52	14.7	7.3	0.50	Nonresponse
37	23F	**Low**	M	85	4	Severe	1	2.80	9	2.73	Nonresponse
38	23F	**Low**	F	85	6	Sepsis	1	3.00	6.96	2.32	Nonresponse
39	23F	**Low**	F	84	4	Sepsis	1	22.40	17.92	0.88	Nonresponse
40	23F	**Low**	M	70	4	Severe	1	1.10	2	1.45	Nonresponse

Paper II

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RESEARCH ARTICLE
Host-Microbe Biology

Corrected and Republished from: A Nonfunctional Opsonic Antibody Response Frequently Occurs after Pneumococcal Pneumonia and Is Associated with Invasive Disease

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PUBLISHER'S NOTE The American Society for Microbiology and *mSphere* would like to inform the readers that this article is a corrected and republished version of https://doi.org/10.1128/mSphere.00925-19, which was retracted (https://doi.org/10.1128/mSphere.01101-20). As stated in the retraction notice, after publication, the authors discovered that "incorrect data were used in the analyses of anticapsular Ig concentrations." This led to a number of inaccuracies in the manuscript, too many to be addressed through a correction. However, as the authors also state, "the main conclusions of the study, related to the results from the OPA, are still valid, i.e., an episode of pneumococcal pneumonia is often an immunizing event, resulting in an improved serotype-specific adaptive immune status as measured with OPA, but a nonfunctional antibody response may occur and is significantly associated with bacteremia." Therefore, the authors were allowed to submit an entire, corrected manuscript for consideration to the journal. The manuscript was editorially reviewed and subsequently accepted.

ABSTRACT Naturally acquired opsonic antipneumococcal antibodies are commonly found in nonvaccinated adults and confer protection against infection and colonization. Despite this, only limited data exist regarding the adaptive immune response after pneumococcal exposure. To investigate the dynamics of naturally acquired antipneumococcal immunity in relation to an episode of infection, opsonic antibody activity was studied with paired acute-phase and convalescent-phase sera obtained from 54 patients with pneumococcal community-acquired pneumonia (CAP) using an opsonophagocytic assay (OPA). Results were compared with clinical characteristics and anticapsular immunoglobulin (Ig) concentrations. Interestingly, a nonfunctional opsonic antibody response (characterized by a decreased convalescent-phase serum OPA titer compared to that of the acute-phase serum or undetectable titers in both sera) was observed in 19 (35%) patients. The remaining individuals exhibited either an increased convalescent-phase OPA titer (n = 24 [44%]) or a detectable, but unchanged, titer at both time points (n = 11 [20%]). Invasive pneumococcal disease (i.e., bacteremia) was significantly more common among patients with a nonfunctional convalescent-phase response than in patients with other convalescent-phase responses. Anticapsular Ig concentrations were higher among patients with detectable convalescent-phase OPA titers (P = 0.003), and the greatest Ig concentration increase was observed among patients with an increased convalescent-phase response (P = 0.002). Our findings indicate that an episode of pneumococcal infection may act as an immunizing event. However, in some cases when patients with CAP also suffer from bacteremia, a nonfunctional opsonic antibody response may occur. Furthermore, the results suggest that factors other than anticapsular Ig concentrations determine opsonic antibody activity in serum.

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IMPORTANCE Numerous reports on the dynamics of antipneumococcal immunity in relation to immunization with pneumococcal vaccines and on the prevalence of naturally acquired immunity in various populations have been published. In contrast, studies on the dynamics of the humoral immune response triggered by pneumococcal infection are scarce. This study provides valuable information that will contribute to fill this knowledge gap. Our main results indicate that a functional immune response may fail after CAP, predominantly among patients with simultaneous bacteremia.

KEYWORDS adaptive immune response, bacteremia, immunoglobulins, opsonization, phagocytosis, pneumonia, *Streptococcus pneumoniae*, adaptive immunity

Streptococcus pneumoniae is a human respiratory tract pathogen responsible for substantial morbidity and mortality on a global scale, causing community-acquired pneumonia (CAP), acute otitis media, and rhinosinusitis as well as invasive pneumococcal disease (IPD) (1, 2). The capsular polysaccharide (CPS), which mediates protection from phagocytosis, is the most important virulence factor of *S. pneumoniae*. CPSs of various chemical compositions may induce the production of specific immunoglobulins (Igs) upon exposure, defining pneumococcal serogroups and serotypes. Serotype-specific anti-CPS Igs that mediate opsonophagocytosis are generally regarded as the most important factor for immunologic protection against pneumococcal infection, and purified CPSs from many serotypes have therefore been used in pneumococcal vaccine formulae for more than half a century (3, 4).

Naturally acquired pneumococcal-antibody-mediated immunity is prevalent in young adults and confers protection against colonization and infection but declines with age (5, 6). Episodes of asymptomatic nasopharyngeal colonization induce protective adaptive immunity, but studies on the dynamics of naturally acquired immunity related to episodes of clinically significant pneumococcal infection are scarce (7). Interestingly, a delayed or absent anti-CPS Ig increase has been described after pneumococcal bacteremia (8–10). This observation raises the question whether infection by pneumococci, unlike an episode of asymptomatic colonization, may fail to induce immunologic boosting. It should, however, be noted that naturally acquired pneumococcal immunity is dependent on antibodies directed against protein antigens and cell-mediated immunity in addition to anti-CPS Ig (11, 12). Thus, the functionality of this response may not be fully evaluated solely by the measurement of anti-CPS Ig concentrations, as has been done in the above-mentioned studies.

Although pneumococcal vaccination has been highly successful in reducing morbidity and mortality, hyporesponsiveness may occur after vaccination in certain clinical situations (13–16). Suboptimal vaccine responses, as well as waning naturally acquired immunity, are linked to reduced opsonic Ig function and diminished B-cell populations (5, 17–21). Consequently, studies on the adaptive immune response after pneumococcal infection are important for increased understanding of a mechanism(s) that may impact vaccine-induced immunity.

In the current study, we investigated the correlation between opsonic antibody activity in serum against the infecting pneumococcal serotype, as measured by an opsonophagocytic assay (OPA), and disease severity as well as other clinical factors in patients with pneumococcal CAP. Serum samples were obtained from a cohort of CAP patients with previously measured total Ig concentrations against CPS of the infecting serotype and pneumococcal DNA load in plasma (22). Interestingly, lower levels of anti-CPS Ig were observed in acute-phase sera from bacteremic patients than nonbacteremic patients, and any distinct Ig increase in convalescent-phase sera did not occur in more than half of the cases in that study. By analyzing these samples with a functional method such as OPA, which may be affected also by Ig directed to non-CPS targets, and by testing the functionality rather than the quantity of antibodies (3, 12), we aimed to further improve the understanding of the naturally occurring immune response to pneumococcal infection.

TABLE 1 Patient characteristics and their relation to outcome in an OPA[a]

Patient characteristic	Values for: All patients	Patients with an acute-phase serum OPA titer of: >1	1	*P*	Patients with a convalescent-phase serum OPA titer that was: Increased	Unchanged	Decreased or undetectable	*P*
No. (%)	54 (100)	28 (52)	26 (48)		24 (44)	11 (20)	19 (35)	
Age [median no. of yrs (range)]	69 (23–91)	75 (23–91)	60 (31–89)	**0.032**	61 (31–90)	78 (46–89)	69 (23–91)	**0.028**
Age >65 yr [no. (%)]	29 (54)	19 (68)	10 (39)	**0.030**	10 (42)	9 (82)	10 (53)	0.086
Female [no. (%)]	25 (46)	13 (46)	12 (46)	0.98	10 (42)	4 (36)	11 (58)	0.43
Current smoking [no. (%)]	16 (30)	8 (29)	8 (31)	0.86	7 (29)	2 (18)	7 (37)	0.56
Comorbidity[b] [no. (%)]	26 (48)	14 (50)	12 (46)	0.78	10 (42)	8 (73)	8 (42)	0.19
CRB-65 [median (range)]	1 (0–4)	1 (0–4)	1 (0–3)	0.41	1 (0–4)	1 (0–3)	1 (0–3)	0.86
Bacteremia [no. (%)]	16 (30)	6 (21)	10 (39)	0.17	5 (21)	1 (9)	10 (53)	**0.019**
Sepsis[c] [no. (%)]	34 (63)	19 (68)	15 (58)	0.44	14 (58)	7 (64)	13 (68)	0.79
SOFA score increase [median (range)]	2 (0–5)	2 (0–5)	2 (0–4)	0.17	2 (0–5)	2 (1–4)	2 (0–4)	0.81
Symptom duration[d] [median no. of days (range)]	3 (0–36)	4 (0–36)	3 (0–11)	0.51	3 (0–21)	1 (0–36)	5 (0–11)	0.078
CRP maximum [median (range)]	298 (42–773)	262 (42–611)	328 (44–773)	0.44	342 (62–611)	171 (42–495)	315 (44–773)	0.050
No. of day between sample collections [median (range)]	31 (20–82)	31 (20–81)	29 (24–82)	0.27	31 (25–81)	33 (25–69)	29 (20–82)	0.29

[a]Characteristics of 54 patients with pneumococcal CAP and results in an opsonophagocytic assay (OPA) for corresponding paired acute-phase and convalescent-phase sera. Patients are grouped according to a detectable (>1) or undetectable (1) acute-phase serum OPA titer, as well as the change of the OPA titer from the acute phase to convalescence. Associations of these outcomes with individual patient characteristics were analyzed. Statistically significant differences between groups ($P < 0.05$) are indicated in bold type. CRB-65, confusion of new onset, respiratory rate of 30 breaths/min, systolic blood pressure of <90 mm Hg or diastolic blood pressure of 60 mm Hg or less, age of 65 years or older; SOFA, sequential organ failure assessment; CRP, C-reactive protein.

[b]One or more of any of the following diagnoses: chronic obstructive pulmonary disease (COPD), heart disease, diabetes mellitus, liver disease, renal insufficiency, neoplasm, or immunosuppression.

[c]Fulfillment of the Sepsis-3 definition (33).

[d]Number of days with symptoms of pneumonia before collection of acute-phase serum.

RESULTS

Available acute-phase and convalescent-phase sera from patients ($n = 54$) infected with a serotype included in the 13-valent pneumococcal conjugate vaccine (PCV13) were obtained from a cohort of patients with radiologically confirmed CAP (22). Demographic and clinical characteristics of the individuals studied are presented in Table 1, and individual data used in analyses are outlined in Table S1 in the supplemental material.

A nonfunctional opsonic antibody response is observed in approximately one-third of patients after CAP. To investigate the adaptive immune response in patients with CAP, an OPA was performed on acute-phase and convalescent-phase sera by measuring opsonic antibody activity against the infecting pneumococcal serotype. OPA titers were calculated based upon the dilution of serum resulting in >50% bacterial killing in the presence of complement and phagocytic cells compared to the level of bacteria in negative controls. If no bacterial killing was observed (i.e., the serum OPA titer was undetectable), the tested serum was assigned an OPA titer of 1. The OPA titers yielded, with corresponding sera, from each individual patient are visualized in Fig. 1. Three divergent convalescent-phase opsonic antibody responses were observed when paired sera were compared; the convalescent-phase serum OPA titer was either increased (44%) (Fig. 1A), unchanged (20%) (Fig. 1B), or undetectable/decreased (35%) (Fig. 1C) compared to the acute-phase serum OPA titer. Patients with decreased or undetectable convalescent-phase serum OPA titers were defined as exhibiting a nonfunctional convalescent-phase opsonic antibody response.

To exclude Ig deficiencies that may explain a low opsonic activity, IgG and IgG2 concentrations were measured in all acute-phase sera, and lower reference limits defined in clinical guidelines (6,700 μg/ml and 1,150 μg/ml, respectively) were used for evaluation. No significant IgG or IgG2 deficiencies were detected in any of the patient sera tested.

Bacteremia is associated with a nonfunctional convalescent-phase opsonic antibody response. To explore whether the opsonic antibody function was associated with any clinical or demographic features, results from OPA were correlated with

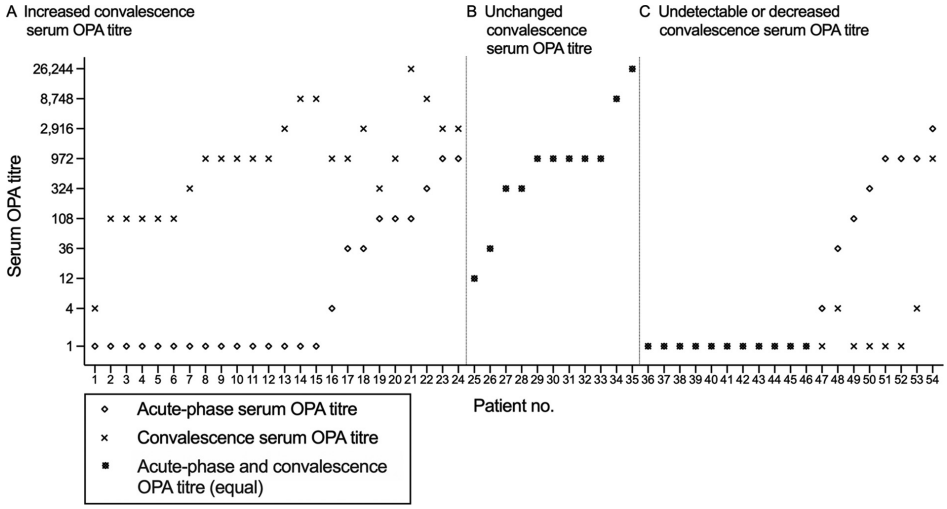

FIG 1 Acute-phase and convalescent-phase opsonic antibody activities in sera from patients with pneumococcal pneumonia. Opsonophagocytic assay (OPA) titers in paired sera during the acute phase and convalescence of 54 patients with pneumococcal community-acquired pneumonia were obtained. Detectable OPA titers (i.e., an OPA titer of >1) were found with sera from 28 (52%) and 38 (71%) patients during their acute phase and convalescence, respectively. The remaining samples did not induce any bacterial killing in the presence of complement and phagocytes (i.e., the OPA titer was 1). (A) Convalescent-phase sera from almost half of the studied patients ($n = 24$ [44%]) yielded increased OPA titers compared to titers in the corresponding acute-phase sera. (B) With sera from 11 patients (20%), equal OPA titers were observed at both sampling times. (C) Finally, OPA titers were undetectable (i.e., their OPA titer was 1) at both sampling times with sera from 11 patients (20%), and decreased OPA titers were yielded with convalescent-phase sera from 8 patients (15%) compared to corresponding acute-phase sera. These patterns were assessed as nonfunctional opsonic antibody responses (35%).

various factors as outlined in Table 1. In these analyses, we compared patients with undetectable or detectable acute-phase serum OPA titers (i.e., an OPA titer of 1 or >1, respectively) and patients with differing convalescent-phase responses (Fig. 1). During the acute phase, undetectable OPA titers were significantly more common among patients younger than 65 years of age (68% versus 39%; $P = 0.032$) than among older patients. However, the lowest median age (61 years) was found in those with an increased convalescent-phase OPA titer ($P = 0.028$). To further investigate any association of opsonic antibody response with age, outcomes in the OPA were compared between stratified age groups (Table 2). No statistically significant differences could be observed using these small groups, but it was noted that 7/10 patients aged <50 years developed an increased convalescent-phase response, whereas only 2/13 patients among those aged ≥80 years developed an increased response.

TABLE 2 Outcome in OPA stratified by age group

Age group (yr)	No. of patients	No. (%) of individuals with: An acute-phase serum OPA titer of: >1	1	A convalescent-phase serum OPA titer that was: Increased	Unchanged	Decreased or undetectable
<50	10	5 (50)	5 (50)	7 (70)	1 (10)	2 (20)
50−59	10	2 (20)	8 (80)	4 (40)	1 (10)	5 (50)
60−69	8	4 (50)	4 (50)	5 (63)	0	3 (38)
70−79	13	7 (54)	6 (46)	6 (46)	4 (31)	3 (23)
≥80	13	10 (77)	3 (23)	2 (15)	5 (38)	6 (46)
P		0.12		0.083[a]		

[a]The chi-square test was performed with dichotomous outcome variables by pooling the "unchanged" and "decreased or undetectable" groups.

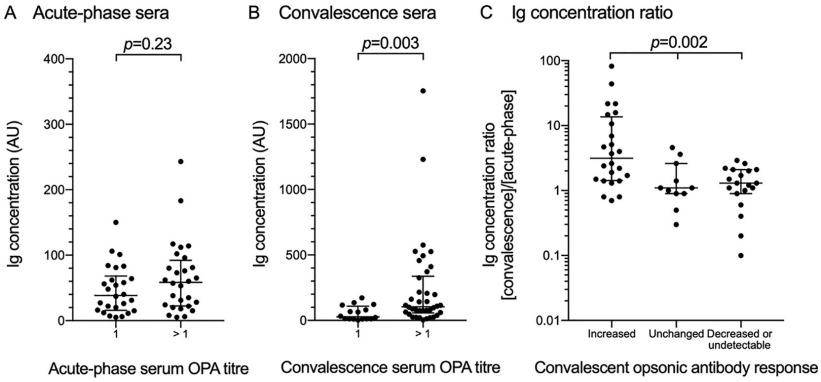

FIG 2 Antipneumococcal Ig concentrations compared to OPA titers and convalescent-phase opsonic antibody responses. Serotype-specific anti-capsular polysaccharide (anti-CPS) total Ig concentrations (in arbitrary units [AU]) (measured by ELISA) in sera from 54 patients with pneumococcal community-acquired pneumonia in relation to corresponding serum OPA titers. Ig concentrations and OPA titers were measured during both the acute phase of infection (A) and during convalescence (B). (C) The Ig concentration fold change from acute phase to convalescence (convalescence/acute-phase Ig concentration ratio) in serum from each patient was compared with their opsonic antibody response. Error bars indicate the interquartile range of median Ig concentration and median Ig concentration ratio.

Importantly, bacteremia was more common among patients with a nonfunctional convalescent-phase opsonic antibody response (53%) (Fig. 1C) than among those with either an increased (21%) (Fig. 1A) or unchanged (9%) (Fig. 1B) convalescent-phase OPA titer (P = 0.019), suggesting an association between IPD and an attenuated immune response against pneumococci after infection.

OPA titers and anticapsular Ig concentrations correlate during the convalescent phase but not the acute phase. To determine whether serotype-specific anti-CPS Ig concentrations may explain the different titers observed in the OPA, total Igs reacting with CPSs were measured by an enzyme-linked immunosorbent assay (ELISA) (22). Acute-phase and convalescent-phase sera from all patients are presented in Fig. 2 and related to the results in the OPA. During the convalescent phase, the median Ig level was higher among individuals with detectable OPA titers compared to those with undetectable OPA titers (105 arbitrary units [AU] versus 28 AU; P = 0.003), while no difference was observed during the acute phase (Fig. 2A and B). In parallel, convalescent/acute-phase Ig concentration ratios (Ig fold change) differed between the groups with diverging convalescent-phase responses, with the greatest median Ig fold change observed among patients with an increased convalescent-phase OPA titer (P = 0.002) (Fig. 2C). Albeit the above associations between outcome in OPA and Ig levels/ratios were observed, low Ig levels were also found with patient sera exhibiting high OPA titers and vice versa. These results indicate that factors other than anticapsular Ig may have influenced opsonic activity of the studied patient sera.

DISCUSSION

In the present study, we used a single-serotype OPA to study the antibody-mediated opsonic activity against pneumococci in sera from adult patients with pneumococcal CAP. By examining paired acute-phase and convalescent-phase sera, the dynamics of the immune response could be presented and assessed. Almost half of the patients (44%) responded with a functional immune response characterized by an improved opsonic antibody function in sera collected approximately 1 to 3 months after the acute phase of infection. Conversely, we found that a surprisingly large proportion of the patients (35%) failed to develop detectable OPA titers or exhibited decreased opsonic activity in serum during convalescence, a response that was considered nonfunctional. The remaining patients (20%) exhibited detectable, but

unchanged, OPA titers at both sampling times, a pattern that is not easily interpreted as functional or nonfunctional. Invasive pneumococcal disease was clearly associated with a nonfunctional opsonic antibody response. Most importantly, our observations reveal that an episode of clinically significant pneumococcal infection often results in immunization and induces an improved humoral immune status against the infecting serotype; however, a nonfunctional opsonic antibody response may occur in some cases.

It is an interesting observation that 28 individuals developed pneumococcal CAP despite exhibiting functional serum opsonic antibody activity during the acute phase, 6 of whom had bacteremia. Protection against mucosal pneumococcal infection is, however, dependent on a well-functioning innate and humoral immunity (11); the latter was not assessed in the current study. Although it is a well-known fact that opsonic antibodies are important for preventing IPD (6, 7, 23), we did not find a significant association between undetectable acute-phase serum OPA titers and bloodstream infection in the current study. This may be due to the use of a low cutoff (i.e., an OPA titer of >1), as an OPA titer as high as 64 has previously been suggested to correlate with protective immunity in adults (3). However, we chose this cutoff to avoid arbitrary grouping of the patients, as more studies are needed to clearly establish a relationship between specific serum OPA titers and protection from pneumococcal infection in adults (3).

Few previous studies on the dynamics of naturally acquired pneumococcal immunity related to an infection episode exist. An association of pneumococcal antigenemia with an attenuated convalescent-phase quantitative anti-CPS Ig response was, however, reported in 1976 (8), and decreased serotype-specific Ig concentrations following pneumococcal infection have been described in case reports (9, 10). In one previous study (24), 88% of convalescent-phase sera from patients with nonbacteremic pneumonia had functional opsonic Ig compared to 50% of sera from bacteremic patients. Similarly, we observed that 82% of nonbacteremic patients and 44% of bacteremic patients had detectable OPA titers in their convalescent-phase sera. These findings suggest that impaired opsonic activity against the homologous serotype may be common subsequent to IPD, but the precise mechanism(s) to this reaction is at present unknown.

Naturally acquired immunity to pneumococci during a life span is characterized by decreasing anti-CPS Ig levels as well as opsonic Ig function with increasing age, explained by infrequent immune boosting due to exposure to the bacterium and immunosenescence (5, 6, 25). Pneumococcal carriage rates among adults are low in northern Europe, thus possibly contributing to infrequent pneumococcal exposure and decreased immunity against pneumococci (26). However, we observed an association between age above 65 years and detectable acute-phase OPA titers, which may contradict the results of these previous studies. On the other hand, the patients who responded with a functional immune response were generally younger, which further supports that high age is linked to impaired immunity against *S. pneumoniae*.

An inability to mount an appropriate antibody response, hyporesponsiveness, has also been seen in studies on pneumococcal vaccines (13–16, 18). This is thought to be associated with an exhausted memory B-cell pool due to high levels of circulating CPS, either at the time of immunization or during previous exposure. Since the T-cell-independent response induced by pure polysaccharide antigens does not result in memory B-cell propagation, adaptive immunity may be reduced after repeated exposures (19–21). Likewise, the impaired immune response observed in the current study might also have been influenced by exposure to a high level of circulating CPS, either during previous colonization or infection or during the studied CAP episode. This is in line with previous studies that suggest an association between antigenemia and a poor quantitative Ig response (8, 10). Moreover, we found that the two patients with the highest pneumococcal DNA concentration in plasma (see Table S1 in the supplemental material) exhibited nonfunctional convalescent-phase responses. This observation further supports the hypothesis that systemic dissemination during infection may prevent a positive adaptive immune response. However, impaired immune responses were also observed in patients with low levels of circulating Spn9802 DNA.

We recently investigated the association between OPA titers and different serotypes in 40 patients with IPD, including 14 of the currently studied patients, and found that serotypes with a thick CPS layer were more prone to cause an impaired convalescent-phase opsonic response (27). This may be due to a high concentration of CPS in the bloodstream caused by serotypes producing a thick capsule and resulting in a hampered convalescent-phase B-cell response. In the current study, the proportions of these infecting serotypes did not differ between bacteremic and nonbacteremic patients (data not shown), and no measurement of free CPS in the bloodstream was available, which is why we cannot conclude whether and to what extent this mechanism may explain observed differences.

We found that serotype-specific Ig concentrations varied considerably at both time points, but a statistically significant correlation with corresponding serum OPA titers was detected during the convalescent phase (Fig. 2). Anti-CPS Ig concentrations and OPA titers in preimmunization sera from adults have previously been reported to weakly correlate but may be improved after pneumococcal vaccination (3, 6, 24, 28). Our results indicate that a similarly improved correlation is induced by an episode of pneumococcal infection. In studies of both naturally acquired and vaccine-induced antibodies, the discrepancy between Ig concentration and OPA titers has been shown to be partly due to IgG with low avidity, a factor that may have contributed to the current results (24, 29, 30). Various concentrations of Ig directed against pneumococcal proteins may contribute to the effectiveness of opsonophagocytosis and might also explain inconsistencies between OPA titers and anti-CPS Ig levels (12). Even if the results of an OPA better reflect the function of antibody-mediated immunity than purely quantitative assays, it is important to mention that nonopsonic anticapsular antibodies (that do not affect readouts in OPA) have been found to prevent pneumococcal colonization as well as dissemination in mice (31). Consequently, it should be kept in mind that functional assays based on opsonophagocytosis do not constitute a perfect surrogate marker for antipneumococcal humoral immune status.

There are a few limitations of this study. First, due to the small number of patients included, the study should be regarded as exploratory; larger studies are needed to confirm our results. Second, based on a low prevalence of asymptomatic pneumococcal carriage in adults in Northern Europe, we regarded a pneumococcal serotype as possible true etiology if it was detected in cultures from the nasopharynx as well as from sputum, in accordance to Swedish clinical guidelines (26, 32). It is, however, possible that CAP was caused by a different etiological agent in patients with positive culture only from the nasopharynx. Furthermore, no information on previous exposure to pneumococci or immunization with the 23-valent pneumococcal polysaccharide vaccine was available for the studied individuals, but prescription rates of this vaccine have been low (1 to 2 doses per 10,000 adults per year) during the last 2 decades (https://sdb.socialstyrelsen.se/if_lak/val.aspx), and it was administered only to certain risk groups during the study period, indicating a low probability that any of the study subjects had received it. Finally, it should be noted that the lack of mass quantity assignments in the ELISA results for Ig concentrations, from which results were provided in arbitrary units (AU), is a limitation. However, the use of AU for comparison of serum Ig concentrations between different patients have been assessed as sufficient for the objectives of the current study.

In conclusion, despite the fact that infection with pneumococci resulted in an improved humoral immune response during convalescence in almost half of the patients in our cohort, approximately one-third of patients did not develop functional opsonic antibodies and even exhibited a decreased immune function in some cases, a response that was significantly associated with IPD. The high systemic CPS load and high age might possibly contribute to the failed immune response observed.

MATERIALS and METHODS

Study population. Study patients were part of a cohort at Örebro University Hospital consisting of 235 adults with CAP admitted to the Department of Infectious Diseases. Inclusion criteria and group

characteristics of the original cohort have been described previously (22). CAP was radiologically verified and defined as acute onset of illness with two of the following signs or symptoms: fever of ≥38°C, dyspnea, cough, pleuritic chest pain, and abnormal lung auscultation. Serum and plasma samples were collected from the patients within 2 days of admission (acute phase) and after approximately 1 to 3 months (convalescence). The duration from onset of illness until hospital admission was based on the history of the patient and was collected from the medical records. To estimate disease severity at admission, the sequential organ failure assessment (SOFA) score was calculated. Patients with an increase in SOFA score of ≥2 from baseline levels were regarded as having sepsis, in accordance with the Sepsis-3 definition (33).

Blood samples and samples from sputum and the nasopharynx were collected at admission. A Bactec blood culturing system (Becton, Dickinson, Sparks, MD) was used for blood culture. The sputum and nasopharyngeal specimens were cultured according to standard microbiological methods. All isolates of *S. pneumoniae* from blood cultures and cultures from respiratory tract secretions were stored at −70°C and transported in a frozen state to the Statens Serum Institut in Copenhagen, Denmark, for serotyping by the Quellung reaction (34). Bacteremic CAP was defined as growth of pneumococci in blood culture, whereas nonbacteremic CAP was defined as pneumococci cultured only from sputum or nasopharyngeal secretions.

We included patients ($n = 54$) from a previous cohort (22) who were infected by a serotype included in PCV13 and from whom paired sera (acute-phase and convalescent-phase sera) were available. The median age was 68.5 years (range, 23 to 91 years). Twenty-five patients were female (46%). Pneumococci grew in blood cultures from 16 (30%) patients, whereas 38 (70%) had nonbacteremic CAP. Among the patients with nonbacteremic CAP, pneumococci were isolated in sputa from 23 patients, whereas the nasopharynx was the only site of isolation for 15 patients. The median time period between paired sera was 30.5 days (range, 20 to 82 days). Infecting serotypes were 3 ($n = 11$), 14 ($n = 10$), 7F ($n = 9$), 23F ($n = 6$), 9V ($n = 4$), 18C ($n = 3$), 19A ($n = 3$), 19F ($n = 3$), 1 ($n = 2$), 4 ($n = 2$), and 6B ($n = 1$). Sera ($n = 13$) from patients with bacteremic CAP included in the present study were recently used in a study on the post-IPD immune response related to pneumococcal serotypes as part of a larger group (27).

Opsonophagocytic assay. A single-serotype opsonophagocytic assay (OPA) based on a Centers for Disease Control and Prevention protocol developed by Romero-Steiner et al. (35) available from the World Health Organization (WHO) Bacterial Respiratory Tract Pathogen Reference Laboratory (University of Alabama [UAB], Birmingham, AL; https://www.vaccine.uab.edu/uploads/mdocs/cdc-ops3.pdf) was performed on all acute-phase and convalescent-phase sera. Some modifications of the method were made according to the more recent UAB multiplexed-OPA protocol (36). Briefly, the *S. pneumoniae* target strain of the infecting serotype (BEI Resources, Manassas, VA) suspended in opsonization buffer B (OBB; Hanks' balanced salt solution with Mg^{2+} and Ca^{2+} supplemented with 0.1% gelatin and 10% heat-inactivated fetal bovine serum) was added to a threefold dilution series (starting at 1:4 dilution of total assay volume) of heat-inactivated patient sera in OBB in duplicates and incubated for 30 min at room temperature (RT) to allow Ig binding to bacteria. Thereafter, samples were incubated with promyelocytic human leukemia (HL-60) cells, differentiated by propagation in 0.8% dimethylformamide for 5 days, suspended in OBB and baby rabbit complement for 45 min at 37°C in 5% CO_2 to facilitate phagocytosis. Finally, phagocytosis was stopped by cooling samples on ice for 20 min, followed by the transfer of samples to blood agar plates and overnight culture at 37°C in 5% CO_2. The number of CFU for each plate was manually counted. The OPA titer of a sample was defined as the inverse ratio for the weakest serum dilution titer that caused >50% killing of bacteria compared to the level of bacteria in a negative control without any serum (i.e., the remaining bacteria, ≤50% CFU). If a sample did not result in >50% killing at any concentration (i.e., the OPA titer was undetectable), it was assigned an opsonic titer of 1 for purpose of analysis and presentation. Acute-phase and convalescent-phase sera from the same individual were tested on the same microtiter plate. Patients whose convalescent-phase serum OPA titer was either undetectable or decreased compared to that of the corresponding acute-phase serum were considered to have a nonfunctional antibody response. A positive-control serum from a PCV13-immunized volunteer was run in the OPA in parallel with sera from each individual and was included in all rounds to ensure the validity of the assay. Some variability of the positive-control serum OPA titer was observed between runs, even with specimens of the same serotype, which prevented direct comparisons of OPA titers between patients (see Table S2 in the supplemental material). Regardless of this fact, the acute phase-to-convalescence dynamic of the serum OPA titer could be assessed as increased, unchanged, or decreased.

Screening for total IgG or IgG2 deficiencies and determination of antipneumococcal Ig. A sandwich enzyme-linked immunosorbent assay (ELISA) for total IgG and IgG2 was performed on all acute-phase sera to exclude Ig deficiencies that may hamper opsonic function of sera as previously described (27). Briefly, MaxiSorp plates (Nunc, Waltham, MA) were coated with rabbit anti-human IgG antibodies (Sigma, Darmstadt, Germany) or mouse anti-human IgG2 antibodies (Sigma) overnight at 4°C. Wells were washed with wash buffer (phosphate-buffered saline [PBS] [pH 7.4], 0.05% Tween 20), followed by incubation with blocking buffer (PBS [pH 7.4], 1% skim milk, 0.05% Tween 20) for 1 h at RT. Thereafter, 10-fold dilution series of patient sera and calibration sera for IgG (Dako, Glostrup, Denmark) or IgG2 (The Binding Site, San Diego, CA) were added to plates in blocking buffer, and the plates were incubated for 1 h at RT. Following an additional wash, horseradish peroxidase (HRP)-conjugated rabbit anti-human IgG antibodies (Dako) were added to all wells, and the plates were incubated for 20 min at RT to allow binding to patient and calibration IgG or IgG2. Finally, optical density was measured at 450 nm, and absorbances of patient sera were compared to those of the calibration sera.

Antipneumococcal CPS total Ig concentrations were determined using a cell wall polysaccharide (CWPS) adsorption ELISA described by Konradsen et al. as recommended by WHO (22, 37). Briefly, sera were adsorped with CWPS before being added to MaxiSorp microtiter plates (Nunc, Roskilde, Denmark)

coated with serotype-specific CPS. Ig binding was compared to that of a standard serum to calculate concentrations in AU.

qPCR for pneumococcal DNA in plasma. Results from examination of acute-phase plasma samples with a quantitative PCR (qPCR) for *S. pneumoniae*-specific Spn9802 DNA were available for 25 of the study patients. Data from these experiments were used to investigate any association with outcome in the OPA. DNA was extracted from plasma samples using an automatic NucliSENS easyMAG instrument (bioMérieux, Marcy-l'Étoile, France). After that, qPCR was used to examine the purified samples for Spn9802 DNA as previously described (38). These results have been published previously (22).

Statistical analyses. All statistical analyses were performed in SPSS v24 (IBM, Armonk, NY). Results were compared between any two groups using the Mann-Whitney U test and between any three groups using the Kruskal-Wallis H test. To test for equality of proportions between groups, the Pearson chi-square test or, if any cells had an expected count less than five, Fisher's exact test was used. Differences were considered statistically significant if two-tailed P values were <0.05.

Ethical approval and consent to participate. The study was approved by the Regional Ethics Board at Lund University Hospital (approval 2012/86) and the Örebro County Council ethical committee (approval 868-1999). All patients provided their informed consent to participate in the study. The study was done in accordance with the Helsinki declaration.

Data availability. The data sets used and/or analyzed during the current study are available from the corresponding author on request.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.02 MB.

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F.U. designed the study, analyzed data, performed experiments, and drafted the manuscript. J.A. designed and initiated the study and revised the manuscript. N.L. did experiments and contributed to the writing. K.S. analyzed patient data, collected patient sera, and revised the manuscript. S.A. designed the study, analyzed patient data, and revised the manuscript. K.R. initiated and designed the study and revised the manuscript.

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Table S1.

Patient No.	Age (years)	Gender	*S. pneumoniae* in blood culture	*S. pneumoniae* in sputum culture	*S. pneumoniae* in nasopharyngeal culture	Infecting serotype	Days between sample collection	Currently smoking	Comorbidity[a]	SOFA score increase	Symptom duration before acute-phase sample	Acute-phase anti-CPS Ig level (AU)	Convalescent-phase anti-CPS Ig level (AU)	Plasma Spn9802 DNA concentration (copies/mL)
1	54	Female	Yes	Yes	Yes	3	45	No	No	1	2	106	494	-
2	71	Male	No	Yes	Yes	3	27	Yes	Yes	1	1	150	116	-
3	57	Female	Yes	Yes	Yes	9V	27	No	No	2	0	31	457	-
4	62	Male	No	Yes	Yes	9V	30	No	No	2	8	83	115	-
5	43	Male	No	Yes	Yes	18C	60	Yes	No	0	0	20	80	0
6	77	Female	No	Yes	Yes	23F	28	No	Yes	2	5	54	81	0
7	59	Female	No	Yes	No	19A	32	No	No	1	6	40	76	0
8	74	Male	No	Yes	No	3	25	No	Yes	3	2	37	87	1.8×10^2
9	39	Male	Yes	No	No	7F	81	No	Yes	3	3	12	525	0
10	31	Male	Yes	No	Yes	7F	46	No	No	0	2	15	1230	0
11	59	Male	No	No	Yes	14	25	Yes	Yes	2	3	6	64	-
12	72	Female	No	No	Yes	19F	28	No	No	0	0	26	410	0
13	31	Male	No	No	Yes	7F	29	No	No	1	4	7	48	4.0×10^2
14	37	Male	No	Yes	Yes	4	28	Yes	No	1	2	58	97	-
15	62	Female	No	No	Yes	7F	28	No	Yes	4	0	56	207	-
16	67	Female	Yes	No	Yes	7F	38	No	No	3	5	112	575	-
17	74	Male	No	No	Yes	3	33	Yes	Yes	2	1	60	77	-
18	90	Male	No	Yes	Yes	14	31	No	Yes	2	8	15	325	0
19	87	Male	No	No	Yes	3	31	No	Yes	5	0	80	61	0

20	40	Male	No	Yes	Yes	1	30	Yes	No	4	4	81	1754	-
21	41	Male	No	Yes	No	14	51	Yes	No	3	2	18	25	-
22	76	Female	No	No	Yes	14	31	No	No	5	8	35	25	-
23	60	Female	No	Yes	Yes	14	28	No	No	0	4	38	100	-
24	68	Female	No	No	Yes	14	81	No	Yes	0	21	65	144	-
25	89	Female	Yes	No	Yes	7F	58	No	Yes	3	7	20	21	1.8×10^4
26	84	Female	No	No	Yes	18C	27	No	Yes	1	1	22	24	0
27	83	Male	No	Yes	Yes	4	38	No	Yes	2	1	76	197	0
28	89	Male	No	Yes	Yes	6B	38	No	No	2	36	8	4	-
29	57	Male	No	Yes	Yes	3	25	No	No	1	0	114	109	0
30	46	Female	No	Yes	Yes	3	69	Yes	No	1	7	243	215	0
31	88	Male	No	No	Yes	9V	33	No	Yes	4	1	102	372	-
32	72	Female	No	No	Yes	14	29	Yes	Yes	1	0	53	76	-
33	78	Male	No	Yes	Yes	19A	33	No	Yes	2	3	24	21	-
34	77	Male	No	Yes	Yes	14	30	No	Yes	3	0	31	142	0
35	76	Male	No	Yes	Yes	14	61	No	Yes	3	1	117	39	-
36	61	Female	Yes	Yes	Yes	1	29	Yes	Yes	2	2	22	23	-
37	54	Male	Yes	Yes	Yes	3	31	Yes	Yes	2	4	81	172	4.5×10^3
38	54	Male	No	Yes	Yes	3	26	No	No	3	6	62	81	-
39	58	Male	No	No	Yes	3	34	Yes	No	2	10	84	122	5.8×10^2
40	79	Female	Yes	No	Yes	9V	24	No	No	3	8	48	64	3.0×10^9
41	54	Female	No	No	Yes	18C	28	No	No	0	5	11	12	-
42	69	Male	Yes	No	No	19A	31	No	Yes	4	3	27	2	-
43	85	Female	Yes	No	No	23F	29	No	Yes	3	0	5	11	0
44	84	Female	Yes	Yes	Yes	23F	82	No	Yes	1	4	16	14	0

45	89	Male	No	Yes	Yes	23F	74	No	Yes	1	1	64	136	-
46	73	Female	No	Yes	Yes	23F	26	Yes	No	2	11	101	118	-
47	85	Male	Yes	No	No	23F	20	No	No	3	3	5	13	-
48	73	Male	No	Yes	Yes	19F	30	No	Yes	3	3	96	162	0
49	83	Female	Yes	No	Yes	19F	26	No	No	3	6	57	33	$1.0\ x\ 10^{10}$
50	62	Female	No	Yes	No	7F	26	Yes	Yes	1	2	6	12	-
51	48	Female	No	No	Yes	3	22	Yes	No	1	6	62	68	-
52	91	Female	Yes	Yes	Yes	14	70	No	No	1	7	28	11	-
53	57	Female	Yes	Yes	Yes	7F	31	Yes	No	2	5	73	12	0
54	23	Male	No	No	Yes	7F	34	No	No	3	11	183	528	$6.2\ x\ 10^{3}$

[a] One or more of any of the following diagnoses: chronic obstructive pulmonary disease, heart disease, diabetes mellitus, liver disease, renal insufficiency, neoplasm, or immunosuppression.

Table S2.

Infecting serotype	Patient No.	Acute-phase serum OPA titer	Convalescent-phase serum OPA titer	Control serum OPA titer
1	36	1	1	2,916
1	20	108	972	2,916
3	37	1	1	108
3	1	1	4	108
3	38	1	1	108
3	39	1	1	108
3	51	972	1	108
3	2	1	108	108
3	19	108	324	108
3	8	1	972	108
3	17	36	972	108
3	29	972	972	108
3	30	972	972	108
4	27	324	324	8,748
4	14	1	8,748	8,748
6B	28	324	324	972
7F	53	972	4	972
7F	25	12	12	972
7F	9	1	972	972
7F	10	1	972	972
7F	16	4	972	972
7F	50	324	1	2,916
7F	54	2,916	972	324
7F	13	1	2,916	324
7F	15	1	8,748	2,916
9V	40	1	1	324
9V	3	1	108	972
9V	4	1	108	972
9V	31	972	972	972
14	52	972	1	972
14	11	1	972	972
14	32	972	972	2,916
14	18	36	2,916	972

14	23	972	2,916	972
14	24	972	2,916	2,916
14	22	324	8,748	972
14	34	8,748	8,748	26,244
14	21	108	26,244	2,916
14	35	26,244	26,244	26,244
18C	41	1	1	972
18C	26	36	36	972
18C	5	1	108	324
19A	42	1	1	2,916
19A	7	1	324	2,916
19A	33	972	972	2,916
19F	49	108	1	108
19F	48	36	4	108
19F	12	1	972	108
23F	43	1	1	972
23F	44	1	1	972
23F	47	4	1	972
23F	45	1	1	972
23F	46	1	1	972
23F	6	1	108	972

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Characterization of *Streptococcus pneumoniae* detected in clinical respiratory tract samples in southern Sweden 2 to 4 years after introduction of PCV13

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SUMMARY

Objective: To determine the serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* associated with mucosal infections in patients of all ages, 2 to 4 years after the transition from a 10-valent pneumococcal conjugate vaccine (PCV10) to PCV13 in the childhood immunization programme.
Methods: Background information and antimicrobial susceptibility data regarding all respiratory tract, middle ear, and conjunctival samples positive for growth of *S. pneumoniae* (n = 2,131) were collected during 18 months in 2016–2018. Available corresponding bacterial isolates were serotyped by PCR and/or antisera (n = 1,858).
Results: In total, 17% of isolates were covered by PCV13, predominantly represented by serotypes 3 (9%) and 19A (5%). The most common nonvaccine serotypes were 11A (10%), 23B (10%), 15A (6%) and 35F (5%). Isolates exhibiting serotype 15A or 23B were often multidrug-resistant (21%) or penicillin nonsusceptible (38%), respectively.
Conclusions: The overall proportion of serotype 19A was halved compared to a previous observation period when PCV10 was used (years 2011–2013), suggesting herd protection related to PCV13. The proportion of serotype 3 was, however, unchanged. Despite most nonvaccine serotypes causing mucosal infections have a low invasive potential, certain antibiotic resistant serotypes may pose a clinical problem.

Introduction

The respiratory tract commensal *Streptococcus pneumoniae* is a transient colonizer of the human nasopharynx and an important etiology of acute otitis media (AOM), community acquired pneumonia (CAP) and invasive pneumococcal disease (IPD), predominantly causing infections in children <5 years of age and the elderly [1]. In Sweden, the 7-valent pneumococcal conjugate vaccine (PCV7) was gradually introduced in the national childhood immunization programme between 2007 and 2009. In 2010, PCV10 and PCV13 replaced that vaccine, with different counties using one or the other. Decreased incidences of IPD, CAP and AOM were seen in children after vaccine introduction, while herd protection against IPD and CAP among the elderly was limited [2–7]. A similar situation was observed in several other European countries since serotype replacement occurs in this age group, leading to a reduced effect on the overall disease incidence [7].

We have previously described the serotype distribution of pneumococci isolated from clinical, disease-related, upper respiratory tract (URT) specimens prior to and after introduction of PCV7 (2009) and PCV10 (2010) in Skåne county [8]. A clear decline of PCV10 serotypes and an overall decrease of the number of samples positive for *S. pneumoniae* was observed in 2011–2013 compared with 2007–2008. In contrast, during the same time period, the proportions of PCV13 serotype 19A, a serotype associated with severe infections and antibiotic resistance [9], and several nonvaccine types (NVTs) increased.

The aim of this study was to describe the spectrum of disease-related *S. pneumoniae* present in the population of Skåne county, southern Sweden, after the transition from using PCV10 to PCV13 in the childhood immunization programme (May 2014), by characterizing pneumococcal isolates detected in clinical respiratory tract cultures (including middle ear and conjunctival samples). The

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serotype distribution and antimicrobial resistance rates of isolated pneumococci were assessed. Additionally, the serotype distribution was compared to that of pneumococcal isolates causing IPD within the county during the same years. While pneumococci causing IPD are monitored nationally in Sweden, the body of knowledge is limited regarding vaccine effects on mucosal infections, and what serotypes that cause them and their corresponding antimicrobial resistance patterns. In addition to PCV13 serotypes 3 and 19A, which were both prevalent previously [8], emerging NVTs were objectives of special interest in our present study.

Methods

Study setting

This study was conducted in southern Sweden (Skåne county) between 1st October 2016 and 31st March 2018. The total population of the county increased from approximately 1,320,000 to 1,349,000 inhabitants during the study period (https://www.scb.se/, accessed 01–03–2020). PCV7 was used in the childhood immunization program in Skåne county between January 2009 and May 2010. Thereafter, PCV10 was used until 1st May 2014 when it was replaced by PCV13. Since 2010, the vaccine uptake of PCV among eligible children nationally as well as in Skåne county has been approximately 97–98% (https://www.folkhalsomyndigheten.se, accessed 30–03–2021). Indications for microbiological sampling of the respiratory tract, middle ear discharge or conjunctiva in primary care settings in Sweden are complicated and/ or therapy-resistant AOM, sinusitis or conjunctivitis, in addition to suspected or confirmed CAP, although any culture may be collected at the discretion of the referring physician. At emergency departments and hospital wards, nasopharyngeal and lower respiratory tract (LRT) culture samples are primarily obtained from patients with suspected LRT infection.

Data collection

Data on all respiratory tract, middle ear and conjunctival culture samples positive for *S. pneumoniae* were obtained from Clinical microbiology (Laboratory medicine, Lund) (n = 2,288). Information included age (range 0–100 years) and sex (51% females) of the sampled patient, collection date, referring unit (69% outpatient settings) and sample type. Specimens from the nasopharynx (n = 1,384), middle ear discharge (n = 85), conjunctival secretions (n = 72), and sinus/nasal discharge (n = 44) were considered URT samples. Samples from the LRT comprised sputum (n = 547) and samples from trachea/bronchi (n = 150). Five samples were of undefined respiratory tract secretions. If multiple samples positive for *S. pneumoniae* were collected within two months from the same patient, and the serotype of the corresponding isolates was identical, only one sample was further analyzed (n = 151 samples excluded). Serotype data regarding pneumococcal isolates from patients with IPD in Skåne county during 2016 to 2018 were obtained from the Public Health Agency of Sweden (n = 519).

Collection of bacterial isolates, culture conditions and species identification

Pneumococcal isolates (n = 1,858), corresponding to 87% of the included culture samples, were stored at −80 °C in glycerol-supplemented horse serum after identification by standard methods. For subsequent analyses, isolates were cultured on blood agar plates for 18 h at 37 °C in 5% CO_2. Five non-pneumococcal isolates, which had initially been classified as *S. pneumoniae*, were excluded from the study.

Antimicrobial susceptibility testing (AST)

Pneumococcal isolates were screened for nonsusceptibility to oxacillin, erythromycin, clindamycin, tetracycline and trimethoprim/sulfamethoxazole using disk diffusion tests (Thermo Fisher Scientific, Waltham, MA). Minimum inhibitory concentrations (MICs) of all oxacillin-resistant isolates were determined with gradient tests for benzylpenicillin, ampicillin and cefotaxime (Etest; BioMérieux, Marcy-l'Étoile, FR). Additional tests for other antibiotics were performed based on the clinical situation. All results from AST were interpreted according to current EUCAST breakpoints at the time of testing (https://eucast.org/clinical_breakpoints/; Clinical breakpoints - bacteria v6.0–8.0). Isolates were considered putatively multidrug-resistant (MDR) or extensively drug-resistant (XDR) if nonsusceptible to ≥3 or ≥5 antimicrobials during screening, respectively. MICs of XDR isolates were determined with broth microdilution (Sensititre Streptococcus STP6F AST Plate; Thermo Fisher Scientific).

Serotyping of pneumococci

Serotyping was performed using a multiplex polymerase chain reaction (PCR) comprising 6 sequential reaction in combination with latex agglutination and the Quellung reaction as described [10]. Isolates that were negative twice in PCRs were serotyped with the ImmuLex Pneumotest Kit (SSI Diagnostica, Copenhagen, DK) and Neufeldt antisera. Isolates that were negative for *cpsA* in PCR twice, or could not be determined through latex agglutination and the Quellung reaction, were considered nontypeable (NT).

Statistical analyses

All statistical tests were performed in SPSS Statistics 26 (IBM, Armonk, NY). To compare proportion between two groups, the Chi-square test and, if the predicted value in any cell was <5, Fisher's exact test were used. To investigate the association of clinical and demographical variables with the isolation of PCV13 serotypes, univariate and multivariate logistic regression analyses were performed. In these analyses, patient age was converted to a dichotomous variable ("PCV13 eligible age") based on birthdate prior to or after the introduction on PCV13 (1st May 2014). Serotype-specific IPD odds ratios (ORs) were defined as $(\frac{a1}{b1})/(\frac{a2}{b2})$, where $a1$ is the number of IPD isolates exhibiting the serotype, $b1$ the number of IPD isolates not exhibiting the serotype, $a2$ the number of respiratory tract isolates exhibiting the serotype, and $b2$ the number of respiratory tract isolates not exhibiting the serotype. Confidence intervals (CI) for frequencies and ORs were calculated as described by Kirkwood and Sterne [11]. Two-tailed p-values < 0.05 were considered statistically significant.

Ethical considerations

The project was approved by the local ethics committee (*Regionala etikprövningsnämnden i Lund*) upon its initiation (approval no. 2012/286) and was updated (approval no. 2016/752) to include the current study.

Results

Clinical samples included

Patient samples were collected 2 to 4 years after the introduction of PCV13 in the national childhood immunization programme. A total of 2,131 samples positive for growth of *S. pneumoniae* were included in our analyses. Background characteristics of the samples and corresponding patients are summarized in Table 1. In addition, frequencies of *S. pneumoniae*-positive URT and LRT samples

Table 1
Background characteristics of samples positive for *S. pneumoniae* and corresponding patients.

Characteristics	Data[a] for:	
	Samples positive for *S. pneumoniae*	Samples with serotyped *S. pneumoniae*
No. of samples	2,131	1,858
Patient age (years)[b]	43 (4–67)	42 (3–67)
Age group		
<2 (years)	350 (16)	311 (17)
2–4	212 (10)	195 (11)
5–9	107 (5)	96 (5)
10–19	50 (2)	46 (3)
20–29	84 (4)	68 (4)
30–39	188 (9)	160 (9)
40–49	167 (8)	155 (8)
50–59	191 (9)	164 (9)
60–69	340 (16)	290 (16)
70–79	284 (13)	242 (13)
≥80	158 (7)	131 (7)
Patient sex		
Female	1,095 (51)	957 (52)
Male	1,036 (49)	901 (49)
Referring department		
Outpatients' department	1,466 (69)	1,281 (69)
Hospital setting	665 (31)	577 (31)
Sample type		
Nasopharyngeal swab	1,336 (63)	1,190 (64)
Sinus/nasal secretion	40 (2)	35 (2)
Middle ear discharge	78 (4)	71 (4)
Conjunctival sample	66 (3)	61 (3)
Sputum	484 (23)	399 (22)
Tracheal/bronchial sample	122 (6)	98 (5)
Unspecified airway secretion	5 (0.2)	4 (0.2)

[a]Numbers are *n* (%) or median (IQR).
[b]Range 0–100 years.

normalized by population size, and the number of samples referred from different health care settings are available in *Supplemental Table 1*.

PCV13 serotypes 3 and 19A are still circulating in the population

The serotype distribution of pneumococcal isolates available for typing is outlined in Fig. 1 (n = 1,858). The overall proportion of serotypes included in PCV13 was 17% (n = 313), and serotypes 3 and 19A comprised 9 and 5%, respectively. In parallel, the percentage of serotypes included in PCV10, and the future vaccines PCV15 (by MSD) and PCV20 (Pfizer) were 3, 21 and 41%, respectively. In *Supplemental Tables 2–4*, frequencies of all serotypes by age group, sample type and calendar quarter are presented. A multivariate regression analysis revealed that PCV13 serotypes were associated with patients not targeted by PCV13 immunization (p<0.001) and with patient samples collected in hospital settings (p = 0.006) (Table 2).

Serotypes detected in respiratory tract samples rarely cause IPD

A total of 519 pneumococci were isolated from patients with IPD in Skåne county 2016–2018 and only 2% (n = 11) and 4% (n = 21) of these originated from children younger than 2 and 15 years of age, respectively. The majority (76%) were from individuals aged 60 years or older. To assess whether the serotypes detected in respiratory tract samples also caused IPD, ORs were calculated comparing their frequencies among IPD and respiratory tract samples from patients of all ages as described in Fig. 2. No significant differences were found between ORs calculated with data from patients aged under 15 years, 15–59 years, or over 59 years for any serotype (*Supplemental Figure 1*). Importantly, most NVTs that were common in respiratory tract infections exhibited IPD/respiratory tract ORs <1.

A small number of NVTs are responsible for antibiotic resistance

Antimicrobial nonsusceptibility rates differed considerably between serotypes, and was highest among several PCV13 serotypes and the NVTs 6C, 15A, 23B, 24 and 35B (*Fig. 3* and Supplemental Table 5). The detected penicillin nonsusceptible pneumococci (PNSP) (n = 237; 11%) exhibited a low median penicillin MIC (0.13 µg/ml, IQR 0.13–0.25 µg/ml), and only a few PNSP were nonsusceptible to ampicillin (13%) or cefotaxime (3%). MICs of putative XDR pneumococci determined with broth microdilution were as indicated in *Supplemental Table 6*, these results showed that gradient tests underestimated the penicillin MIC with 1–2 dilution steps in 16 out of 26 (62%) cases.

Discussion

We characterized *S. pneumoniae* isolated from clinical respiratory tract samples at a single laboratory serving an entire county in southern Sweden 2 to 4 years after PCV13 introduction in the childhood immunization programme. Serotypes 3 and 19A were still prevalent, but less in the immunized age group, while remaining PCV13 serotypes were rarely detected. Most frequently isolated

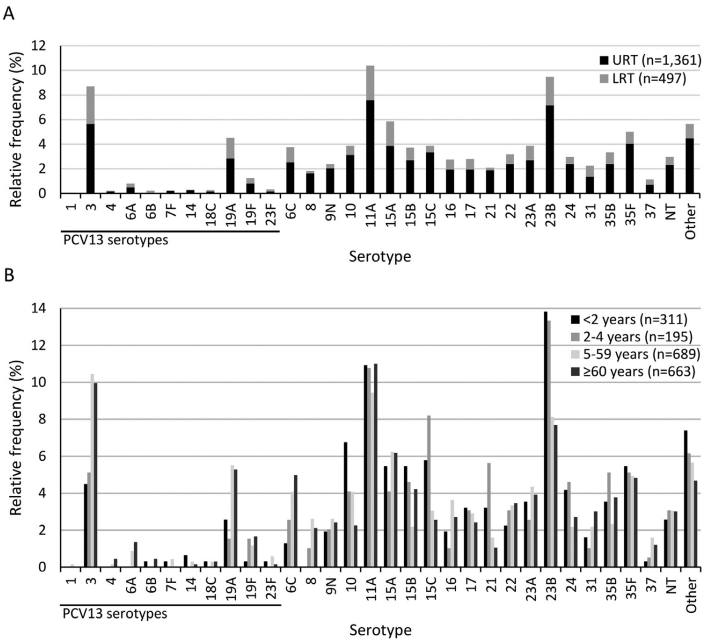

Fig. 1. Serotype distribution of *S. pneumoniae* isolates (n = 1,858; 87% of all samples positive for growth of *S. pneumoniae*) detected in clinical respiratory tract samples in Skåne county 2 to 4 years after PCV13 introduction. (A) Overall serotype distribution of pneumococcal isolates grown from upper and lower respiratory tract samples. (B) Serotype distribution of pneumococci detected in samples from patients of different ages. Serotypes included in PCV13 are indicated by the black bars below the x-axes. Non-vaccine types that represent <1% of all isolates are pooled as "Other". URT, upper respiratory tract; LRT, lower respiratory tract.

Table 2
Univariate and multivariate logistic regression analyses of factors associated with pneumococcal serotypes included in PCV13. The overall frequency of PCV13 serotypes was 17%. Significant associations are indicated in bold. OR, odds ratio; CI, confidence interval; LRT, lower respiratory tract.

	PCV13-included serotype/total	Univariate analysis			Multivariate analysis[a]		
Characteristics	*n*/*n* (%)	OR	95% CI	*p*	OR	95% CI	*p*
Patient age[b]		**1.01**	**1.005–1.014**	**<0.001**	–		
PCV13 eligible age[c]	38/440 (9)	**0.39**	**0.28–0.56**	**<0.001**	**0.46**	**0.32–0.67**	**<0.001**
Female sex	158/957 (17)	0.95	0.75–1.21	0.69	–		
Hospital setting	125/577 (22)	**1.61**	**1.25–2.07**	**<0.001**	**1.43**	**1.11–1.85**	**0.006**
LRT sample	111/497 (22)	**1.64**	**1.27–2.13**	**<0.001**	1.31	1.00–1.71	0.051

[a] A total of 1,854 samples were included in the multivariate analysis.
[b] Continuous variable.
[c] Patients born after 1st May 2014.

NVTs seldomly caused IPD during the study period, but some specific serotypes were often PNSP (15A, 23B and 35B) or MDR/XDR (15A and serogroup 24).

Pneumococcal serotype epidemiology in relation to PCV use is often investigated through carriage studies of asymptomatic individuals or by examining isolates causing IPD. Studies of clinical non-invasive isolates are scarcer but may be beneficial as they reflect the spectrum of disease-associated pneumococci circulating in the population. A limitation of this approach is although that we cannot fully determine whether the detected isolates represent the true etiology of respiratory infections. Secondly, we did not have information about the diagnoses of the sampled patients. Lastly, penicillin gradient tests were used which may result in underestimated penicillin MIC values, which was indicated by a selection of isolates analyzed with broth microdilution (*Supplemental Table 6*) [12].

Compared to our previous report on the years following PCV10 introduction the number of URT samples positive for *S. pneumoniae* collected from children <2 years decreased from 1,250 (95% CI 1,161–1,341) to 724 (95% CI 651–803) per 100,000 person years (*Supplemental Table 1*) [8]. An in-depth analysis of nasopharyngeal samples collected from children in Skåne county during 14 years also described this decline and showed that it was associated with increasing PCV-coverage [13]. This is likely partially explained by the decreased incidences of AOM-related diagnoses and CAP in Swedish children during the study years, but it may also be the re-

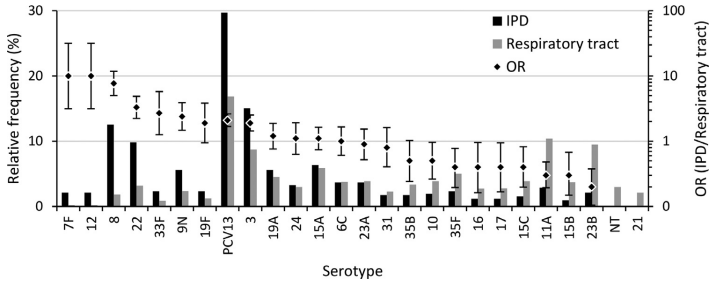

Fig. 2. Serotypes of pneumococcal isolates detected in respiratory tract samples (n = 1,712), compared with samples from sterile sites (IPD, n = 477) in Skåne county 2 to 4 years after PCV13 introduction. The relative frequency of the individual serotypes, as well as PCV13 serotypes pooled, and their IPD/respiratory tract odds ratio are presented. Error bars represent 95% confidence intervals. Serotypes representing <2% of isolates of both respiratory tract and IPD samples are not shown in the figure. CI, confidence interval; IPD, invasive pneumococcal disease; OR, odds ratio.

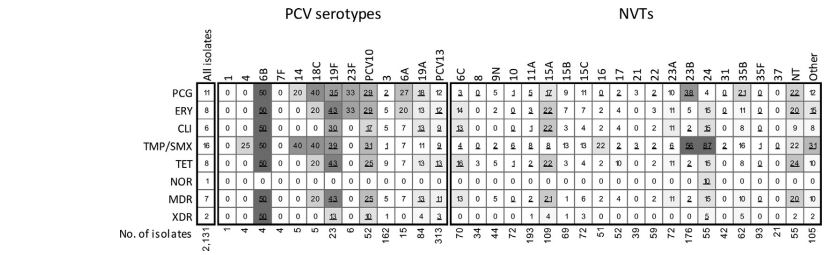

Fig. 3. Antimicrobial nonsusceptibility rates of pneumococci detected in clinical respiratory tract samples in Skåne county, 2 to 4 years after the introduction of PCV13 (n = 2,131). Numbers shown are the percentage of isolates of each serotype, or pooled PCV13 serotypes that were nonsusceptible to the specified substance or exhibited an MDR/XDR phenotype. Gray fields indicate that the nonsusceptibility rate of the corresponding serotype is higher than the overall proportion detected. Underscored numbers indicate a statistically significant difference of the nonsusceptibility rate of a serotype compared to that of all other serotypes combined. Only a minority of isolates (n = 389) were screened for norfloxacin nonsusceptibility and the presented numbers are the nonsusceptibility rates among those tested. Multidrug-resistance and extensive drug-resistance was defined as nonsusceptibility to ≥3 and ≥5 of the tested substances, respectively. Non-vaccine types that represent <1% of all serotyped isolates are pooled as "Other". CLI, clindamycin; ERY, erythromycin; MDR, multidrug-resistant; NOR, norfloxacin; NT, nontypeable; PCG, benzylpenicillin; PCV, pneumococcal conjugate vaccine; TET, tetracycline; TMP/SMX, trimethoprim-sulfamethoxazole; XDR, extensively drug-resistant.

sult of changes or geographical differences in clinical practice [5,6]. However, no guidelines regarding collection of respiratory cultures have changed since the previous study (2011–2013) and the reduction of *S. pneumoniae* in nasopharyngeal samples was shown to be independent of the overall reduction of culture referrals supporting the hypothesis that this is an effect of PCV usage [13].

Compared to 2011–2013, the proportion of *S. pneumoniae* serotype 19A in respiratory tract samples was halved from 10% to 5%, while no overall reduction of serotype 3 (9%) was observed [8]. The proportions of both serotypes were lower among children immunized with PCV13 in the current study. This pattern was also observed 2011–2013 regarding serotype 3 (Riesbeck K. et al., unpublished data), suggesting that it might not be an effect of PCV13 implementation. In contrast, the age association of serotype 19A in respiratory tract samples appears to have been established after the switch from PCV10 to PCV13. Taken together, our findings indicate a direct effect of PCV13 on respiratory infections in the vaccinated age group and emerging herd-protection for serotype 19A, while no effect on the occurrence of serotype 3 was seen. In parallel, after the introduction of PCV13 in 2010 in Stockholm (Sweden), serotype 19A decreased in carriage among children <5 years [14]. Moreover, serotype 3 slightly increased despite it was uncommon at baseline. Studies of PCV10 and PCV13 herd effects on adult noninvasive pneumonia in Iceland and the United States, respectively, also showed declining numbers of infection by the vaccine targeted serotypes, except serotype 3 in the American study [15,16]. Continued transmission of serotype 3 is likely due to a limited vaccine effect on nasopharyngeal colonization [17]. Similar incomplete herd protection for serotype 19A and serotype 19F, has been proposed as a possible mechanism explaining why they are not eliminated from the population as readily and completely as many other PCV serotypes in some areas, but this has not been confirmed [18]. Persistent circulation of serotype 19A in the population may cause resurgence of its incidence in IPD among children if vaccination against it is halted, which was observed in Belgium after the transition from PCV13 to PCV10 [19]. Bearing this in mind, the high prevalence of this serotype observed requires continued surveillance to detect potential re-emergence in IPD, as PCV10 replaced PCV13 in several Swedish counties, including Skåne county, in 2019 when it was procured nationally.

The most frequently isolated NVTs (11A, 23B, 15A, 35F, and the closely related 15B and 15C) are common replacement serotypes detected in carriage in Sweden (Stockholm) [3,14]. They also increased in adult non-invasive pneumonia after the introduction of PCV10 and PCV13 in Iceland and the USA, respectively [15,16]. While these serotypes have low-medium invasive potential, they may cause a considerable proportion of IPD cases in both children and adults if they become sufficiently prevalent in the population that is evident from the numbers outlined in *Fig. 2* [14,20]. The majority of IPD cases were, however, caused by PCV13 serotypes (mostly 3 and 19A) and NVTs that were seldom detected in respiratory tract samples (serotypes 8 and 9N, and serogroup 22), which

is similar to the situation in Germany among patients aged 60 years or older [18]. Serotype 15A was an exception, being the only NVT that was among the most prevalent in both patient groups. Even if the IPD ORs calculated for serotypes in the current study do not represent invasive disease potential by definition, they largely agree with the invasive disease potentials of serotypes detected in Stockholm 2011–2015 [14]. This further indicated that the spectrum of serotypes causing mucosal infections is more similar to that found in carriage than in IPD. However, because only a small number of IPD samples were from children, the ORs should be interpreted with caution for this particular age group. Finally, the fact that 76% of the IPD samples used in the analyses were collected from individuals aged 60 years or older clearly illustrates how the age distribution of IPD patients has changed in Sweden since PCV introduction, the incidence declined among children <2 years but was unaltered among the elderly [3].

In parallel to our results, the association of NVTs 6C, 15A, 23B, 24F and 35B to antibiotic resistance has previously been reported [21–24]. Further studies are needed to comment on the clinical significance of the antimicrobial resistance patterns of these serotypes in Sweden. Data from Norway and Germany, however, showed an increase in IPD caused by serotypes 15A and 23B after introduction of PCV13, replacement serotypes that were often MDR or PNSP, respectively [21,25]. Serotype 19A has been the subject of concern in many regions since PCV implementation due to antibiotic resistance, which further emphasizes the importance of monitoring this serotype after the replacement of PCV13 with PCV10 in 2019 [26].

In conclusion, this study indicated that the disease burden of pneumococcal respiratory tract infections has continued to decline after PCV13 introduction. Furthermore, serotype 19A has become less prevalent among patients of all ages, which suggests a direct as well as herd effect of PCV13, but is still circulating in the population. In contrast, the prevalence of serotype 3 remains unchanged. Importantly, reversion to the use of PCV10 in the childhood immunization program may lead to an increased incidence of pediatric IPD caused by serotype 19A due to the circulating reservoir of this serotype. Finally, the NVTs that dominated in respiratory tract samples are generally low invasive, but certain serotypes, such as 15A and 23B, may constitute a future clinical problem due to high rates of PNSP or multidrug-resistance.

Declaration of Competing Interest

Hans-Christian Slotved and Jonas Ahl report grants from Pfizer outside the submitted work. Kristian Riesbeck reports grants from Pfizer, during the conduct of the study; personal fees from GSK, personal fees from MSD, and grants from Pfizer, outside the submitted work. Remaining authors have nothing to disclose.

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Access to data

All data used in the analyses are available from the authors upon request.

Contribution

JA and KR conceived and planned the study. FU and KR administered the project. FU, HCS and KF developed methodology. FU performed experiments. FU, ER and HCS analyzed the results. FU drafted the manuscript with support from ER. All authors contributed to reviewing and editing the manuscript, and have approved the final version.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2021.05.031.

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Supplemental materials

Characterization of *Streptococcus pneumoniae* detected in clinical respiratory tract samples in southern Sweden 2 to 4 years after introduction of PCV13

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Supplemental Table 1. Frequencies of *S. pneumoniae*-positive upper and lower respiratory tract samples normalized by population size and number of samples referred from different health care settings. URT, upper respiratory tract; LRT, lower respiratory tract.

	Positive samples per year[a]			No. of positive samples referred from[b]		
Patient age group (yrs)	Total	URT samples	LRT samples	All settings	Outpatient departments	Hospital settings[c]
All ages	105 (101-110)	75 (71-79)	30 (28-32)	2126 (29)	1462 (25)	664 (37)
<2	730 (657-810)	724 (651-803)	6 (2-19)	350 (1)	290 (1)	60 (2)
2-4[d]	281 (246-322)	271 (236-311)	9 (4-19)	211 (3)	175 (2)	36 (11)
5-9	84 (70-102)	75 (61-92)	9 (5-17)	107 (11)	89 (4)	18 (44)
10-19	22 (17-29)	20 (15-26)	2 (1-5)	50 (10)	40 (5)	10 (30)
20-29	32 (26-39)	26 (20-33)	6 (4-10)	84 (19)	65 (14)	19 (37)
30-39	70 (61-81)	53 (45-62)	18 (13-23)	188 (25)	152 (22)	36 (39)
40-49	64 (55-75)	43 (36-52)	21 (16-27)	167 (32)	126 (27)	41 (49)
50-59[d]	76 (66-87)	38 (32-47)	37 (30-45)	190 (49)	120 (48)	70 (51)
60-69[d]	157 (141-174)	81 (70-94)	75 (65-88)	339 (48)	202 (51)	137 (44)
70-79[d]	149 (133-167)	70 (59-83)	78 (66-91)	282 (52)	146 (62)	136 (43)
≥80	142 (121-166)	90 (74-109)	52 (40-67)	158 (37)	57 (44)	101 (33)

[a]Numbers are presented as no. of samples per 100,000 person years (95% CI). To calculate frequencies the number of inhabitants at the end of the study period was used (March 2018). URT samples comprise nasopharyngeal, middle ear, nasal/sinus and conjunctival samples. LRT samples comprise sputum and samples from larynx or bronchi.
[b]Numbers are presented as total no. of samples (% LRT samples).
[c]Emergency departments and hospital wards.
[d]A total of 5 samples of unspecified airway secretions have been excluded from the numbers in these age groups.

Supplemental Table 2. Frequency distribution of pneumococcal serotypes detected in samples from patients of different ages. The total number of isolates of every serotype and their relative frequency within each age group is presented.

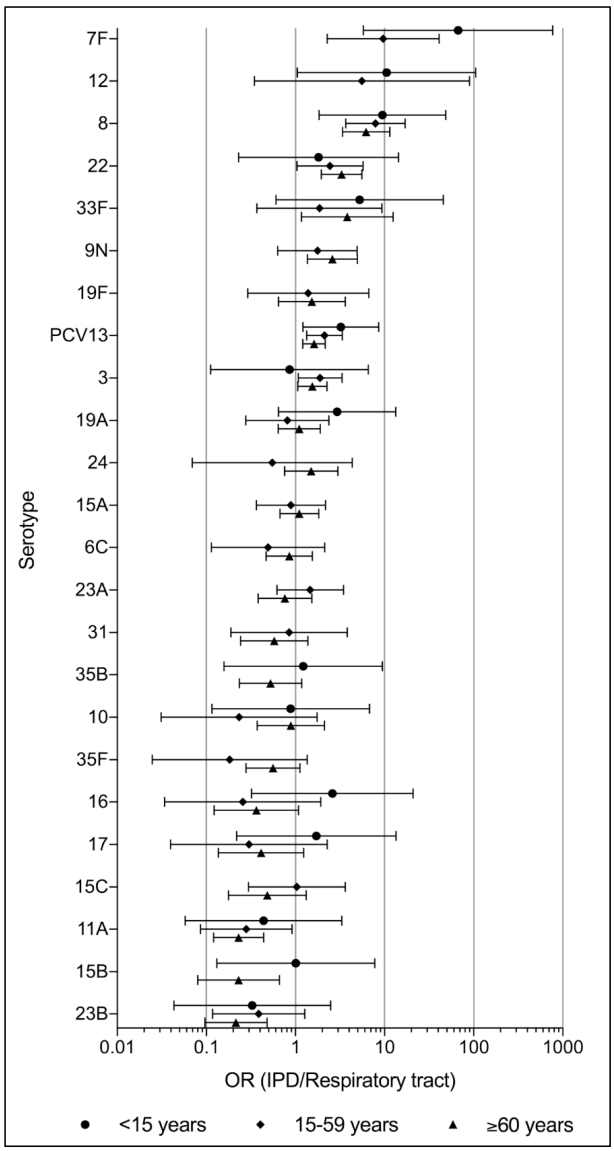
	All ages	<2 years	2-4 years	5-59 years	≥60 years
Serotype	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
1	1 (0.1)			1 (0.1)	
3	162 (8.7)	14 (4.5)	10 (5.1)	72 (10.4)	66 (10)
4	4 (0.2)			1 (0.1)	3 (0.5)
6A	15 (0.8)			6 (0.9)	9 (1.4)
6B	4 (0.2)	1 (0.3)			3 (0.5)
7F	4 (0.2)	1 (0.3)		3 (0.4)	
14	5 (0.3)	2 (0.6)		2 (0.3)	1 (0.2)
18C	5 (0.3)	1 (0.3)		2 (0.3)	2 (0.3)
19A	84 (4.5)	8 (2.6)	3 (1.5)	38 (5.5)	35 (5.3)
19F	23 (1.2)	1 (0.3)	3 (1.5)	8 (1.2)	11 (1.7)
23F	6 (0.3)	1 (0.3)		4 (0.6)	1 (0.2)
6C	70 (3.8)	4 (1.3)	5 (2.6)	28 (4.1)	33 (5)
6D	1 (0.1)				1 (0.2)
7A	1 (0.1)			1 (0.1)	
7B	3 (0.2)	1 (0.3)		2 (0.3)	
7C	4 (0.2)			4 (0.6)	
8	34 (1.8)		2 (1)	18 (2.6)	14 (2.1)
9A	2 (0.1)			1 (0.1)	1 (0.2)
9L	1 (0.1)				1 (0.2)
9N	44 (2.4)	6 (1.9)	4 (2.1)	18 (2.6)	16 (2.4)
10	72 (3.9)	21 (6.8)	8 (4.1)	28 (4.1)	15 (2.3)
11A	193 (10.4)	34 (10.9)	21 (10.8)	65 (9.4)	73 (11)
11D	1 (0.1)	1 (0.3)			
12	4 (0.2)	1 (0.3)	2 (1)	1 (0.1)	
13	6 (0.3)		1 (0.5)	2 (0.3)	3 (0.5)
15A	109 (5.9)	17 (5.5)	8 (4.1)	43 (6.2)	41 (6.2)
15B	69 (3.7)	17 (5.5)	9 (4.6)	15 (2.2)	28 (4.2)
15C	72 (3.9)	18 (5.8)	16 (8.2)	21 (3)	17 (2.6)
16	51 (2.7)	6 (1.9)	2 (1)	25 (3.6)	18 (2.7)
17	52 (2.8)	10 (3.2)	6 (3.1)	20 (2.9)	16 (2.4)
18A	2 (0.1)			1 (0.1)	1 (0.2)
18B	1 (0.1)	1 (0.3)			
20	7 (0.4)	3 (1)		1 (0.1)	3 (0.5)
21	39 (2.1)	10 (3.2)	11 (5.6)	11 (1.6)	7 (1.1)
22	59 (3.2)	7 (2.3)	6 (3.1)	23 (3.3)	23 (3.5)
23A	72 (3.9)	11 (3.5)	5 (2.6)	30 (4.4)	26 (3.9)
23B	176 (9.5)	43 (13.8)	26 (13.3)	56 (8.1)	51 (7.7)
24	55 (3)	13 (4.2)	9 (4.6)	15 (2.2)	18 (2.7)
28	3 (0.2)			2 (0.3)	1 (0.2)
29	2 (0.1)				2 (0.3)
31	42 (2.3)	5 (1.6)	2 (1)	15 (2.2)	20 (3)
33A	2 (0.1)			1 (0.1)	1 (0.2)
33F	16 (0.9)	4 (1.3)	2 (1)	6 (0.9)	4 (0.6)
33?	1 (0.1)			1 (0.1)	
34	16 (0.9)	2 (0.6)	1 (0.5)	8 (1.2)	5 (0.8)
35A	1 (0.1)			1 (0.1)	
35B	62 (3.3)	11 (3.5)	10 (5.1)	16 (2.3)	25 (3.8)
35C	1 (0.1)				1 (0.2)
35F	93 (5)	17 (5.5)	10 (5.1)	34 (4.9)	32 (4.8)
36	1 (0.1)	1 (0.3)			
37	21 (1.1)	1 (0.3)	1 (0.5)	11 (1.6)	8 (1.2)
38	16 (0.9)	5 (1.6)	5 (2.6)	4 (0.6)	2 (0.3)
40	10 (0.5)	3 (1)	1 (0.5)	3 (0.4)	3 (0.5)
41	1 (0.1)				1 (0.2)
42	1 (0.1)	1 (0.3)			
47	1 (0.1)				1 (0.2)
NT	55 (3)	8 (2.6)	6 (3.1)	21 (3)	20 (3)

Supplemental Table 3. Frequency distribution of pneumococcal serotypes isolated from different sample types. The total number of isolates of every serotype and their relative frequency within each sample type is presented.

Serotype	Total *n (%)*	Nasopharynx sample *n (%)*	Nasal/sinus sample *n (%)*	Middle ear discharge *n (%)*	Conjunctival sample *n (%)*	Sputum *n (%)*	Tracheal/ bronchi sample *n (%)*	Unspecified respiratory tract secretions *n* (%)
1	1 (0.1)	1 (0.1)						
3	162 (8.7)	73 (6.1)	4 (11)	26 (37)	2 (3.3)	49 (12)	8 (8.2)	
4	4 (0.2)	3 (0.3)					1 (1.0)	
6A	15 (0.8)	7 (0.6)	1 (2.9)		1 (1.6)	4 (1.0)	2 (2.0)	
6B	4 (0.2)	1 (0.1)				3 (0.8)		
7F	4 (0.2)	4 (0.3)						
14	5 (0.3)	4 (0.3)			1 (1.6)			
18C	5 (0.3)	2 (0.2)		1 (1.4)		1 (0.3)	1 (1.0)	
19A	84 (4.5)	43 (3.6)	2 (5.7)	4 (5.6)	4 (6.6)	26 (6.5)	5 (5.1)	
19F	23 (1.2)	14 (1.2)			1 (1.6)	4 (1.0)	4 (4.1)	
23F	6 (0.3)	3 (0.3)				2 (0.5)	1 (1.0)	
6C	70 (3.8)	41 (3.4)	3 (8.6)		2 (3.3)	18 (4.5)	5 (5.1)	1 (25)
6D	1 (0.1)					1 (0.3)		
7A	1 (0.1)	1 (0.1)						
7B	3 (0.2)	3 (0.3)						
7C	4 (0.2)		1 (2.9)			2 (0.5)	1 (1.0)	
8	34 (1.8)	29 (2.4)		1 (1.4)		4 (1.0)		
9A	2 (0.1)	1 (0.1)					1 (1.0)	
9L	1 (0.1)						1 (1.0)	
9N	44 (2.4)	33 (2.8)	1 (2.9)	1 (1.4)	3 (4.9)	3 (0.8)	3 (3.1)	
10	72 (3.9)	53 (4.5)	2 (5.7)	1 (1.4)	2 (3.3)	11 (2.8)	3 (3.1)	
11A	193 (10.4)	127 (11)	8 (23)	2 (2.8)	4 (6.6)	42 (11)	10 (10)	
11D	1 (0.1)	1 (0.1)						
12	4 (0.2)	4 (0.3)						
13	6 (0.3)	5 (0.4)				1 (0.3)		
15A	109 (5.9)	70 (5.9)	1 (2.9)		1 (1.6)	31 (7.8)	6 (6.1)	
15B	69 (3.7)	43 (3.6)	2 (5.7)	4 (5.6)	1 (1.6)	16 (4.0)	3 (3.1)	
15C	72 (3.9)	57 (4.8)	1 (2.9)	2 (2.8)	2 (3.3)	8 (2.0)	2 (2.0)	
16	51 (2.7)	30 (2.5)		3 (4.2)	1 (1.6)	15 (3.8)		2 (50)
17	52 (2.8)	30 (2.5)	1 (2.9)	4 (5.6)	1 (1.6)	13 (3.3)	3 (3.1)	
18A	2 (0.1)	1 (0.1)				1 (0.3)		
18B	1 (0.1)	1 (0.1)						
20	7 (0.4)	6 (0.5)			1 (1.6)			
21	39 (2.1)	32 (2.7)	1 (2.9)	1 (1.4)	1 (1.6)	2 (0.5)	2 (2.0)	
22	59 (3.2)	39 (3.3)	1 (2.9)	3 (4.2)	1 (1.6)	12 (3.0)	3 (3.1)	
23A	72 (3.9)	43 (3.6)	2 (5.7)	2 (2.8)	3 (4.9)	19 (4.8)	3 (3.1)	
23B	176 (9.5)	113 (9.5)	1 (2.9)	9 (13)	10 (16)	33 (8.3)	10 (10)	
24	55 (3)	42 (3.5)		2 (2.8)		9 (2.3)	2 (2.0)	
28	3 (0.2)	2 (0.2)					1 (1.0)	
29	2 (0.1)	2 (0.2)						
31	42 (2.3)	19 (1.6)	2 (5.7)	2 (2.8)	2 (3.3)	14 (3.5)	3 (3.1)	
33A	2 (0.1)	2 (0.2)						
33F	16 (0.9)	12 (1.0)				2 (0.5)	2 (2.0)	
33?	1 (0.1)					1 (0.3)		
34	16 (0.9)	13 (1.1)				3 (0.8)		
35A	1 (0.1)	1 (0.1)						
35B	62 (3.3)	40 (3.4)		1 (1.4)	3 (4.9)	14 (3.5)	4 (4.1)	
35C	1 (0.1)	1 (0.1)						
35F	93 (5)	72 (6.1)	1 (2.9)	2 (2.8)		14 (3.5)	4 (4.1)	
36	1 (0.1)	1 (0.1)						
37	21 (1.1)	13 (1.1)				6 (1.5)	2 (2.0)	
38	16 (0.9)	13 (1.1)			1 (1.6)	1 (0.3)		1 (25)
40	10 (0.5)	8 (0.7)				2 (0.5)		
41	1 (0.1)					1 (0.3)		
42	1 (0.1)	1 (0.1)						
47	1 (0.1)					1 (0.3)		
NT	55 (3)	30 (2.5)			13 (21)	10 (2.5)	2 (2.0)	

Supplemental Table 4. Frequency distribution of pneumococcal serotypes isolated during the six calendar quarters of the study period. The total number of isolates of every serotype and their relative frequency during each quarter is presented.

	Total	2016 Q4	2017 Q1	2017 Q2	2017 Q3	2017 Q4	2018 Q1
Serotype	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*	*n (%)*
1	1 (0.1)		1 (0.2)				
3	162 (8.7)	20 (8.8)	39 (8.8)	20 (8.3)	8 (4.6)	14 (5)	61 (12.3)
4	4 (0.2)		1 (0.2)		2 (1.1)		1 (0.2)
6A	15 (0.8)	2 (0.9)	3 (0.7)	1 (0.4)	2 (1.1)	2 (0.7)	5 (1)
6B	4 (0.2)				1 (0.6)	3 (1.1)	
7F	4 (0.2)		1 (0.2)	1 (0.4)	1 (0.6)		1 (0.2)
14	5 (0.3)	3 (1.3)				1 (0.4)	1 (0.2)
18C	5 (0.3)		1 (0.2)	2 (0.8)	1 (0.6)		1 (0.2)
19A	84 (4.5)	8 (3.5)	31 (7)	13 (5.4)	4 (2.3)	7 (2.5)	21 (4.3)
19F	23 (1.2)	1 (0.4)	6 (1.4)	4 (1.7)	5 (2.9)	5 (1.8)	2 (0.4)
23F	6 (0.3)	1 (0.4)	3 (0.7)		1 (0.6)	1 (0.4)	
6C	70 (3.8)	6 (2.7)	23 (5.2)	13 (5.4)	10 (5.7)	6 (2.2)	12 (2.4)
6D	1 (0.1)					1 (0.4)	
7A	1 (0.1)		1 (0.2)				
7B	3 (0.2)					2 (0.7)	1 (0.2)
7C	4 (0.2)	1 (0.4)			1 (0.6)		2 (0.4)
8	34 (1.8)	4 (1.8)	7 (1.6)	5 (2.1)	3 (1.7)	4 (1.4)	11 (2.2)
9A	2 (0.1)				1 (0.6)		1 (0.2)
9L	1 (0.1)	1 (0.4)					
9N	44 (2.4)	2 (0.9)	6 (1.4)	6 (2.5)	8 (4.6)	10 (3.6)	12 (2.4)
10	72 (3.9)	9 (4)	18 (4.1)	12 (5)	5 (2.9)	9 (3.2)	19 (3.8)
11A	193 (10.4)	18 (8)	47 (10.6)	26 (10.7)	15 (8.6)	30 (10.8)	57 (11.5)
11D	1 (0.1)					1 (0.4)	
12	4 (0.2)	1 (0.4)	1 (0.2)			2 (0.7)	
13	6 (0.3)		3 (0.7)	1 (0.4)	1 (0.6)	1 (0.4)	
15A	109 (5.9)	15 (6.6)	30 (6.8)	11 (4.5)	12 (6.9)	15 (5.4)	26 (5.3)
15B	69 (3.7)	10 (4.4)	12 (2.7)	11 (4.5)	5 (2.9)	13 (4.7)	18 (3.6)
15C	72 (3.9)	12 (5.3)	21 (4.7)	4 (1.7)	6 (3.4)	8 (2.9)	21 (4.3)
16	51 (2.7)	1 (0.4)	8 (1.8)	9 (3.7)	7 (4)	9 (3.2)	17 (3.4)
17	52 (2.8)	5 (2.2)	11 (2.5)	9 (3.7)	8 (4.6)	5 (1.8)	14 (2.8)
18A	2 (0.1)	1 (0.4)			1 (0.6)		
18B	1 (0.1)				1 (0.6)		
20	7 (0.4)	1 (0.4)	2 (0.5)	1 (0.4)	1 (0.6)		2 (0.4)
21	39 (2.1)	5 (2.2)	10 (2.3)	8 (3.3)	2 (1.1)	7 (2.5)	7 (1.4)
22	59 (3.2)	9 (4)	10 (2.3)	7 (2.9)	3 (1.7)	7 (2.5)	23 (4.7)
23A	72 (3.9)	7 (3.1)	19 (4.3)	13 (5.4)	6 (3.4)	7 (2.5)	20 (4)
23B	176 (9.5)	25 (11.1)	38 (8.6)	18 (7.4)	14 (8)	43 (15.5)	38 (7.7)
24	55 (3)	4 (1.8)	13 (2.9)	12 (5)	6 (3.4)	8 (2.9)	12 (2.4)
28	3 (0.2)		2 (0.5)		1 (0.6)		
29	2 (0.1)		1 (0.2)		1 (0.6)		
31	42 (2.3)	3 (1.3)	12 (2.7)	4 (1.7)	5 (2.9)	5 (1.8)	13 (2.6)
33A	2 (0.1)	2 (0.9)					
33F	16 (0.9)	4 (1.8)	2 (0.5)		1 (0.6)	5 (1.8)	4 (0.8)
33?	1 (0.1)						1 (0.2)
34	16 (0.9)	3 (1.3)	3 (0.7)	2 (0.8)	1 (0.6)	4 (1.4)	3 (0.6)
35A	1 (0.1)	1 (0.4)					
35B	62 (3.3)	7 (3.1)	12 (2.7)	10 (4.1)	7 (4)	17 (6.1)	9 (1.8)
35C	1 (0.1)		1 (0.2)				
35F	93 (5)	14 (6.2)	20 (4.5)	6 (2.5)	7 (4)	14 (5)	32 (6.5)
36	1 (0.1)		1 (0.2)				
37	21 (1.1)	5 (2.2)	4 (0.9)	2 (0.8)	3 (1.7)	3 (1.1)	4 (0.8)
38	16 (0.9)		3 (0.7)		4 (2.3)	3 (1.1)	6 (1.2)
40	10 (0.5)	4 (1.8)	4 (0.9)	2 (0.8)			
41	1 (0.1)		1 (0.2)				
42	1 (0.1)		1 (0.2)				
47	1 (0.1)	1 (0.4)					
NT	55 (3)	10 (4.4)	10 (2.3)	9 (3.7)	4 (2.3)	6 (2.2)	16 (3.2)



Supplemental Figure 1. Age group specific invasive pneumococcal disease/ respiratory tract odds ratios with 95% confidence intervals of the most common pneumococcal serotypes detected during the study period. IPD, invasive pneumococcal disease; OR, odds ratio; PCV13; 13-valent pneumococcal conjugate vaccine included serotypes.

Supplemental Table 5. Antimicrobial resistance rates of pneumococcal serotypes. non-S, nonsusceptible; PCG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; TMP/SMX, trimethoprim/sulfamethoxazole; MDR, multidrug-resistant; XDR, extensively drug-resistant.

Serotype (*n*)	PCG non-S *n (%)*	ERY non-S *n (%)*	CLI non-S *n (%)*	TET non-S *n (%)*	TMP/SMX non-S *n (%)*	NOR non-S *n/n*[a]	MDR *n (%)*	XDR *n (%)*
1								
3	4 (2.5)	8 (4.9)	8 (4.9)	15 (9.3)	2 (1.2)	0/15	8 (4.9)	1 (0.6)
4					1 (25)			
6A	4 (26.7)	3 (20)	1 (6.7)	1 (6.7)	1 (6.7)	0/4	1 (6.7)	
6B	2 (50)	2 (50)	2 (50)	2 (50)	2 (50)	0/2	2 (50)	2 (50)
7F								
14	1 (20)				2 (40)	0/1		
18C	2 (40)	1 (20)		1 (20)	2 (40)	0/2	1 (20)	
19A	15 (17.9)	11 (13.1)	11 (13.1)	11 (13.1)	9 (10.7)	0/22	11 (13.1)	3 (3.6)
19F	8 (34.8)	10 (43.5)	7 (30.4)	10 (45.5)	9 (39.1)	0/11	10 (43.5)	3 (13)
23F	2 (33.3)	2 (33.3)				0/2		
6C	2 (2.9)	10 (14.3)	9 (12.9)	11 (15.7)	3 (4.3)	0/12	9 (12.9)	
6D		1 (100)	1 (100)	1 (100)		0/1	1 (100)	
7A								
7B	2 (66.7)	3 (100)	3 (100)	3 (100)	2 (66.7)	0/3	3 (100)	2 (66.7)
7C	3 (75)			2 (50)	3 (75)	0/3	2 (50)	
8				1 (2.9)		0/2		
9A	2 (100)				2 (100)	0/2		
9L		1 (100)			1 (100)			
9N	2 (4.5)	1 (2.3)		2 (4.5)	1 (2.3)	0/3	2 (4.5)	
10	1 (1.4)			1 (1.4)	4 (5.6)	0/5		
11A	10 (5.2)	5 (2.6)	1 (0.5)	3 (1.6)	15 (7.8)	0/24	4 (2.1)	1 (0.5)
11D								
12								
13								
15A	19 (17.4)	24 (22)	24 (22)	24 (22)	9 (8.3)	0/26	23 (21.1)	4 (3.7)
15B	6 (8.7)	5 (7.2)	2 (2.9)	2 (2.9)	9 (13)	0/9	1 (1.4)	1 (1.4)
15C	8 (11.1)	5 (6.9)	3 (4.2)	3 (4.2)	9 (12.5)	0/10	4 (5.6)	2 (2.8)
16		1 (2)	1 (2)	1 (2)	11 (21.6)	0/5	1 (2)	
17	1 (1.9)	2 (3.8)	2 (3.8)	5 (9.6)	1 (1.9)	0/9	2 (3.8)	
18A	2 (100)				2 (100)	0/2		
18B	1 (100)	1 (100)		1 (100)	1 (100)		1 (100)	
20	1 (14.3)				1 (14.3)	0/2		
21	1 (2.6)				1 (2.6)	0/5		
22	1 (1.7)	2 (3.4)	1 (1.7)	1 (1.7)	1 (1.7)	0/3	1 (1.7)	
23A	7 (9.7)	8 (11.1)	8 (11.1)	8 (11.1)	4 (5.6)	0/10	8 (11.1)	
23B	66 (37.5)	8 (4.5)	3 (1.7)	3 (1.7)	98 (55.7)	0/94	4 (2.3)	
24	2 (3.6)	8 (14.5)	8 (14.5)	8 (14.5)	48 (87.3)	1/10	8 (14.5)	3 (5.5)
28								
29								
31					1 (2.4)	0/1		
33A					1 (50)			
33F		6 (37.5)	1 (6.3)		6 (37.5)			
33?	1 (100)	1 (100)		1 (100)	1 (100)	0/1	1 (100)	
34	1 (6.3)	1 (6.3)	1 (6.3)	1 (6.3)	7 (43.8)	0/3	1 (6.3)	
35A								
35B	13 (21)	7 (11.3)	5 (8.1)	5 (8.1)	10 (16.1)	0/17	6 (9.7)	3 (4.8)
35C								
35F					1 (1.1)	0/4		
36								
37						0/2		
38					1 (6.3)			
40		2 (20)	2 (20)	2 (20)	5 (50)	0/2	2 (20)	
41								
42								
47								
NT	12 (21.8)	11 (20)	5 (9.1)	13 (23.6)	12 (21.8)	0/14	11 (20)	1 (1.8)

[a]Presented as *n* resistant/*n* tested within each serotype because only 389 of all isolated pneumococci were screened for norfloxacin resistance.

Supplemental Table 6. Results from MIC determination with broth microdilution of putative XDR *S. pneumoniae* isolates detected during AST screening. MIC, minimum inhibitory concentration; XDR, extensively drug-resistant; PCG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; TMP/SMX, trimethoprim-sulfamethoxazole; LEV, levofloxacin.

						Broth microdilution MIC for:						
Isolate no.	Year	Patient age	Sample type	Serotype	Gradient test PCG MIC	PCG	ERY	CLI	TET	TMP/SMX	LEV	XDR phenotype confirmed
1	2017	4	Nasopharynx	19F	0.5	1 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
2	2017	34	Nasopharynx	3	1	1 (I)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
3	2018	2	Nasopharynx	19A	1	4 (R)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
4	2018	0	Conjunctiva	19A	1	4 (R)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
5	2016	1	Nasopharynx	19F	1	4 (R)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
6	2018	42	Nasopharynx	19A	0.5	4 (R)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
7	2017	88	Nasopharynx	19F	1	4 (R)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
8	2017	37	Nasopharynx	15A	0.25	0.5 (I)	>2 (R)	>1 (R)	>8 (R)	1 (S)	1 (S)	No
9	2017	3	Nasopharynx	15A	0.13	0.13 (I)	>2 (R)	>1 (R)	>8 (R)	≤0.5 (S)	1 (S)	No
10	2017	7	Sputum	15C	1	0.5 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
11	2017	89	Sputum	15A	0.13	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	1 (S)	1 (S)	No
12	2017	32	Sputum	15A	0.13	0.13 (I)	>2 (R)	>1 (R)	≤1 (S)	1 (S)	≤0.5 (S)	No
13	2018	1	Nasopharynx	24	0.25	1 (I)	>2 (R)	>1 (R)	>8 (R)	≤0.5 (S)	1 (S)	No
14	2017	2	Middle ear	15B	0.25	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	1 (S)	1 (S)	No
15	2017	9	Sputum	24	0.5	0.5 (I)	>2 (R)	>1 (R)	>8 (R)	≤0.5 (S)	1 (S)	No
16	2017	1	Nasopharynx	7B	1	2 (I)	>2 (R)	>1 (R)	>8 (R)	1 (S)	1 (S)	No
17	2018	27	Nasopharynx	7B	1	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	1 (S)	1 (S)	No
18	2017	2	Nasopharynx	15C	1	4 (R)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
19	2017	1	Nasopharynx	11A	0.13	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
20	2017	68	Sputum	6B	0.25	0.25 (I)	>2 (R)	0.25 (S)	>8 (R)	1 (S)	≤0.5 (S)	No
21	2016	41	Conjunctiva	NT	1	2 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	≤0.5 (S)	Yes
22	2017	68	Sputum	35B	0.13	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	2 (I)	1 (S)	Yes
23	2017	58	Bronchi	35B	0.13	0.5 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
24	2017	0	Nasopharynx	35B	0.13	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	4 (R)	1 (S)	Yes
25	2017	0	Nasopharynx	6B	0.25	0.25 (I)	>2 (R)	>1 (R)	>8 (R)	>4 (R)	1 (S)	Yes
26	2016	76	Sputum	24	0.06	0.13 (I)	>2 (R)	0.25 (S)	>8 (R)	4 (R)	>4 (R)	Yes

Paper IV

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Extensive/Multidrug-Resistant Pneumococci Detected in Clinical Respiratory Tract Samples in Southern Sweden Are Closely Related to International Multidrug-Resistant Lineages

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Background/Objective: The frequencies of non-susceptibility against common antibiotics among pneumococci vary greatly across the globe. When compared to other European countries antibiotic resistance against penicillin and macrolides has been uncommon in Sweden in recent years. Multidrug resistance (MDR) is, however, of high importance since relevant treatment options are scarce. The purpose of this study was to characterize the molecular epidemiology, presence of resistance genes and selected virulence genes of extensively drug-resistant (XDR) (*n*=15) and MDR (*n*=10) *Streptococcus pneumoniae* detected in clinical respiratory tract samples isolated from patients in a southern Swedish county 2016-2018. With the aim of relating them to global MDR pneumococci.

Methods: Whole genome sequencing (WGS) was performed to determine molecular epidemiology, resistance genes and presence of selected virulence factors. Antimicrobial susceptibility profiles were determined using broth microdilution testing. Further analyses were performed on isolates from the study and from the European nucleotide archive belonging to global pneumococcal sequence cluster (GPSC) 1 (*n*=86), GPSC9 (*n*=55) and GPSC10 (*n*=57). Bacteria were analyzed regarding selected virulence determinants (pilus islet 1, pilus islet 2 and Zinc metalloproteinase C) and resistance genes.

Results: Nineteen of 25 isolates were related to dominant global MDR lineages. Seventeen belonged to GPSC1, GPSC9 or GPSC10 with MDR non-PCV serotypes in GPSC9 (serotype 15A and 15C) as well as GPSC10 (serotype 7B, 15B and serogroup 24). Pilus islet-1 and pilus islet-2 were present in most sequence types belonging to

GPSC1 and in two isolates within GPSC9 but were not detected in isolates belonging to GPSC10. Zinc metalloproteinase C was well conserved within all analyzed isolates belonging to GPSC9 but were not found in isolates from GPSC1 or GPSC10.

Conclusions: Although MDR *S. pneumoniae* is relatively uncommon in Sweden compared to other countries, virulent non-PCV serotypes that are MDR may become an increasing problem, particularly from clusters GPSC9 and GPSC10. Since the incidence of certain serotypes (3, 15A, and 19A) found among our MDR Swedish study isolates are persistent or increasing in invasive pneumococcal disease further surveillance is warranted.

Keywords: antimicrobial resistance (AMR), extensive drug resistance (XDR), multidrug-resistant (MDR), global pneumococcal sequence cluster, mucosal infection, respiratory tract, streptococcus pneumoniae, serotype

INTRODUCTION

Streptococcus pneumoniae is a commensal of the human respiratory tract and its primary niche is the nasopharynx. Dissemination from the nasopharynx can lead to different types of pneumococcal disease including pneumonia, acute otitis media and sinusitis, of which the bacterial species is considered the most common etiology (Weiser et al., 2018). *S. pneumoniae* also causes invasive pneumococcal disease (IPD), characterized as meningitis, sepsis, and bacteriemic pneumonia that contributes to a high morbidity and mortality globally (Troeger et al., 2018). The most important virulence factor of the pneumococcus is the capsule and around 100 different serotypes have been identified (Ganaie et al., 2020). The first conjugated pneumococcal polysaccharide vaccine PCV7 was introduced in the year of 2000 in the US (covering serotypes 6B, 7F, 9V, 14, 18C, 19F and 23F). Serotype replacement then led to the development of higher valency vaccines, and PCV10 (PCV7 serotypes with addition of 1, 4 and 5) and PCV13 (PCV7 serotypes with addition of 1, 3, 4, 5, 6A and 19A) are mainly administrated. In southern Sweden, PCV7 was introduced in the child immunization program in 2009. PCV10 was implemented one year later with a shift to PCV13 in 2014, and a further change back to PCV10 in 2019.

In a meta-analysis performed by (Andrejko et al., 2021) on 312,783 pneumococci isolated from children, it was shown that the implementation of PCV in child immunization campaigns has resulted in an absolute significant decrease of penicillin non-susceptible pneumococci (PNSP). This reduction was mainly hypothesized to be attributed to reduction of non-susceptible vaccine serotypes (VT), suggesting the possibility to use vaccines also in control of antimicrobial resistance. Local changes with increased antimicrobial resistance due to serotype replacement is, however, a phenomenon to be aware of as has been observed in Sweden regarding IPD isolates in the post-PCV era. An increase of PNSP from 3.3% in 2007 to 5.6% in 2013-2016 has been observed, primarily due to the increase of non-vaccine types (NVT) PNSP (Naucler et al., 2017). Serotype switch events in already multidrug-resistant (MDR; nonsusceptibility to 3 or more different antimicrobial classes) VT lineages is another way for new clones to expand after vaccine implementation. Examples of serotype switch events have been noted in the post PCV era but the general survival rate of the resulting strains in the population is to be followed through further studies (Savinova et al., 2020; Scherer et al., 2021).

In a study by Gladstone et al. (2019) global pneumococcal sequence clusters (GPSC) were defined as a new tool for epidemiological analysis of *S. pneumoniae* in the post PCV era. It was shown that some GPSCs have an increased accumulation of MDR isolates as for example GPSC1. Close surveillance of the GPSCs is of importance for the evaluation of effect of PCVs on the overall antimicrobial resistance (AMR) within different lineages.

The pattern of non-susceptibility to common antibiotics among pneumococci vary greatly across the globe (Andrejko et al., 2021). In Sweden, AMR to penicillin and macrolides has been low in later years compared to other European countries. The European center of disease control (ECDC) estimated that Sweden, in 2019, had 6.5% PNSP and 6.5% macrolide non-susceptible invasive *S. pneumoniae* compared to the EU average of 12.1 and 14.5% (ECDC, 2020a; ECDC, 2020b). During the years 2016-2018 *S. pneumoniae* detected in clinical respiratory tract samples in Skåne County, southern Sweden, were investigated regarding serotype distribution and antimicrobial susceptibility. Of the 2,131 included isolates, 11% were PNSP and 8% macrolide non-susceptible. Furthermore, 7% were MDR and 2% were extensively drug resistant (XDR; nonsusceptibility to 5 or more different antimicrobial classes) (Golden et al., 2015; Uddén et al., 2021). MDR and XDR isolates included both VT and NVT pneumococci (Uddén et al., 2021).

The purpose of this study was to do whole genome sequencing (WGS) on pneumococcal MDR isolates detected in Skåne County between 2016-2018 with the aim of studying their resistance genes and molecular epidemiology in a global context. We present data on GPSC, resistance genes and selected virulence genes. We also show data regarding the virulence factor zinc metalloproteinase C (ZmpC) and the presence of PI-1 and PI-2 islets among different MDR GPSC as possible contributors to virulence or increased transmission among pneumococci.

MATERIALS AND METHODS

Streptococcus pneumoniae Isolates

In our recent study, a total of 2,131 pneumococci were detected among clinical respiratory tract samples during 18 months in 2016-2018 (Uddén et al., 2021). Serotyping was performed as described in (Uddén et al., 2018). Briefly a multiplex polymerase chain reaction (PCR) scheme in combination with latex agglutination and the Quellung reaction was used. Of the 1,858 isolates that were available for further analyses, 26 isolates were classified as XDR based on non-susceptibility to ≥5 antimicrobial classes during screening with disk diffusion tests (oxacillin, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole and norfloxacin) and a gradient test for benzylpenicillin (Etest®; BioMérieux, Marcy-l'Étoile, FR), and were included in the current study. Minimum inhibitory concentrations (MIC) of the currently studied isolates were confirmed with broth microdilution (BMD) (Sensititre Streptococcus STP6F AST Plate; Thermo Fisher Scientific, Waltham, MA) and interpreted according to breakpoints provided by EUCAST 2021 to determine MDR (*n*=10) or XDR (*n*=16) phenotype (Uddén et al., 2021). One of 26 isolates was, however, excluded based on low quality of WGS, and not included in further analyses.

Whole Genome Sequencing

Illumina sequencing described by (Kavalari et al., 2019) was used for WGS. Species identification was performed using ribosomal multi locus sequence typing (rMLST) provided on PubMLST (https://pubmlst.org) (Jolley et al., 2018). Capsular loci were analyzed to determine capsular genotype using the PneumoCaT tool (Kapatai et al., 2016).

Molecular Epidemiology and Resistance Genes

Assembled genomes were uploaded to PathogenWatch (https://pathogen.watch) to assign MLST and GPSC (Jolley et al., 2018). Corresponding clonal complex (CC) of the detected sequence types, and additional epidemiological information regarding GPSC and MLST were acquired from the Global Pneumococcal Sequencing Project database (https://www.pneumogen.net/gps/GPSC_lineages.html, accessed 1-07-2021) and PubMLST. Clonal complexes were assigned by a single locus threshold (6/7) (Gladstone et al., 2019). PathogenWatch was additionally used to screen for antimicrobial resistance genes (*ermB, mefA, tetM*) and mutations (*folA/folP, gyrA/parC*) conferring resistance to commonly used antibiotics for pneumococcal disease and mosaic penicillin binding protein (PBP) profiles conferring resistance to β-lactam antibiotics in pneumococci (Li et al., 2017; Gladstone et al., 2019).

Determination of Virulence Factors

Genes encoding the virulome were screened for using NCBI Genome Workbench version 3.6.0 and BlastN was performed for comparison of nucleotide sequences. Selected sequences for virulence genes of interest related to *S. pneumoniae* were collected from the Virulence finder database nucleotide dataset_B (downloaded 4/8-2021) (Weiser et al., 2018; Liu et al., 2019). Additional sequences were selected for detection of pilus islet 1 (PI-1) (GenBank accession numbers: EF560625–EF560637) and pilus islet 2 (PI-2) (GenBank accession number: EU311539.1) (Bagnoli et al., 2008; Moschioni et al., 2008). A 95% identity and 80% coverage were used for identification of a sequence (Kavalari et al., 2019).

Phylogeny, Resistance Determinants and Selected Virulome of Isolates Within GPSC1, GPSC9 and GPSC10

Three phylogenetic trees were constructed with the sequenced isolates belonging to GPSC1, GPSC9 and GPSC10 together with additional reference isolates described by Gladstone et al. (Gladstone et al., 2019) provided from ENA (https://www.ebi.ac.uk/ena/browser/home) (ENA run accessions in **Supplemental Material**). The isolates were selected to represent different sequence types within GPSC1 (*n*=79), GPSC9 (*n*=50) and GPSC10 (*n*=52). MinTyper 1.0 were used for the SNP distance matrices and creation of the phylogenetic trees (Hallgren et al., 2021). The trees were rooted to concatenated sequences of different reference strains, which are specified in each figure. The phylogenetic trees were visualized using Interactive tree of life (iTol v.5) (Letunic and Bork, 2021). Assembled genomes of the ENA isolates were downloaded from PathogenWatch together with metadata including PBP-profiles, resistance genes (*ermB, mefA, tetM*) and mutations (*folA/folP*) for the isolates (date 8/8-2021). The presence of selected virulence factors (ZmpC, PI-1 and PI-2) was analyzed as previously described.

RESULTS

Three Different Global Pneumococcal Sequence Clusters Dominated in the Catchment Area

Included isolates were serotyped and assigned to MLST, GPSC and CC by extraction from WGS data (**Table 1**). All isolates (*n*=25) were identified as *S. pneumoniae* using rMLST. Three different GPSC dominated among the samples, GPSC1 (*n*=7), GPSC9 (*n*=5) and GPSC10 (*n*=5). The dominating serotypes were 15A (*n*=4), 19A (*n*=3), 19F (*n*=3), 35B (*n*=3) and in total 10 different serotypes (3, 6B, 7B, 11A, 15A, 15B, 15C, 19A, 19F and 35B) and 1 serogroup (24) were identified and in general consistent with WGS. Isolates belonging to the same GPSC often carried identical resistance genes and closely related PBP profiles.

ZmpC and Pilus Islets Were Present Among Only a Fraction of Isolates

The isolates were analyzed for virulence factors known to be important for pneumococcal pathogenesis and colonization. Selected virulence factors, ZmpC and pilus islets, were present among only a selection of the isolates **(Table 1)**. The virulence

TABLE 1 | XDR and MDR isolates were closely related to international MDR lineages.

Isolate	Year	Age	Sample type	Serotype	GPSC[a]	MLST	CC	Resistance	PCV coverage				Virulence		
ID		Years		Phenotype/Genotype				Profile	PCV10	PCV13	PCV15	PCV20	*PI-1*	*PI-2*	*zmpC*
1	2017	4	Nasopharynx	19F/19F	**1**	236[b]	–	XDR	+	+	+	+	+	+	–
2	2017	34	Nasopharynx	3/3	**1**	271[b]	CC320	XDR	–	+	+	+	+	+	–
3	2018	2	Nasopharynx	19A/19A	**1**	320	CC320	XDR	–	+	+	+	+	+	–
4	2018	0	Conjunctiva	19A/19A	**1**	320	CC320	XDR	–	+	+	+	+	+	–
5	2016	1	Nasopharynx	19F/19F	**1**	2920[b]	–	XDR	+	+	+	+	+	+	–
6	2018	42	Nasopharynx	19A/19A	**1**	4768	CC320	XDR	–	+	+	+	+	+	–
7	2017	88	Nasopharynx	19F/19F	**1**	8359	–	XDR	+	+	+	+	+	+	–
8	2017	37	Nasopharynx	15A/15A	**9**	63[c]	CC63	MDR-4	–	–	–	–	–	–	+
9	2017	3	Nasopharynx	15A/15A	**9**	63[c]	CC63	MDR-4	–	–	–	–	–	–	+
10	2017	7	Sputum	15C/15C	**9**	782[c]	CC63	XDR	–	–	–	–	–	–	+
11	2017	89	Sputum	15A/15A	**9**	3816[c]	CC63	MDR-4	–	–	–	–	–	–	+
12	2017	32	Sputum	15A/15A	**9**	3816[c]	CC63	MDR-3	–	–	–	–	–	–	+
13	2018	1	Nasopharynx	24/24	**10**	230[d]	CC230	MDR-4	–	–	–	–	–	–	–
14	2017	2	Middle ear	15B/15B	**10**	4253[d]	CC230	MDR-4	–	–	–	+	–	–	–
15	2017	9	Sputum	24/24	**10**	6227[d]	CC230	MDR-4	–	–	–	–	–	–	–
16	2017	1	Nasopharynx	7B/7B	**10**	Novel1	–	MDR-4	–	–	–	–	–	–	–
17	2018	27	Nasopharynx	7B/7B	**10**	Novel1	–	MDR-4	–	–	–	–	–	–	–
18	2017	2	Nasopharynx	15C/15C	**16**	83[e]	CC81	XDR	–	–	–	–	–	–	–
19	2017	1	Nasopharynx	11A/11A	43	8605	–	XDR	–	–	–	+	–	–	–
20	2017	68	Sputum	6B/6E	**47**	2040[f]	CC315	MDR-3	+	+	+	+	+	–	–
21	2016	41	Conjunctiva	NT/NT	**81**	4149	–	XDR	–	–	–	–	–	–	–
22	2017	68	Sputum	35B/35B	91	373	–	XDR	–	–	–	–	–	–	–
23	2017	58	Bronchi	35B/35B	91	373	–	XDR	–	–	–	–	–	–	–
24	2017	0	Nasopharynx	35B/35B	91	373	–	XDR	–	–	–	–	–	–	–
25	2017	0	Nasopharynx	6B/6E	**115**	135	CC1348	XDR	+	+	+	+	+	–	–
									20%	**36%**	**36%**	**44%**	**36%**	**28%**	**20%**

The major GPSC identified were GPSC1 (n=7), GPSC9 (n=5) and GPSC10 (n=5) and 21/25 isolates belong to GPSC known to carry a high degree of resistance. Most of the isolates (13/25) were PMEN clones or single locus variants (SLV) of PMEN clones. Major clonal complexes identified were CC320 (n=4), CC230 (n=3) and CC63 (n=5). Some isolates were reclassified as MDR due to changes in resistance pattern upon BMD testing, and updated breakpoints by EUCAST in 2019, but 15/25 showed an XDR resistance profile. PCV coverage of the sequenced isolates was low; 20% for PCV10 and 36% for PCV13. Major non-PCV serotypes detected include MDR serotype 15A,15C, serogroup 24, 7B and 35B. Additional coverage would not be increased with the use of PCV15 and only slightly with the use of PCV20, 44% with addition of serotype 11A and 15B. Pilus islet 1 (PI-1) and pilus islet-2 were present among 36% and 28% of the isolates respectively. PI-1 and PI-2 was carried by all isolates belonging to GPSC1. Zinc metalloproteinase C (ZmpC) was only present among isolates belonging to GPSC9 20% (n=5/25).

a- Bold - >2.5 in mean no of resistant classes in GPSC.

b- Identical or SLV of PMEN Taiwan19F-14 ST236.

c- Identical or SLV of PMEN Sweden15A-25 ST63.

d- Identical or SLV of PMEN Denmark14-32 ST230.

e- SLV of PMEN Spain23F-1 ST81.

f- SLV of PMEN Poland6B-20 ST315.

GPSC, Global pneumococcal sequence cluster; MLST, Multi locus sequence type; CC, Clonal complex (defined as 6/7 identical alleles); XDR, Extensively drug resistant; MDR, Multidrug resistant; PMEN, Pneumococcal molecular epidemiology network.

gene *zmpC* was only carried by the 5 isolates belonging to GPSC9. PI-1 was present in 36% (9/25) of the isolates and PI-2 in 28% (7/25) of the isolates. All isolates belonging to GPSC1 carried both PI-1 and PI-2 (*n*=7). All isolates were equipped with *ply*, *lytA*, *lytC*, *cbpE*, *pavA*, *hysA*, *eno*, *piuA*, *psaA*, *cppA*, *htrA* and *tig/ropA* that are common virulence genes.

High Correlation Between Genotypic and Phenotypic Antimicrobial Susceptibility Among MDR Pneumococci

All the isolates (*n*=25) were selected for further investigation including MIC test using BMD and WGS (**Table 2**). All isolates harbored *ermB* and *tetM*, and a minority (*n*=7) also carried *mefA* belonging to GPSC1. Trimethoprim-sulfamethoxazole resistance was observed in 15/25 isolates, although 24/25 carried genes related to antibiotic resistance to sulfamethoxazole with an amino acid insertion in *folP*. The genotype correspondence with phenotype regarding β-lactam resistance was high with 23/25 isolates, having a benzylpenicillin MIC corresponding within 1 dilution step to their predicted MIC in relation to their PBP profile. Two isolates harboring *ermB* were susceptible to clindamycin upon testing with BMD, and one isolate was susceptible to tetracycline although harboring *tetM*. Two isolates harbored *cat* with phenotypic resistance to chloramphenicol. None of the included isolates carried mutations within *gyrA*/*parC* and were all susceptible to levofloxacin (**Supplemental Material**). All included isolates were susceptible to linezolid, vancomycin and meropenem (**Supplemental Material**). Based upon MICs determined with BMD, 10/25 isolates did not exhibit XDR phenotypes, because they were reclassified as susceptible to trimethoprim-sulfamethoxazole following updated EUCAST breakpoints in 2019.

TABLE 2 | Phenotypic and genotypic antimicrobial susceptibility correspondence was high.

Isolate	β-lactam antibiotics					Macrolide/Lincosamide			Tetracycline		Trimethoprim		Chloramphenicol	
ID	PBP profile	Benzylpenicillin		Cefotaxime			Ery	Cli			Sulfamethoxazole			
	1a-2b-2x	Inferred MIC	BMD MIC	Inferred MIC	BMD MIC	*ermB/ mefA*	BMD MIC	BMD MIC	*tetM*	BMD MIC	*folA/ folP*	BMD MIC	*cat*	BMD MIC
1	13-16-47	2	1 I	1	0.25 S	+/+	>2 R	>1 R	+	>8 R	+/+	4 R	–	2 S
2	17-16-47	2	1 I	1	1 I	+/+	>2 R	>1 R	+	>8 R	+/+	>4 R	–	4 S
3	13-11-16	4	4 R	2	2 I	+/+	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
4	13-11-16	4	4 R	2	2 I	+/+	>2 R	>1 R	+	>8 R	+/+	>4 R	–	4 S
5	13-16-New	4	4 R	8	2 I	+/+	>2 R	>1 R	+	>8 R	+/+	>4 R	–	8 S
6	13-11-16	4	4 R	2	2 I	+/+	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
7	13-14-20	4	4 R	1	1 I	+/+	>2 R	>1 R	+	>8 R	+/+	>4 R	–	4 S
8	New-7-138	0.5	0.5 I	0.5	0.5 S	+/-	>2 R	>1 R	+	>8 R	-/+	1 S	–	4 S
9	24-27-28	0.25	0.12 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	-/-	≤0.5 S	–	4 S
10	17-53-36	2	0.5 I	1	0.25 S	+/-	>2 R	>1 R	+	>8 R	-/+	4 R	–	4 S
11	24-27-13	0.25	0.25 I	0.25	0.12 S	+/-	>2 R	>1 R	+	>8 R	-/+	1 S	–	4 S
12	24-27-13	0.25	0.12 I	0.25	0.12 S	+/-	>2 R	>1 R	+	≤1 S	-/+	1 S	–	4 S
13	New-15-22	0.5	1 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	-/+	≤0.5 S	–	8 S
14	New-15-367	0.25	0.25 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	-/+	1 S	–	≤1 S
15	17-15-22	0.5	0.5 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	-/+	≤0.5 S	–	2 S
16	17-144-8	2	2 I	0.5	1 I	+/-	>2 R	>1 R	+	>8 R	-/+	1 S	–	4 S
17	17-144-8	2	0.25 I	0.5	1 I	+/-	>2 R	>1 R	+	>8 R	-/+	1 S	–	4 S
18	15-12-18	2	4 R	1	1 I	+/-	>2 R	>1 R	+	>8 R	+/+	>4 R	+	16 R
19	7-12-135	0.25	0.25 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
20	New-53-35	0.5	0.25 I	0.25	0.25 S	+/-	>2 R	0.25 S	+	>8 R	-/+	1 S	–	4 S
21	25-7-56	2	2 I	1	1 I	+/-	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
22	7-1-New	0.25	0.25 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	+/+	2 I	–	2 S
23	7-1-242	0.25	0.5 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
24	7-1-242	0.25	0.25 I	0.12	0.12 S	+/-	>2 R	>1 R	+	>8 R	+/+	4 R	–	4 S
25	8-67-103	0.25	0.25 I	0.25	0.25 S	+/-	>2 R	>1 R	+	>8 R	+/+	>4 R	+	16 R

Similar patterns of resistance were observed based on the GPSC. GPSC1 isolates (ID 1-7) showed a higher penicillin MIC (1-4 mg/ml), carriage of ermB/mefA/tetM as well as mutations conferring resistance to co-trimoxazole. GPSC9 isolates (ID 8-12) had a lower grade of penicillin nonsusceptibility (MIC 0.12-0.5 mg/ml) and carried ermB and tetM. One isolate (ID 10) was also resistant to co-trimoxazole with the folA I100L mutation and amino acid insertions in folP 57-70. Isolates belonging to GPSC10 (ID 13-17) had a low to high grade of penicillin nonsusceptibility (MIC 0.25-2 mg/ml) and carried ermB and tetM. Remaining isolates were of varied resistance patterns but were all non-susceptible to penicillin and macrolides.

Related Resistance Genes and Presence of Selected Virulence Genes Among Closely Associated Clones Within GPSC1, GPSC9 and GPSC10

To study selected virulence factors of interest and resistance genes, GPSC specific phylogenetic trees were constructed together with metadata. PI-1 and/or PI-2 was present among all the included isolates belonging to GPSC1 (**Figure 1**) but was only found in two isolates (PI-1) belonging to GPSC9 (**Figure 2**). No isolates belonging to GPSC10 carried PI-1 or PI-2 (**Figure 3**). All isolates belonging to GPSC9 carried *zmpC* but none of the GPSC1 or GPSC10 isolates carried the gene. Isolates that were closely related in the SNP tree carried similar PBP profiles, serotypes, and resistance genes in all the GPSC.

DISCUSSION

WGS was performed on clinical MDR isolates detected in Skåne County, southern Sweden during 18 months in 2016-2018. They belonged to dominant GPSCs that carry high frequencies of antibiotic resistance, namely GPSC1, GPSC9 and GPSC10 (Gladstone et al., 2019).

GPSC1 is globally dominated by serotype 19A and 19F (Gladstone et al., 2019). After the introduction of PCV7/PCV10, including serotype 19F, in child immunization programs, an increased incidence of IPD caused by MDR serotype 19A CC320, GPSC1, was observed (Schroeder et al., 2017; Cassiolato et al., 2018). In contrast, introduction of PCV13 has decreased the incidence of IPD related to serotype 19A CC320 (Isturiz et al., 2017; Schroeder et al., 2017). Serotype 19A is, however, persisting both in Skåne County and among IPD cases in Sweden, 11% in 2020 (Public Health Agency of Sweden, 2021; Uddén et al., 2021). Of note is also that a variant of serotype 19A CC320 have been associated with PCV13 failures and breakthroughs in Ireland (Corcoran et al., 2021).

Serotypes 19A and 19F were the most common serotypes in GPSC1 in our study with one exception: a serotype 3 ST271 isolate. In 2015-2017, three serotype 3 ST271 MDR isolates were detected in two different states in the US and the serotype switch within the ST271 was described by Scherer *et al.* (Scherer et al., 2021). Two additional serotype 3 ST271 pneumococci are also described in PathogenWatch, isolated in South Africa 2013.

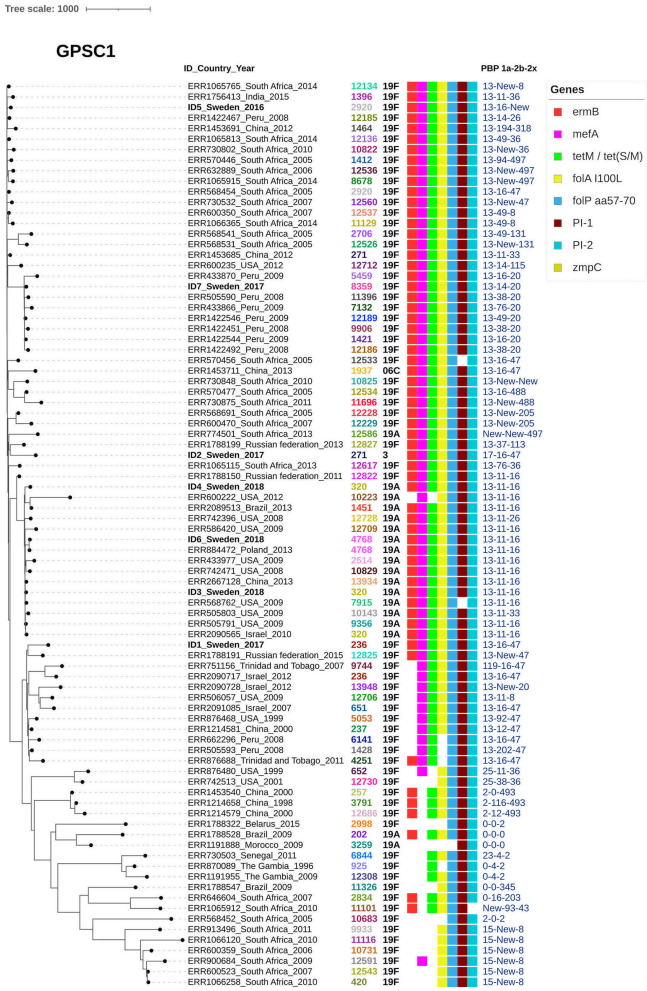

FIGURE 1 | Dual presence of both Pilus islet 1 and 2 is common within GPSC1. GPSC1 tree including 86 isolates showing ID number, country and year followed by serotype, MLST, gene presence and PBP-profile (1a-2b-2x). As a reference isolate ID 1 was used. Almost all isolates (83/86) carry both Pilus islet 1 (PI-1) and Pilus islet 2 (PI-2). The three isolates that were negative for PI-1 or PI-2 however contained larger fragments of the corresponding pilus islets. All the Swedish isolates and many of the included (55/86) carry dual macrolide resistance mechanisms with both *ermB* and *mefA*. Regarding the PBP profile PBP-1a allele 13 is common as well as the profile 13-11-16. Zinc metalloproteinase C (ZmpC) was not found among either of the isolates included.

The presence of an additional similar isolate in Sweden 2017, carrying identical resistance genes and PBP profile, may indicate global spread of the clone. The finding of this isolate, that is closely related to the widely distributed 19F ST271 clone, is of concern since the use of PCV13 has not reduced serotype 3 IPD incidence in Sweden (Naucler et al., 2017; Public Health Agency of Sweden, 2021). Post PCV13 introduction in many Swedish counties, *S. pneumoniae* serotype 3 has remained the most common serotype causing IPD in Sweden, accounting for 15% of cases in 2020 (Public Health Agency of Sweden, 2021).

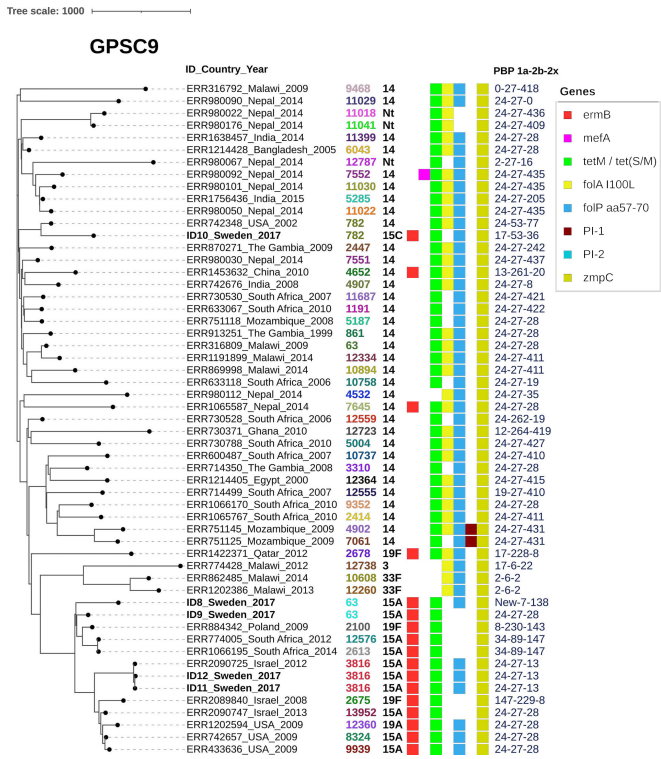

FIGURE 2 | Zinc metalloproteinase C is highly preserved among isolates belonging to GPSC9. GPSC9 tree including 55 isolates showing ID number, Country and Year followed by serotype, MLST, gene presence and PBP-profile (1a-2b-2x). As a reference isolate ID 9 was used. All the Swedish isolates and all the included 100% harbor the gene for zinc metalloproteinase C (*zmpC*). Macrolide resistance gene *ermB* was mostly present in a subclade with serotype 19A, 19F and 15A isolates. Most of the Swedish isolates (4/5), belonged to this subclade with one exception being the ST782 serotype 15C isolate more closely related to other pneumococci within GPSC9. This 15C isolate diverged in both serotype and PBP profile from other closely related pneumococci. Regarding the PBP profile PBP-1a allele 24 is common within the GPSC among our included isolates. Only two isolates carried Pilus islet 1 (PI-1) that are closely related from Mozambique. Pilus islet-2 was not found among either of the isolates included.

All currently studied GPSC1 isolates carried both PI-1 and PI-2, conferring increased adherence to host cells (Barocchi et al., 2006; Bagnoli et al., 2008). Our findings are in concordance with other studies in which the presence of PI-1 has been linked to MDR lineages with penicillin resistance, exemplified by CC320 that was also present among our MDR isolates (Dzaraly et al., 2020). In addition, GPSC1 and, specifically, CC320 have been shown to carry both PI-1 and PI-2 (Dzaraly et al., 2020; Nagaraj et al., 2021). Taken together the presence of MDR GPSC1 still warrants surveillance to follow serotype switch variants and expansion of MDR following PCV10 reintroduction in 2019 as previously discussed (Uddén et al., 2021).

GPSC10 has been described to be of concern regarding both multidrug resistance and serotype replacement in the post-PCV era (Nagaraj et al., 2021). Several isolates in our study belonged to GPSC10, including some NVTs including 7B, 15B and serogroup 24. None of these serotypes are included in currently available PCVs, and to the best of our knowledge only 15B will be included in a future multivalent PCV (Greenberg et al., 2018; Essink et al., 2020).

GPSC9 has mostly been dominated by VT 14 but serotype 15A was the most common serotype found in our study. Serotype 15A has also increased in several parts of the world following the implementation of PCV13 (van der Linden et al., 2015; Nakano et al., 2016; Wu et al., 2020), and it is not targeted by any PCV currently in development to our knowledge (Greenberg et al., 2018; Essink et al., 2020). In both Norway and the US serotype 15A has increased in the post PCV era or been related to MDR

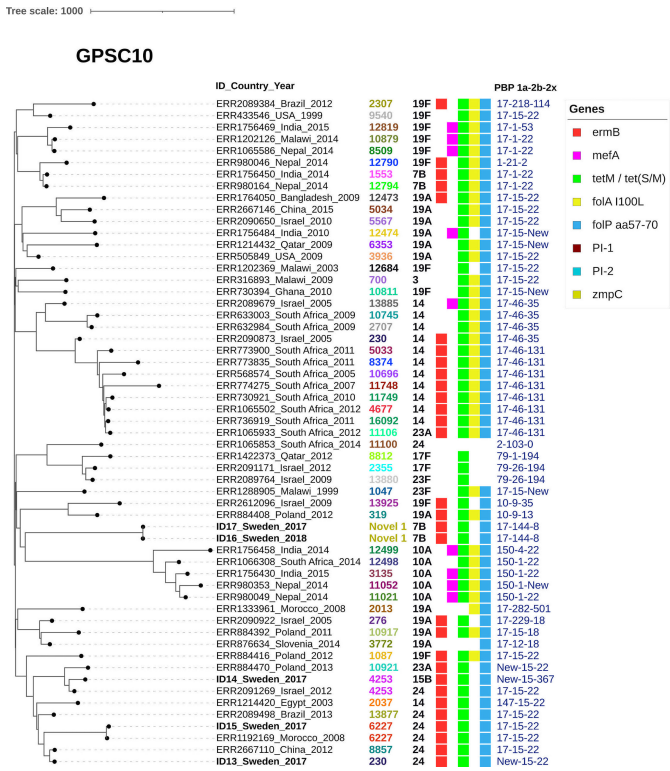

FIGURE 3 | GPSC10 lack both pilus islets and ZmpC. GPSC10 tree including 57 isolates showing ID number, Country and Year followed by serotype, MLST, gene presence and PBP-profile (1a-2b-2x). As a reference isolate ID 13 was used. All the Swedish isolates and all the included 100% harbored the zink metalloproteinase C gene (*zmpC*). Macrolide resistance mechanism *ermB* was mostly present in different sub-clades with serogroup 24, serotype 14 or 7B isolates. Regarding the PBP profile PBP-1a allele 17 was common within the GPSC among our included isolates. None of the isolates carried either Pilus islet 1, Pilus islet 2 or *zmpC*.

IPD cases (Beall et al., 2018; Siira et al., 2020). In the US CC63, belonging to GPSC9, was also found to be the main contributor to MDR invasive 15A isolates in 2015-2016 (Beall et al., 2018). In 2020, serotype 15A was one of the more common serotypes (7%) among IPD cases in Sweden and in 2016 serotype 15A together with 23B were the main contributors to MDR IPD (Naucler et al., 2017; Public Health Agency of Sweden, 2021). This NVT was also common among respiratory tract isolates and frequently MDR in Skåne County 2016-2018 (Uddén et al., 2021). Although the currently studied isolates are not from IPD cases, our results indicate that serotype 15A, GPSC9 MDR pneumococci are present in the population and is likely of importance regarding invasive pneumococcal disease also in Sweden.

Of the isolates detected in Skåne County, the *zmpC* gene was only present in GPSC9 (n=5/25). The gene was also present among all GPSC9 isolates included from ENA. The protease encoded by *zmpC* cleaves human matrix metalloproteinase 9 and has, in mouse pneumonia models, been correlated to increased virulence in comparison to knockout strains (Oggioni et al., 2003) and inhibit neutrophil influx to the lung by cleavage of P-Selectin Glycoprotein 1 (Surewaard et al., 2013). Other targets of the protease include syndecan-1 and membrane-associated mucin MUC16 that are shed from the epithelium by the protease, increasing virulence (Chen et al., 2007; Govindarajan et al., 2012). ZmpC has in an observational study also been associated to a more severe clinical presentation among IPD patients with increased rates of ICU admission (Cremers et al., 2014). Previously ZmpC has been linked to serotypes 11A, 8 and 33F of ST53 and ST62, both belonging to GPSC3 (Camilli et al., 2006; Gladstone et al., 2019; Hansen et al., 2021).

In our study, the conservation within GPSC9 is presented. The protease importance for the success in dissemination and virulence of different GPSCs is something that can be of further interest to study due to its conservation in certain lineages recurring in pneumococcal disease like GPSC9 and most likely also GPSC3.

Irregularities between genotypic and phenotypic resistance were present although the concordance in general was high. One isolate carried *tetM* while still being sensitive, this has previously been noted and might be due to mutations within *tetM* not addressed in our study (Grohs et al., 2012). Overall β-lactam resistance showed a high correspondence pointing at the possibilities of using sequencing as a diagnostic tool for identifying resistance mechanisms in clinical isolates not detected by culture. Isolates included in the study were detected in routine clinical diagnostic testing. Isolated primarily from the nasopharynx (15/25) with an unknown indication for sampling. We thus cannot exclude that pneumococcus isolated might represent carriage strains rather than disease causing strains. However, they all represent highly resistant pneumococci that are present in the population of southern Sweden.

CONCLUSION

The majority of the sequenced XDR and MDR pneumococcal isolates detected in Skåne County belonged to a limited number of GPSCs, primarily GPSC1, GPSC9 and GPSC10. Many *S. pneumoniae* were NVTs belonging to internationally widespread pneumococcal lineages of which many also cause invasive pneumococcal disease. The prevalence of XDR and MDR clones causing disease that are not targeted by current PCVs is of concern, such as those detected within GPSC10 and GPSC9. The most prevalent serotypes found are also common or increasing among IPD cases in Sweden post PCV10/13. Further surveillance is of importance as this may affect both treatment and prophylactic measures regarding IPD and mucosal pneumococcal disease.

DATA AVAILABILITY STATEMENT

The data presented in the article will be made available by the authors upon request. The genomic sequence data for the 25 isolates are deposited in the European nucleotide archive (https://www.ebi.ac.uk/ena) (ENA project accession: PRJEB41999).

ETHICS STATEMENT

The study was approved by the local ethics committee (Regionala etikprövningsnämnden i Lund) 341 (approval no. 2012/286) and the ethical approval was updated (approval no. 2016/752) to include the current study period.

AUTHOR CONTRIBUTIONS

FU, JA, and KR contributed to conception of the study. LY, FU, H-CS, and KF developed methodology. LY and FU administered the project. LY, FU, and H-CS performed the bioinformatic analysis. LY wrote the first draft of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.824449/full#supplementary-material

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Paper V

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Pneumococcal carriage among children aged 4 – 12 years in Angola 4 years after the introduction of a pneumococcal conjugate vaccine

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ABSTRACT

Children in Angola are affected by a high burden of disease caused by pneumococcal infections. The 13-valent pneumococcal conjugate vaccine (PCV13) was introduced in the childhood immunization programme in 2013 but the serotype distribution of *Streptococcus pneumoniae* and antimicrobial susceptibility patterns are unknown. We did a cross-sectional nasopharyngeal carriage study in Luanda and Saurimo, Angola (PCV13 3rd dose coverage 67% and 84%, respectively) during November to December 2017 comprising 940 children aged 4–12 years. The main objective was to assess vaccine serotype coverage and antimicrobial susceptibility rates for *S. pneumoniae*. Our secondary aim was to characterize colonizing strains of *Haemophilus influenzae* and *Moraxella catarrhalis*. Pneumococcal colonization was found in 35% (95% CI 32–39%) of children (n = 332), with 41% of serotypes covered by PCV13. The most common serotypes were 3 (8%), 18C (6%), 23F (6%), 11A (6%), 34 (6%), 19F (5%) and 16 (5%). Carriage of *H. influenzae* and *M. catarrhalis* was detected in 13% (95% CI 11–15%) and 15% (95% CI 13–17%) of children, respectively. Non-susceptibility to penicillin was common among pneumococci (40%), particularly among PCV13-included serotypes (50% vs. 33%; p = 0.003), although the median minimal inhibitory concentration was low (0.19 μg/mL, IQR 0.13–0.25 μg/mL). Most pneumococci and *H. influenzae* were susceptible to amoxicillin (99% and 88%, respectively). Furthermore, resistance to trimethoprim-sulfamethoxazole was>70% among all three species. Multidrug-resistant pneumococci (non-susceptible to ≥ 3 antibiotics; 7% [n = 24]) were further studied with whole genome sequencing to investigate clonality as an underlying cause for this phenotype. No clearly dominating clone(s) were, however, detected. The results indicate that continued use of PCV13 may have positive direct and herd effects on pneumococcal infections in Angola as carriage of vaccine serotypes was common in the non-vaccinated age group. Finally, amoxicillin is assessed to be a feasible empirical treatment of respiratory tract infections in Angola.

1. Introduction

Streptococcus pneumoniae cause substantial mortality and morbidity among children in low- and middle-income countries due to community acquired pneumonia (CAP), invasive pneumococcal disease (IPD) and acute otitis media (AOM) [1,2]. The bacterium frequently colonizes the nasopharynx of children, a niche it shares with *Haemophilus influenzae* and *Moraxella catarrhalis*, which also are important causes of AOM, and in the case of *H. influenzae* CAP, bacteraemia and meningitis [3,4]. General infant immunization with pneumococcal conjugate vaccines (PCV) has resulted in decreasing incidences of pneumococcal infections among children, with some herd effects in adults through reduced transmission of

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vaccine type (VT) pneumococci [2,5]. However, serotype replacement with non-vaccine serotypes (NVT) has in some cases caused emergence of invasive and/or antibiotic resistant NVTs and partially diminished herd effects [5–6]. Studies of pneumococcal carriage are of great importance for evaluation of PCV implementation [7].

In Angola, pneumococcal infections are a major health issue with a mortality rate of 216 per 100,000 children aged 1 to 59 months in 2015, predominantly related to CAP and IPD [2]. Furthermore, the high incidence of AOM among children represents an important public health concern leading to extensive morbidity due to complications such as chronic suppurative otitis media (CSOM) and hearing loss [8,9]. PCV13 was introduced in the Angolan childhood immunization programme in 2013 (3 + 0 schedule, administered at 2, 4 and 6 months) and the WHO-UNICEF estimate of national immunization coverage (WUENIC) for the 3rd PCV dose was 45–59% during 2014 to 2018 [10]. The *H. influenzae* type b (Hib) conjugate vaccine was introduced in Angola in 2006 (3 + 0 schedule) with a WUENIC between 43% and 59% since 2007 [10]. We have previously studied the bacterial spectrum associated with CSOM in Angola and detected a small number of pneumococci (n = 34), of which 35% were covered by PCV13 and 53% were penicillin non-susceptible (PNSP) [11]. Furthermore, recent studies of bacterial meningitis at a paediatric hospital in Luanda have shown that *S. pneumoniae* is the currently dominating etiological agent, and that 48% (10/21) of serotyped pneumococci were VT in 2016–2017 [12,13]. In addition to these reports, the current pneumococcal serotype distribution and antimicrobial susceptibility rates are unknown. To enable future evaluation of the immunization programme, more data on the present situation when vaccinations commenced are urgently needed.

With the main objective to characterize the spectrum of *S. pneumoniae* circulating in the Angolan population 4 years after the introduction of PCV13, we conducted a cross-sectional nasopharyngeal carriage study in children aged 4 to 12 years in Angola during November to December 2017. The study individuals belonged to an age group untargeted by routine PCV immunization and the detected serotype distribution was therefore thought to be largely unaffected by direct vaccine effects. Associations between background factors of the studied individuals and pneumococcal carriage were also investigated. A secondary aim was to determine carriage rates and antimicrobial susceptibility of *H. influenzae* and *M. catarrhalis*. The current study was part of a project initiated by the Department of Otorhinolaryngology at Hospital Josina Machel, Luanda and the Faculty of Medicine, Agostinho Neto University, Luanda to investigate etiology, epidemiology and treatment strategies of otitis media in Angola.

2. Materials and methods

2.1. Study setting

Enrolment of study participants was conducted during November to December 2017 in Luanda, the capital of Angola, and in villages surrounding Saurimo, the province capital of Lunda Sul in north-eastern Angola. Participating children, accompanied by their parent(s) or guardian(s), were recruited at sampling stations set up outdoors after church gatherings, at local community centers and schools. A small proportion (13%; n = 126) of the samples were collected from children visiting outpatient departments at Hospital Josina Machel and Hospital Pediátrico David Bernardino, Luanda. These children were recruited before seeing a physician and the diagnosis leading to their visit could therefore not be recorded. Enrolment of participants to the study was organized and implemented by personnel of the Department of Otorhinolaryngology at Hospital Josina Machel, Luanda. Upon inclusion, the parent(s) or guardian(s) of the children provided oral consent for the child to participate in the study. According to data obtained from the Angolan Ministry of Health (personal communication, MF) coverages of the 3rd dose of PCV13 (67% and 84%, respectively) and the Hib vaccine (65% and 83%, respectively) were slightly lower in Luanda as compared to Lunda Sul in 2017, but do not indicate any substantial difference in immunization patterns. These numbers are official administrative coverage estimates, which may explain the fact that they differ from the national WUENIC [10].

2.2. Collection of nasopharyngeal samples

Trans-nasal nasopharyngeal sampling was performed with flexible mini-tip flocked swabs (FLOQSwabs®; Copan Italia, Brescia, Italy) and collected in skimmed milk-tryptone-glucose-glycerine (STGG) medium [7]. The samples were transported in cooler-bags with ice-blocks and stored at −80 °C at the National Institute for Health Research, Luanda for a maximum of 1 month until transportation to Sweden (in cooler-bags with ice-blocks). Upon arrival at Clinical Microbiology, Department of Translational Medicine, Lund University (Malmö, Sweden) samples were frozen at −80 °C until further processing.

2.3. Collection of participant background information

In connection with the microbiological sampling, background information of the included children was collected through an interview of their parent or guardian. No medical/vaccination records were available and the data was based on the history provided by the parent or guardian. Questions concerned age and sex of the child, ongoing chronic or acute medical conditions, recent antibiotic therapy and vaccination status (*i.e.*, whether the child received routine vaccines or not), number of children in the household, presence of any tobacco smoker in the household, and access to electricity/water as well as cooking method in the household. In response to questions regarding ongoing medical conditions various replies were given, comprised of both unspecific symptoms (*e.g.* "fever", "headache", "otalgia") as well as diagnoses (*e.g.* CSOM, malaria). Replies that were sufficiently specific were used to assess if children had signs of ongoing infections or any chronic ear or respiratory tract condition. Unspecific answers (*e.g.* "yes") were excluded from the analyses and treated as missing answers. Height and weight of the children was recorded and BMI calculated.

2.4. Culture conditions and species identification

All samples were cultured on blood agar and chocolate blood agar at 35 °C in 5% CO_2 for approximately 48 h. An optochin disk was included on the blood agar to facilitate identification of *S. pneumoniae*. Single colonies of suspected *S. pneumoniae*, of the most common colony morphology if multiple were present, were selected and re-cultured on blood agar for 16–24 h and tested for optochin susceptibility to confirm species. Suspect colonies of *H. influenzae* and *M. catarrhalis* were re-cultured on chocolate blood agar and species identification of these isolates was confirmed with Matrix-assisted laser desorption/ionization - time of mass spectrometry (MALDI-TOF MS). Confirmed isolates of *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* were stored at −80 °C in glycerol-supplemented horse serum.

2.5. Antimicrobial susceptibility testing

Isolates were screened for resistance to clinically relevant antibiotics using disk diffusion tests (Oxoid, Basingstoke, UK). Benzylpenicillin and amoxicillin minimum inhibitory concentrations

(MICs) of pneumococci were determined with gradient tests (Etest; bioMérieux, Marcy-l'Étoile, France). Pneumococcal isolates that were non-susceptible to ≥ 3 of antibiotics (benzylpenicillin, erythromycin, clindamycin, tetracycline, trimethoprim-sulfamethoxazole or norfloxacin) during screening were regarded as possible multidrug-resistant (MDR) pneumococci and were further studied with broth microdilution to determine MDR phenotype (Sensititre; Thermo Fisher Scientific, Waltham, MA). All results from susceptibility testing were interpreted according to the European Committee of Antimicrobial Susceptibility Testing (EUCAST) 2020 clinical breakpoint tables [14].

2.6. Serotyping of pneumococci

All pneumococcal isolates, except 4 that were lost due to contamination, were serotyped (n = 328) according to a sequential 6-reaction multiplex polymerase chain reaction (PCR) protocol combined with latex agglutination and the Quellung reaction, as previously described [11]. Briefly, crude DNA extracts were produced by heating overnight growth from blood agar plates in sterile water to 99 °C for 10 min followed by centrifugation to remove cellular debris. PCR was performed as specified by da Gloria Carvalho *et al.* [15] with primers published by the CDC [16] distributed in 6 sequential reactions. When required, the serotype of isolates positive in any PCR reaction was further specified through the Quellung reaction with Neufeldt antisera (SSI Diagnostica, Denmark). Isolates that were negative in all PCR reactions twice were serotyped with the ImmuLex Pneumotest kit (SSI Diagnostica) and Neufeldt antisera. Isolates that were negative for the *cpsA* gene (included in all PCR reactions) twice or whose serotype could not be confirmed through latex agglutination, were regarded non-typeable (NT).

2.7. Capsule typing of H. Influenzae

H. influenzae capsule types were determined by MALDI-TOF MS [17,18]. All results were confirmed by PCR as previously described [19–21]. In cases of discrepancies between the two methods the results from PCR typing are reported.

2.8. Whole genome sequencing of antibiotic non-susceptible pneumococci

Whole genome sequencing (WGS) described by Kavalari *et al.* [22], was performed on possible MDR pneumococcal isolates to investigate whether these phenotypes could be explained by clonality and to determine resistance mechanisms. Briefly, genomic DNA was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) and fragment libraries were constructed using a Nextera XT Kit (Illumina, Little Chesterford, UK) followed by 250-bp paired-end sequencing (MiSeqTM; Illumina) according to the manufacturer's instructions. The paired-end Illumina data were *de novo* assembled using SKESA assembler [23]. Bioinformatic analyses were done using NCBI Genome Workbench v 3.3.1 (NCBI, Bethesda, MD) if not otherwise specified. Identification of the 16S rRNA position 203 was performed as previously described by Scholz *et al.* [24] to confirm pneumococcal species based on the presence of cytosine at this position. When needed, multilocus sequence analysis (MLSA) was used to assign species [25]. Capsular loci were analysed to determine capsular genotype using the PneumoCaT tool [26]. To assign multilocus sequence type (MLST) [27] and global pneumococcal sequence cluster (GPSC) [28] of isolates, and to identify penicillin binding protein (PBP) profiles [29] and additional resistance genes, assembled genomes were uploaded to Pathogenwatch [30]. The genomic sequence data for the 26 isolates are deposited in the Genbank (https://www.ebi.ac.uk/ena) (ENA accession no. is: PRJEB39312).

2.9. Statistical analyses

All statistical analyses were performed in SPSS Statistics version 26 (IBM, Armonk, NY). Background information on included individuals, colonization rates and antimicrobial susceptibility patterns are presented descriptively. The Chi-square test or Fisher's exact test (when appropriate) and the Mann-Whitney U test were used to compare proportions and medians between groups, respectively. Two-tailed p-values < 0.05 were considered statistically significant. If a significant difference was indicated in a contingency table with more than two rows or columns, *post-hoc* testing was done with two-by-two Chi-square tests with an adjusted significance level depending on the number of analyses performed (Bonferroni correction). Univariate and multivariate logistic regression was performed to investigate associations between background variables and pneumococcal carriage rate, calculating odds ratios (OR) with 95% confidence intervals (95% CI). In these analyses BMI and the number of siblings in the household were converted to binary variables with cut-offs set as BMI < 5th percentile (age and sex specific) as defined by the WHO [31] and ≥ 5 children (*i.e.,* > the overall median), respectively.

2.10. Ethical considerations

Accompanying parent(s) or guardian(s) of the included children gave their informed consent for participation after receiving information about the study. Authorization for the study was obtained from the Ethical committee at the Medical National Council of Angola, the Scientific Council of the Faculty of Medicine of Agostinho Neto University and the General Director of Hospital Josina Machel.

3. Results

3.1. Study population

Nasopharyngeal culture samples were collected from a total of 940 children aged 4 to 12 years (median 8 years) in community settings in Luanda (n = 654; 70%) and Saurimo, Lunda Sul (n = 160; 17%), and at 2 hospitals in Luanda (n = 126; 13%). Background characteristics of the included individuals are listed in Table 1.

3.2. Carriage of S. pneumoniae occurs in one third of children

S. pneumoniae, *H. influenzae*, and *M. catarrhalis* were isolated from 35% (95% CI 32–39%; n = 332), 13% (95% CI 11–15%; n = 121), and 15% (95% CI 13–17%; n = 139) of samples, respectively. Simultaneous carriage of pneumococci and *H. influenzae* was detected in 6% of samples, pneumococci and *M. catarrhalis* in 5%, *H. influenzae* and *M. catarrhalis* in 1%, and all 3 bacterial species in 2%. Median age of carriers and non-carriers differed only regarding *M. catarrhalis* (7 [IQR 6–9] and 9 [IQR 7–11] years, respectively; p < 0.001). Colonization rates at the different study sites are presented in Fig. 1. Univariate and multivariate regression analysis was performed to investigate association of demographic and clinical variables with pneumococcal carriage (Table 2).

3.3. Nearly half of isolated pneumococci consists of vaccine serotypes

In total 328 pneumococcal isolates were serotyped, and the overall and site-specific serotype distributions are presented in

Table 1
Demographical and clinical background characteristics of sampled children at the different inclusion sites. Values are presented as *n* (%) for categorical variables and median (IQR) for continuous variables.

Characteristic[a]	Missing data (*n*)	Total	Luanda (community)	Luanda (hospital)	Saurimo (community)
Number (*n*)		940	654	126	160
Age (years)	0	8 (7–10)	9 (7–10)	8 (6–10)	9 (7–11)
Female sex	0	444 (47)	323 (49)	44 (35)	69 (43)
BMI	1	15.6 (14.4–16.8)	15.6 (14.5–16.8)	15.4 (14.2–17.2)	15.7 (14.5–16.7)
Chronic ear/auditory or respiratory tract symptoms	167	49 (6)	12 (2)	21 (19)	16 (11)
Ongoing infectious symptoms	134	59 (7)	8 (1)	39 (66)	12 (8)
Antibiotic treatment last month	142	62 (8)	19 (4)	43 (35)	0
Vaccinated according to schedule	144	642 (81)	403 (79)	114 (93)	125 (78)
Number of children in the household	4	4 (3–5)	4 (3–5)	3 (3–5)	4 (3–5)
Smoker in the household	2	122 (13)	64 (10)	14 (11)	44 (28)
Access to grid electricity	21	696 (76)	579 (92)	117 (93)	160 (100)
Access to running water	490	76 (17)	40 (13)	30 (73)	6 (6)
Cooking method in household	166				
- Coal		2 (0.3)	2 (0.3)	0	0
- Gas		771 (99.6)	643 (99.5)	124 (100)	4 (100)
- Electricity		1 (90.1)	1 (0.2)	0	0

[a] All data except BMI is based on the history provided by the children's parent or guardian.

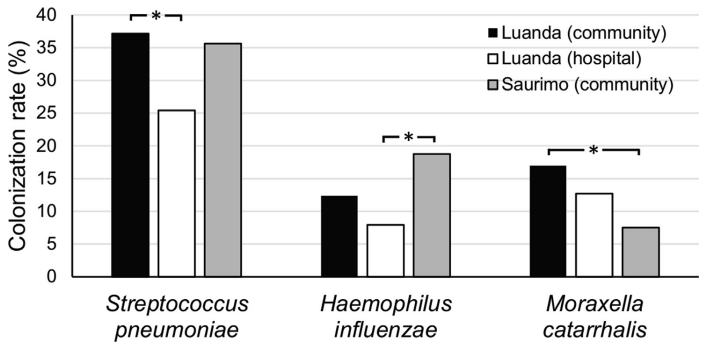

Fig. 1. Nasopharyngeal colonization of *S. pneumoniae, H. influenzae* and *M. catarrhalis* among Angolan children aged 4–12 years in community settings in Luanda and Saurimo, Lunda Sul, and 2 hospitals in Luanda. A statistically significant difference (with Bonferroni correction; $p < 0.017$) between any two collection sites is indicated with an asterisk.

Table 2
Logistic regression analysis assessing association of clinical and demographical variables with pneumococcal carriage. ORs with 95% CIs indicating statistical significance are shown in bold. BMI, body mass index.

Variable	Missing data (*n*)	Frequency of variable (carriers/total)	Univariate regression (OR [95% CI])	Multivariate regression (OR [95% CI])[a]
Age[b]	0		0.95 (0.89–1.01)	0.95 (0.88–1.02)
Female sex	0	158/444	1.02 (0.78–1.34)	1.06 (0.77–1.46)
BMI < 5th percentile	1	47/113	1.35 (0.91–2.02)	1.10 (0.69–1.76)
Site of inclusion	0			
- Luanda (community)		243/654	= reference	= reference
- Luanda (hospital)		32/126	**0.58 (0.37–0.89)**	0.43 (0.17–1.07)
- Saurimo (community)		57/160	0.94 (0.65–1.34)	0.61 (0.30–1.24)
Chronic ear/auditory or respiratory tract symptoms	167	18/49	0.91 (0.50–1.65)	1.59 (0.77–3.29)
Ongoing infectious symptoms	134	18/59	0.70 (0.40–1.25)	1.14 (0.48–2.72)
Antibiotic treatment last month	142	8/62	**0.22 (0.10–0.47)**	**0.27 (0.09–0.83)**
Vaccinated according to schedule	144	244/642	0.99 (0.69–1.42)	1.09 (0.73–1.61)
≥ 5 children in the household	4	139/381	1.09 (0.83–1.43)	0.90 (0.65–1.24)
Smoker in the household	2	44/122	1.03 (0.70–1.54)	1.55 (0.96–2.51)
Access to grid electricity	21	243/696	0.87 (0.64–1.19)	0.97 (0.52–1.83)
Access to running water	490	28/76	0.95 (0.57–1.59)	–
H. influenzae colonization	0	70/121	**2.92 (1.98–4.31)**	**2.37 (1.51–3.72)**
M. catarrhalis colonization	0	67/139	**1.88 (1.31–2.71)**	1.43 (0.92–2.20)

[a] The total number of cases included in the multivariate model was 688 (73%). All variables except "access to running water", which was excluded due to the large proportion of missing data, were included in the multivariate model.
[b] Continuous variable.

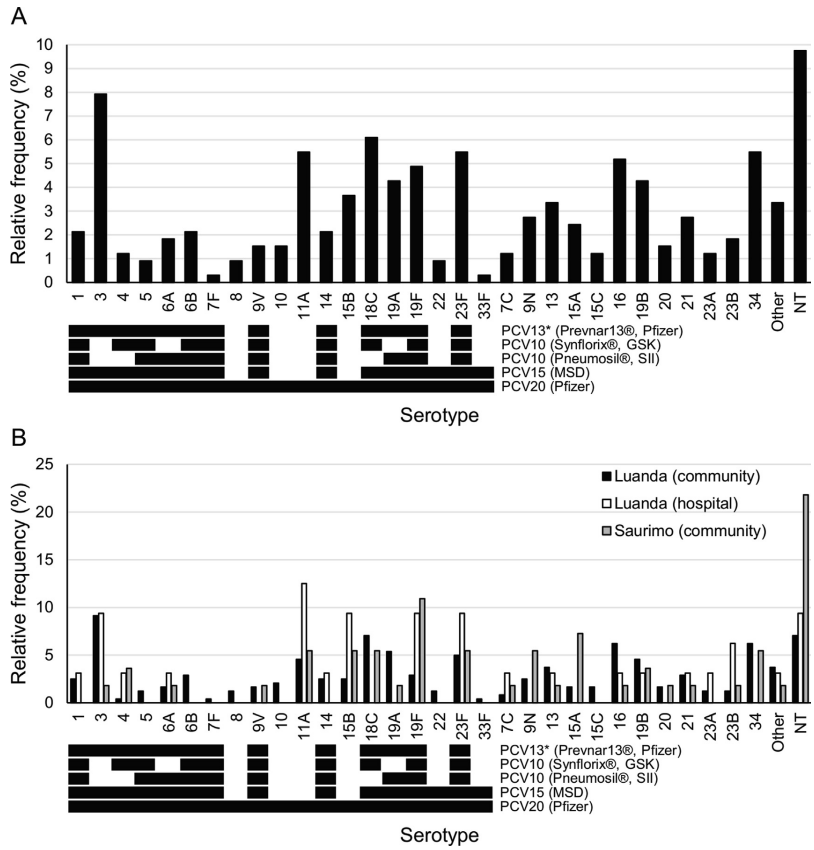

Fig. 2. Serotype distribution of *S. pneumoniae* detected in nasopharyngeal samples from Angolan children aged 4–12 years in community settings in Luanda and Saurimo, Lunda Sul, and 2 hospitals in Luanda. The overall distribution of pneumococcal serotypes (A) and their relative frequency at each study site (B) are presented. Serotypes included in different currently marketed or upcoming PCVs are grouped to the left on the x-axis indicated by the black bars below. The proportion of serotyped pneumococci covered was 41% for the PCV13 (Prevnar13® [Pfizer, New York City, NY]; indicated by asterisk) currently used in Angola compared to 27% ($p < 0.001$) for Synflorix® (GlaxoSmithKline, Brentford, UK), 26% ($p < 0.001$) for Pneumosil® (Serum Institute of India, Pune, IN), both PCV10, and 42% ($p = 0.8$) for PCV15 (Merck Sharp & Dohme, Kenilworth, NJ) and, finally, 54% ($p = 0.001$) for PCV20 (Pfizer). NVTs representing < 1% of all isolates are pooled as "Other". SII, Serum Institute of India; GSK, GlaxoSmithKline; MSD, Merck Sharp & Dohme.

Fig. 2. A large proportion of analyzed serotypes were included in PCV13 (n = 134; 41%), representing 5 of the 10 most common serotypes (serotypes 3 [8%], 18C [6%], 23F [6%], 19F [5%] and 19A [4%]), and we conclude a VT carriage rate of 14%. The most common NVTs were 11A (6%), 34 (6%), 16 (5%), 19B (4%) and 15B (4%). The proportions of serotypes covered by other current or proposed PCVs are indicated in Fig. 2. Most *H. influenzae* were determined to be NTHi (n = 109; 90%). The 12 (10%) encapsulated strains comprised 7 Hif, 2 Hie, and 1 isolate each of Hib, Hic and Hid. The results from MALDI-TOF MS typing were consistent with those from PCR typing except for a few cases of NTHi (n = 4), which were misclassified as Hie by MALDI-TOF MS typing [17,18].

3.4. Non-susceptibility to penicillin is common among pneumococci

Antimicrobial susceptibility rates of the isolated bacteria are presented in Table 3. Penicillin non-susceptibility was common among *S. pneumoniae* (40%), but all PNSP isolates exhibited only intermediate resistance with a median MIC of 0.19 μg/mL (IQR 0.13–0.25 μg/mL), and no differences were noted between the study sites. PCV13-included serotypes were significantly associated with penicillin non-susceptibility (50% vs. 33%; $p = 0.003$). Prevalence of PNSP within specific serotypes is outlined in Fig. 3. Twenty-four (7%) MDR *S. pneumoniae* isolates, *i.e.*, non-susceptible to ≥ 3 antibiotics, were detected and further characterized with WGS and broth microdilution (Table 4).

Table 3
Antimicrobial susceptibility patterns of isolated bacteria. Figures are presented as *n* (%).

	S. pneumoniae	*H. influenzae*	*M. catarrhalis*
Benzylpenicillin[a]			
- S	195 (60)	103 (85)[b]	3 (2)[b]
- I	130 (40)		
- R		18 (15)	136 (98)
Amoxicillin			
- S	322 (99)	106 (88)	
- I	1 (0.3)		
- R	2 (0.6)	15 (12)	
Erythromycin			
- S	322 (99)		
- I	1 (0.3)		
- R	2 (0.6)		
Clindamycin			
- S	232 (99)		
- R	2 (0.6)		
Tetracycline			
- S	257 (79)	105 (87)	135 (97)
- I	12 (4)		
- R	56 (17)	16 (13)	4 (3)
Trimethoprim-sulfamethoxazole			
- S	57 (18)	19 (16)	41 (29)
- I	6 (2)		
- R	262 (81)	102 (84)	98 (71)
Norfloxacin			
- S	324 (99.7)		
- R	1 (0.3)		

[a] Intravenous non-meningitis EUCAST breakpoints for *S. pneumoniae*; 0.06 < I ≤ 2 μg/mL.
[b] Screening substance for betalactamase-production.

3.5. Several global pneumococcal sequence clusters (GPSC) occur among MDR pneumococci but no dominating cluster was found

To further investigate molecular epidemiology and resistance mechanisms of pneumococcal MDR isolates, these were subjected to WGS. Identified GPSCs, MLST sequence types (STs), capsular genotypes, and resistance genes are listed in Table 4. The two most common GPSCs found were 94 (n = 7) and 10 (n = 5), predominantly comprising serogroup 23 and serotype 3, respectively. Two isolates (*S. pneumoniae* #110 and #152) shared 6/7 alleles with Pneumococcal Molecular Epidemiology Network (PMEN; https://www.pneumogen.net/pmen/) clones Spain^{9V}-3 ST156 and Taiwan^{19F}-14 ST236, respectively. Importantly, identification of cytosine at the 203 position of 16S rRNA sequence confirmed *S. pneumoniae* species in 25/26 analysed isolates, while the remaining isolate (#123) was revealed to be *Streptococcus pseudopneumoniae* through MLSA. Results corresponding to this isolate have been omitted from previous sections but are included in Table 4 for the sake of completeness.

4. Discussion

Previous studies on the microbiology of pneumococci in Angola are scarce, a fact that limits the possibilities to forecast vaccine effects and to make future evaluations. We have conducted a nasopharyngeal carriage study in children that have not been targeted by PCV immunization (aged 4–12 years in 2017). Importantly, our results reveal that 41% of colonizing pneumococci were covered by PCV13 4 years after the introduction of the vaccine and that these serotypes were more frequently PNSP (50% vs. 33%).

An overall pneumococcal carriage rate of 35% was detected, slightly lower than the 43% reported among 5 to 15-year-old children in a systematic review of pneumococcal carriage in sub-Saharan Africa, but the rates varied considerably in the included studies that were performed prior to introduction of PCV [32]. In a study including unvaccinated 5 to 15-year-old children after introduction of PCV in Malawi, pneumococcal carriage was detected in 37% of 89 individuals [33]. Colonization by *H. influenzae* and recent antibiotic use were the only two studied variables that were significantly associated with pneumococcal carriage in a multivariate logistic regression (Table 2). A positive but non-significant trend (OR 1.43) was also seen in the association between *M. catarrhalis* and pneumococci. The positive association between colonization with the three studied species has been described earlier [34]. This finding may both be explained by inter-species "cooperation" or be dependent on other host or environmental factors [4]. Unfortunately, many answers recorded regarding medical conditions and symptoms of the studied individuals were inconclusive which is a limitation for these analyses.

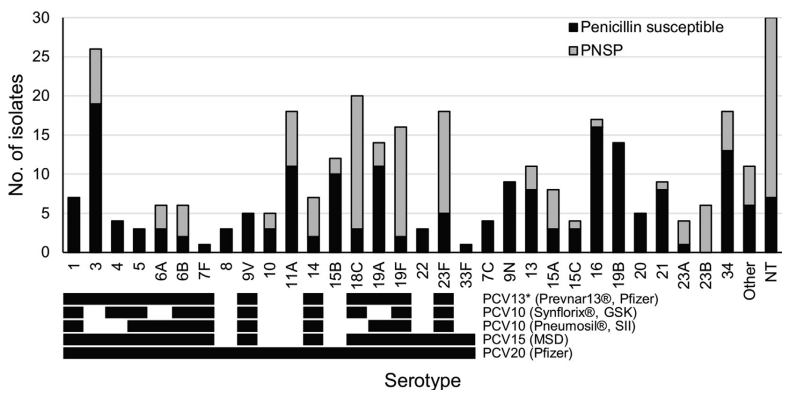

Fig. 3. Prevalence of PNSP among detected pneumococcal serotypes. Serotypes included in different currently marketed or upcoming PCVs are grouped to the left on the x-axis indicated by the black bars below. Penicillin non-susceptibility was more frequent among serotypes included in the PCV13 currently used in Angola (indicated by asterisk) than non-PCV13 serotypes (50% vs. 33%; p = 0.003). Penicillin non-susceptibility is defined as a minimal inhibitory concentration > 0.06 mg/L. NVTs representing < 1% of all isolates are pooled as "Other". SII, Serum Institute of India; GSK, GlaxoSmithKline; MSD, Merck Sharp & Dohme.

Table 4
Findings regarding species and serotype determination, molecular epidemiology, resistance patterns and resistance mechanisms of pneumococcal MDR isolates analysed with WGS. Twenty-four (7%) isolates exhibited MDR phenotype according to EUCAST 2020 clinical breakpoints and were analysed with WGS and broth microdilution. Two additional isolates were included in the analyses, but were revealed to be a *Streptococcus pseudopneumoniae* (#123) and a non-MDR *S. pneumoniae* (#795), respectively. GPSC, global pneumococcal sequence cluster; MLST, multi-locus sequence type; PcG, benzylpenicillin; ERY, erythromycin; CLI, clindamycin; TET, tetracycline; TMP-SMX, trimethoprim-sulfamethoxazole; MOX, moxifloxacin; NR, not relevant.

No.	Serotype	Capsular genes	16S rRNA position 203	GPSC	MLST[a]	PcG[b]	ERY	CLI	TET	TMP-SMX	MOX	*ermB*	*mefA/E*	*tetM*	PBP1a	PBP2b	PBP2x	Inferred PcG MIC
16	10	10F	C	Novel	Novel1 (2–57-62–3-6–88-309)	I (0.12)	I (0.5)	S	R (>8)	R (4)	S	–	+	+ (Tn916)	2	183	242	0.12
31	6B	6E	C	129	3207	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	Novel	Novel	Novel	0.12
110	15C	15C	C	6	166	I (2.0)[e]	R (>2)	R (>1)	S	R (>4)	S	+	–	NR	15	12	7	2
123[c]	NT	–	A	–		I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	–	–	–	–
152	19F	19F	C	1	271	I (2.0)[e]	R (>2)	R (>1)	R (>8)	R (>4)	S	+	+	+ (Tn5251)	13	49	8	4
153	38	38	C	117	6103	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	2	Novel	Novel	0.25
186	NT	NT	C	Novel	Novel2 (1–9-novel-154–15-1–262)	I (0.5)	S	S	R (>8)	R (>4)	R (2)	NR	NR	+ (Tn916)	Novel	176	Novel	0.25
214	11A	11A	C	Novel	Novel3 (1–16-54–1-9–1-824)	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	Novel	Novel	Novel	0.25
229	38	38	C	117	6103	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	2	Novel	Novel	0.25
312	23A	10A/23A	C	Novel	Novel4 (2–5-54–63-?-6–8)	I (0.25)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	150	1	242	0.25
329	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	19	Novel	Novel	0.25
505	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
535	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
543	6A	6A	C	62	912	I (0.5)	S	S	R (8)	R (>4)	S	NR	NR	+ (Tn916)	192	Novel	Novel	0.5
561	23F	23F	C	94	Novel6 (7–16-8–8-6–60-17)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	Novel	0.25
570	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	17	15	22	0.5
585	19F	19F	C	Novel	9716	I (0.12)	S	S	R (>8)	I (2)	S	NR	NR	+	19	Novel	Novel	0.25
595	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
631	23F	23F	C	94	Novel6 (7–16-8–8-6–60-17)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	Novel	0.25
632	10	10A	C	10	3135	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	150	1	22	0.25
689	3	3	C	10	700	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	17	15	Novel	0.5
705	19F	19F	C	94	Novel7 (7–16-8–8-6–142-9)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	19	Novel	Novel	0.25
706	15A	15A	C	Novel	Novel8 (1–8-73–47-36–1-6)	I (0.5)	S	S	R (>8)	R (>4)	S	NR	NR	+	Novel	Novel	Novel	0.25

Table 4 (*continued*)

No.	Serotype	Capsular genes	16S rRNA position 203	GPSC	MLST[a]	PcG[b]	ERY	CLI	TET	TMP-SMX	MOX	*ermB*	*mefA/E*	*tetM*	PBP1a	PBP2b	PBP2x	Inferred PcG MIC
795[d]	23F	23F	C	94	Novel5 (7–16-8–8-6–60-14)	I (0.25)	S	S	R (>8)	S	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
879	23F	23F	C	94	Novel9 (7–16-novel-8–6-60–14)	I (0.25)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn916)	12	Novel	104	0.12
886	NT	NT	C	Novel	Novel10 (7–9-1–38-13–1-74)	I (0.12)	S	S	R (>8)	R (>4)	S	NR	NR	+ (Tn1545)	Novel	0	Novel	0.12

[a] Allele signatures of novel MLSTs are presented as (*aroE-gdh-gki-recP-spi-xpt-ddl*).
[b] Intravenous non-meningitis EUCAST breakpoints; $0.06 < I \leq 2$ μg/mL
[c] Identified as *S. pseudopneumoniae* using MLSA.
[d] Isolate *S. pneumoniae* #795 was initially classified as MDR but was revealed to be susceptible to trimethoprim-sulfamethoxazole and is therefore not classified as MDR.

In the present study, it is unknown to which extent the detected serotype distribution has been indirectly affected by the infant immunization programme. Nevertheless, nasopharyngeal carriage of VT pneumococci was confirmed to still exist in children (14%) at our study sites, where the vaccine coverage of PCV13 (3 dosages) was 52–84% in 2017 (according to WHO/UNICEF and official Angolan estimates). In recent studies performed in Malawi, Ghana and Tanzania after PCV-introduction it has been noted that VT carriage persists in children even if a decrease is observed [35–37]. Several factors may contribute to these findings, including incomplete vaccine coverage, lack of a booster dose during the second year of life resulting in waning serotype-specific immunity, and transmission of VT pneumococci from parents and older siblings [35,37,38]. Taken together, it is crucial that the vaccine coverage of PCV in Angolan children is increased, but an alternate vaccination schedule (2 + 1 or 3 + 1) is likely also to be of importance to eliminate VT transmission [39].

The two most common serotypes found, VTs 3 (8%) and 18C (6%), have been identified in Malawi [33], Ghana [35], Tanzania [36], The Gambia [38] and, Uganda [40], but generally less frequently than in our study. Both serotypes are common causes of IPD [41]. However, the effect of PCV13 on serotype 3 carriage is likely lower than for other included serotypes which may contribute to its continued transmission [42]. The NVTs presented in Fig. 2 generally have low invasive disease potential but do cause substantial numbers of IPD worldwide as they become more prevalent in carriage [41]. Specifically, this applies to serotypes 9N, 10A, 11A, 15A/B/C, 23A and 23B [6]. Future higher-valency PCVs may become impotant to prevent emerging NVTs [43]. Among currently marketed and proposed PCVs, Pfizer's PCV20 candidate exhibited a higher coverage (54%) of the detected serotype distribution than the currently used PCV13 (41%) (Fig. 2).

Penicillin non-susceptibility was more common among VT strains (50% vs. 33%), explained by high rates among serotypes 6A, 6B, 14, 18C, 19F, and 23F. Further use of PCV may therefore result in decreasing rates of PNSP in Angola which has been observed in Ghana [35]. However, some NVTs were also frequently PNSP, specifically serotypes 15A, 23A, and 23B which has been previously reported and risk to attenuate vaccine effects on total PNSP prevalence [6,44]. Despite the high frequency of PNSP, virtually all pneumococci (99%), as well as the majority of *H. influenzae* (88%), were susceptible to amoxicillin. Most bacteria were non-susceptible against trimethoprim-sulfamethoxazole (83%) which was similarly reported recently from Tanzania and may be related to high use of the substance as prophylactic treatment in HIV-positive individuals [36]. Based on the results, amoxicillin is a feasible empirical treatment of respiratory tract infections and AOM in outpatient settings in Angola while tetracycline and, in particular, trimethoprim-sulfamethoxazole are unfavourable choices. Broth microdilution was only used to determine MICs of MDR isolates which is a limitation of the study as gradient tests may underestimate the MICs of penicillin for *S. pneumoniae* 1–2 dilution steps [45]. However, even though this may affect the presented median penicillin MIC, it is unlikely that the SIR classifications were affected as most MICs of penicillin detected were in the low-intermediate range.

Studies of the molecular epidemiology of *S. pneumoniae*, in addition to serotyping, in relation to PCV implementation are of importance as virulence of specific strains may be as dependent on genetic lineage as serotype, and the latter may switch within a lineage [28]. We performed a limited, explorative investigation with WGS of molecular epidemiology and resistance genes among MDR pneumococci. All serotype 3 and serogroup 23 isolates belonged to GPSCs 10 and 94, respectively. According to the Global Pneumococcal Sequencing Project database [46] GPSC10 has been identified globally and exhibit several serotypes, but all serotype 3 isolates have been reported from sub-Saharan Africa and, similar to our results, exhibit ST700. All GPSC94 isolates previously reported have been found in sub-Saharan Africa and mostly exhibit serotypes 19A or 23F. These findings suggest that clonality may explain multi-resistant phenotypes among *S. pneumoniae* in Angola. Many different GPSCs and STs were seen, however, and too few isolates were studied to draw any conclusions. Further studies on the molecular epidemiology of pneumococci, including both carriage and disease-associated isolates, are needed to clarify which genetic lineages may be of significant clinical importance in Angola.

Whole genome sequencing revealed that one isolate identified as *S. pneumoniae* belonged to the closely related species *S. pseudopneumoniae*. The incorrect classification of this isolate exemplifies

the difficulty to discriminate *S. pneumoniae* from other mitis group streptococci [47]. The fact that no additional, but labour intense, methods were used to confirm pneumococcal species is a limitation of this study. It is possible that the high number of NT pneumococci observed may include additional incorrectly identified *S. pseudopneumoniae*.

Capsule typing of *H. influenzae* revealed that the majority of colonizing strains were indeed NTHi (90%). The high agreement in typing results for MALDI-TOF MS and PCR underlines the strength of MALDI-TOF MS as a rapid method for capsule typing of *H. influenzae*. A few NTHi were misclassified as Hie by MALDI-TOF MS. This is a known limitation of the MALDI-TOF MS typing method and isolates classified as Hie should be confirmed by PCR typing [18]. Only a single isolate of Hib was detected, a finding that is consistent with a Kenyan study of Hib carriage detecting carriage in only 2 of 1408 children aged > 5 years, after the introduction of the conjugated Hib vaccine [48]. Even though the estimated coverage of Hib-immunization, alike PCV-coverage, has been suboptimal since its introduction in 2006 [10], the low carriage prevalence and previously reported decline of meningitis and empyema caused by *H. influenzae* may most likely be related to a vaccine effect [13,49].

5. Conclusions

The current study has provided a first description of the spectrum of pneumococci circulating among unvaccinated children in Angola four years after the introduction of PCV13 in the national childhood immunization programme. Enduring nasopharyngeal carriage of VT pneumococci, which were more often PNSP, indicate that continued, and more comprehensive administration of PCV13 may have positive effects on pneumococcal disease in the country. Additionaly, a vaccine schedule including a booster dose may reduce transmission of VT pneumococci further and future higher-valency vaccines may have a broader impact. Further investigations of phenotypic and genetic characteristics of colonizing and infecting pneumococci in Angola are of great importance for assessing effects of the vaccination programme and for formulating future effective treatment and prevention strategies.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: FU, HCS and KR participate in projects supported by Pfizer. TP has participated in projects supported by Sanofi Pasteur. Remaining authors declare no conflicts of interest.

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Paper VI

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Infectious Diseases of Poverty

RESEARCH ARTICLE Open Access

Aerobic bacteria associated with chronic suppurative otitis media in Angola

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Abstract

Background: Chronic suppurative otitis media (CSOM) is an important cause of hearing loss in children and constitutes a serious health problem globally with a strong association to resource-limited living conditions. Topical antibiotics combined with aural toilet is the first-hand treatment for CSOM but antimicrobial resistance and limited availability to antibiotics are obstacles in some areas. The goal of this study was to define aerobic pathogens associated with CSOM in Angola with the overall aim to provide a background for local treatment recommendations.

Methods: Samples from ear discharge and the nasopharynx were collected and cultured from 152 patients with ear discharge and perforation of the tympanic membrane. Identification of bacterial species was performed with matrix-assisted laser desorption/ionization-time of flight mass spectrometry and pneumococci were serotyped using multiplex polymerase chain reactions. Antimicrobial susceptibility testing was done according to EUCAST.

Results: One hundred eighty-four samples from ear discharge and 151 nasopharyngeal swabs were collected and yielded 534 and 289 individual isolates, respectively. In all patients, correspondence rate of isolates from 2 ears in patients with bilateral disease was 27.3% and 9.3% comparing isolates from the nasopharynx and ear discharge, respectively. *Proteus* spp. (14.7%), *Pseudomonas aeruginosa* (13.2%) and *Enterococcus* spp. (8.8%) were dominating pathogens isolated from ear discharge. A large part of the remaining species belonged to *Enterobacteriaceae* (23.5%). Pneumococci and *Staphylococcus aureus* were detected in approximately 10% of nasopharyngeal samples. Resistance rates to quinolones exceeded 10% among *Enterobacteriaceae* and was 30.8% in *S. aureus*, whereas 6.3% of *P. aeruginosa* were resistant.

Conclusions: The infection of the middle ear in CSOM is highly polymicrobial, and isolates found in nasopharynx do not correspond well with those found in ear discharge. Pathogens associated with CSOM in Angola are dominated by gram-negatives including *Enterobacteriaceae* and *P. aeruginosa,* while gram-positive enterococci also are common. Based on the results of antimicrobial susceptibility testing topical quinolones would be the preferred antibiotic therapy of CSOM in Angola. Topical antiseptics such as aluminium acetate, acetic acid or boric acid, however, may be more feasible options due to a possibly emerging antimicrobial resistance.

Keywords: Chronic suppurative otitis media, Enterobacteria, Infection, Otitis media, *Proteus*, *Pseudomonas aeruginosa*

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Multilingual abstracts

Please see Additional file 1 for translation of the abstract into the six official working languages of the United Nations.

Background

Chronic suppurative otitis media (CSOM) is a prolonged and often recurring bacterial infection of the middle ear defined by perforation of the tympanic membrane and otorrhoea lasting more than 2 weeks according to the World Health Organization (WHO), although a commonly used clinical definition is 6 weeks. The infection usually develops in early childhood, with a peak around 2 years of age, but can persist until adulthood [1, 2]. WHO estimates that 65 to 330 million people suffer from CSOM worldwide. The greatest burden of disease is found in low-income countries in Sub-Saharan Africa and Oceania, where incidence rates over 0.7% have been reported, with higher numbers among children under 5 years of age [1, 3]. Furthermore, certain ethnical groups are particularly affected, including the Inuit of Greenland, Native Americans and Aboriginal Australians [2]. Risk factors associated with CSOM include frequent episodes of acute otitis media (AOM), other respiratory tract infections, and traumatic tympanic rupture as well as factors correlating with resource-limited living conditions such as overcrowding, poor nutrition and hygiene, and chronic infectious diseases. Even if the pathogenesis is multifactorial, the clinical onset is frequently an episode of AOM complicated by tympanic membrane perforation and a subsequent superinfection of the middle ear with bacteria entering through the outer ear channel. Additionally, perforated tympanic membrane causes Eustachian tube dysfunction, allowing for pathogens to ascend to the middle ear through reflux of nasopharyngeal secretions [2, 4]. Formation of biofilm has been implicated to sustain the infection and reduce the efficacy of antibiotic treatment [5]. CSOM is an important cause of conductive as well as sensorineural hearing loss in children, which may be the result in more than 50% of patients. Moreover, facial nerve paralysis, sinus thrombosis, labyrinthitis, meningitis and brain abscesses are other rare complications. The WHO estimates that up to 28 000 yearly deaths can be attributed to CSOM on a global basis [1].

Microbiological findings in CSOM vary between studies. However, the bacterial spectrum most often identified in the CSOM-affected middle ear is dominated by *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterobacteriaceae* such as *Proteus* spp. and *Klebsiella pneumoniae* [1, 2, 4]. Anaerobic bacteria are commonly detected in studies of CSOM applying suitable anaerobic methods for isolation. Infections are often polymicrobial and a synergistic relationship between aerobes and anaerobes has been suggested [2, 6]. *Mycobacterium tuberculosis* is a rare cause of chronic infection of the middle ear, but tuberculous otomastoiditis has to be considered in a patient presenting with chronic ear discharge [7].

Being an important cause of pediatric hearing loss in developing countries that is potentially preventable, studies facilitating efficient care of CSOM are highly desirable. As the pathogenesis and varying microbiology of CSOM are not fully understood, it is of importance to investigate the prevalence of various pathogens in different areas [2]. Knowledge on the local incidence as well as spectrum of bacteria present and their antimicrobial susceptibility patterns is imperative for effective empirical treatment as well as contributing to the general understanding of the disease [6].

Luanda, the capital of Angola, has a population of approximately 6 million in 2014, and is one of the fastest growing cities in Africa, where a large part of the population lives in conditions lacking sanitation, fresh drinking water and with a high mortality rate among children under 5 years of age [8, 9]. Studies during 1981–1982 showed a CSOM prevalence of 2–4% in school children in Luanda [10]. Moreover, studies from 2011 reported CSOM in 4% of healthy children in Luanda and higher numbers in those with comorbidities such as HIV and tuberculosis [11, 12]. Studies on antimicrobial susceptibility of pathogens involved in CSOM from Angola are scarce, and generally do not regard the antibiotics commonly used to treat this particular infection. A high incidence of methicillin resistant *S. aureus* (MRSA) and carbapenemase-producing *Enterobacteriaceae* has, however, been reported [13, 14]. In the current study, we present microbiological findings from middle ear discharge and the nasopharynx in patients with CSOM in Luanda and three other provinces in Angola with the aim to provide a background for recommendations on the treatment of the disease in the public health care system.

Methods

Study design

The present study is a part of a project at the Ear, nose and throat (ENT)-department at Hospital Josina Machel (HJM) in Luanda to improve otitis media care and prevent hearing loss. AOM and CSOM are among the most frequent reasons for attending this clinic, which until 2016 was the only public ENT-clinic in Luanda. Clinical samples and patient information were collected from patients of all ages with CSOM at the outpatient section of the HJM ENT-clinic and at health care centers in three other Angolan provinces (Lunda Sul, Namibe, Zaire) from January to December 2016. CSOM was defined as perforation of the tympanic membrane confirmed by otoscopy and purulent ear discharge lasting more than 14 days.

Sampling and culture conditions

Sampling for microbiological cultures was conducted by experienced clinicians using standard techniques. After cleaning the auditory channel with 70% ethanol, ear discharge samples were collected with a swab. For nasopharyngeal sampling, a swab was introduced into nasopharynx through the nostril crossing the choana until it touched the wall of the nasopharynx. Samples from both loci were collected in skim milk-tryptone-glucose-glycerol (STGG) medium and stored at − 70 °C at the Public Health Laboratory (Luanda) prior to transport to the Riesbeck laboratory (Malmö, Sweden). Clinical specimens were cultured on hematin agar, Columbia CNA agar (Oxoid, Hampshire, UK), and UriSelect agar supplemented with vancomycin (Bio-Rad, Hercules, CA) and incubated at 35.5 °C in 5% CO_2 (hematin and Columbia CNA agar) or at aerobic conditions (UriSelect) for 16–18 h.

Species identification and serotyping of pneumococci

Bacterial species identification was done by Matrix-assisted laser desorption/ionization – time of flight mass spectrometry (MALDI-TOF MS) [15]. Briefly, bacteria from a single colony of each isolate were applied to a MALDI target plate (Bruker Daltonics, Bremen, Germany) in duplicates and were overlaid with 1 μl HCCA matrix (Bruker Daltonics) and let dry completely. Mass spectra were then acquired with a microflex MALDI-TOF mass spectrometer with flexControl software (Bruker Daltonics, Bremen, Germany) using default settings (mass range of spectra, m/z 2000 to 20 000 in linear positive-ionization mode), and species were identified using the MALDI Biotyper 4.1 software with Bruker taxonomy library ($N = 6903$) (Bruker Daltonic). *Streptococcus pneumoniae* were serotyped using multiplex polymerase chain reaction (mPCR) [16]. Briefly, DNA was prepared from each pneumococcal isolate by boiling bacteria in Tris-EDTA-buffer and thereafter analysed in 6 sequential mPCRs containing, in total, 32 primer pairs identifying genes specific to different serotypes or groups of serotypes. A primer pair identifying the pneumococcus *cpsA* gene was included in all reactions as internal positive control indicating successful reaction. Primer sequences and the distribution of primer pairs to the different reactions, as well as PCR cycling conditions are presented in Additional file 2. If a pneumococcal serotype could not be identified by mPCR or further serotyping was needed to distinguish individual serotypes the Pneumotest Latex Kit and Neufeldt Antisera (Statens Seruminstitut, Copenhagen, Denmark), both based on the Quellung-reaction of capsular swelling, were used according to the manufacturer's instructions.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed with disc diffusion according to European Committee on Antimicrobial Susceptibility Testing for all antibiotics except colistin, for which broth microdilution was performed on MICRONAUT-S plates (Merlin Diagnostika, Bornheim, Germany) according to the manufacturer's instructions. Susceptibility testing was done on the species that are generally regarded as being relevant for the pathogenesis of CSOM or AOM and the results were interpreted according to Breakpoint Tables of the European Committee on Antimicrobial Susceptibility Testing [17]. A number of isolates of the relevant species were not available for susceptibility testing. These isolates were, however, missing at random.

Statistical analysis

Descriptive statistics are used to present demographics, species distribution and microbial susceptibility. All data were computerized and analysed using Microsoft Excel version 15.37 (Microsoft, Redmond, WA) and are presented as absolute numbers and percentages.

Results

CSOM is mainly found in children less than 12 years of age

In total, 152 patients with tympanic membrane perforation and ear discharge lasting more than 14 days, attending the ENT-clinic or health care centers, were included in the study. A majority were from Luanda (77%; $N = 111$), whereas 17% ($N = 24$), 5% ($N = 7$) and 1% ($N = 2$) of patients were from Lunda Sul, Zaire and Namibe, respectively (Fig. 1). For 8 individuals, information about enrolment site was missing. Patient age ranged from 0 to 77 years and the median age was 12.6 years. Sixty-two percent ($N = 91$) of the patients were male while 38% ($N = 56$) were female. A list of all patients, including demographical data and a listing of all microbiological findings is supplied in Additional file 3.

Co-colonization by several different species is common in the CSOM-affected middle ear

Samples from ear discharge from one or both ears ($N = 184$) and nasopharyngeal swabs ($N = 151$) were collected. A total of 823 microbes were identified, 534 in samples from ear discharge and 289 in nasopharyngeal samples. The mean number of isolates per sample was slightly higher in ear discharge samples than in nasopharyngeal samples (2.9 vs. 2.4, respectively). Only 16 ear samples yielded a single isolate as compared to 55 nasopharyngeal swabs. Of 187 isolates recovered from the middle ears in patients with bilateral disease, 52 (27.3%) were isolates of the same species present in both ears of the same individual. On the other hand, 9.3% ($N = 27$) of the nasopharyngeal isolates corresponded to an

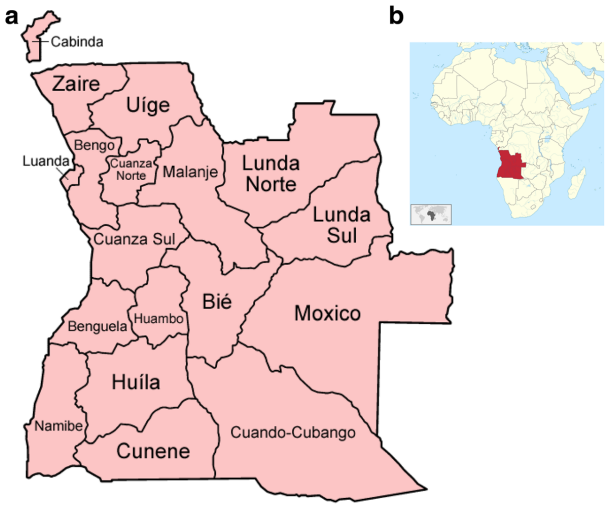

Fig. 1 **a** Map of Angola and its provinces [39] (by Golbez used under CC BY 2.5, https://creativecommons.org/licenses/by/2.5/). **b** Location map of Angola in Africa [40] (by TUBS used under CC BY-SA 3.0, https://creativecommons.org/licenses/by-sa/3.0/)

isolate simultaneously present in ear discharge from the same patient. The number of samples and isolates with regard to age group are described in Table 1.

Proteus spp., *Pseudomonas aeruginosa* and enterococci dominate in CSOM

A summary of the microbiological findings is presented in Table 2. In ear discharge samples, 87 different species were identified; *Proteus* spp. (14.7%), *P. aeruginosa* (13.2%) and *Enterococcus* spp. (8.8%) were, however, dominating. Other *Enterobacteriaceae* constituted a large group, representing 23.5% of isolates, in which *Providencia* spp., *Morganella morganii, Citrobacter* spp. and *Klebsiella* spp. were the most prevalent bacteria. In contrast, *Pseudomonas* spp. other than *P. aeruginosa* (16.0%), pneumococci (10.9%) and *S. aureus* (8.2%) were dominating in nasopharyngeal samples, beside the abundant occurence of coagulase-negative staphylococci (CoNS) (30.7%). Of 34 pneumococcal isolates, 35.3% ($N = 12$) were identified as serotypes included in the 13-valent pneumococcal conjugate vaccine (PCV13). Serotype 19F ($N = 4$) was the most common serotype followed by serotype 6A ($N = 3$) and non-PCV13 serotype 17F ($N = 3$) (Table 3).

Table 1 Demographics of the study population and numbers of samples collected and pathogens isolated with regard to age group

Age group (years)	*N* (%)	Gender (male/female)[1]	Ear discharge samples		Nasopharyngeal samples		Total		Corresponding isolates[3]	
			N	Isolates[2]	*N*	Isolates[2]	*N*	Isolates[2]	Ear/Ear	Ear/Nph
< 5	34 (22)	20/14	44	124 (2.8 [0–5])	34	104 (3.1 [1–6])	78	194 (2.5 [0–6])	10 (18.2)	7 (10)
5–9	23 (15)	15/8	26	81 (3.1 [0–6])	23	52 (2.3 [0–3])	49	117 (2.4 [0–6])	7 (36.8)	3 (8.3)
10–14	24 (16)	16/8	31	97 (3.1 [1–7])	24	51 (2.1 [0–5])	55	144 (2.6 [0–7])	15 (33.3)	6 (12.8)
≥ 15	55 (36)	33/22	66	184 (2.8 [1–6])	54	109 (2.0 [0–4])	120	286 (2.4 [0–6])	18 (29.5)	11 (10.8)
No age data	16 (11)	7/4	17	48 (2.8 [1–4])	16	45 (2.8 [1–4])	33	96 (2.9 [1–4])	2 (28.6)	0
Total	152	91/56	184[4]	534 (2.9 [0–7])	151[5]	361 (2.4 [0–6])	335	823 (2.5 [0–7])	52 (27.3)	27 (9.3)

No data about age or gender was available for 16 and 5 patients, respectively
[1] Gender data was missing for 5 patients
[2] Presented as *N* (mean number of isolates per sample [range])
[3] Number of species present in two ear discharge samples, or in samples from ear discharge and the nasopharynx, from the same patient. Presented as N (%)
[4] Bilateral samples were collected from 32 patients
[5] No nasopharyngeal sample was collected from 1 patient

Table 2 Microbiological findings in samples from ear discharge and the nasopharynx

Isolates from ear discharge (*N* = 534)		Isolates from nasopharynx (*N* = 289)	
Pathogen	*N* (%)	Pathogen	*N* (%)
Proteus spp.	79 (14.7)	CoNS	90 (30.7)
Pseudomonas aeruginosa	71 (13.2)	*Pseudomonas* spp.	47 (16.0)
Enterococcus spp.	47 (8.8)	*Streptococcus pneumoniae*	32 (10.9)
Providencia spp.	42 (7.8)	*Staphylococcus aureus*	24 (8.2)
CoNS	39 (7.3)	*Proteus* spp.	12 (4.1)
Corynebacterium spp.	31 (5.8)	*Moraxella catarrhalis*	10 (3.4)
Morganella morganii	24 (4.5)	*Enterococcus* spp.	10 (3.4)
Alcaligenes faecalis	19 (3.5)	*Arthrobacter* spp.	8 (2.7)
Citrobacter spp.	18 (3.4)	*Acinetobacter* spp.	6 (2.0)
Klebsiella spp.	16 (3.0)	*Alcaligenes faecalis*	6 (2.0)
Arthrobacter spp.	15 (2.8)	*Haemophilus influenzae*	5 (1.7)
Staphylococcus aureus	14 (2.6)	*Corynebacterium* spp.	4 (1.4)
Other *Streptococcus* spp.	12 (2.2)	Fungi	4 (1.4)
Fungi	12 (2.2)	*Pantoea agglomerans*	4 (1.4)
Kerstersia gyiorum	12 (2.2)	*Streptococcus pyogenes*	3 (1.0)
Escherichia coli	11 (2.0)	*Enterobacter* spp.	3 (1.0)
Enterobacter spp.	11 (2.0)	Other[2]	21 (7.2)
Pseudomonas spp.	10 (1.9)	No growth	4 (1.4)
Achromobacter spp.	7 (1.3)		
Streptococcus pyogenes	6 (1.1)		
Other[1]	38 (7.1)		
No growth	3 (0.6)		

Pathogens representing less than 1% of isolates in each group have been pooled as "Other". CoNS = coagulase-negative staphylococci

[1] Included pathogens listed from most to least prevalent: *Stenotrophomonas maltophilia*, *Acinetobacter* spp., *Gemella morbillorum*, *Bordetella trematum*, *Globicatella sulfidifaciens*, *Aeromonas caviae*, *Escherichia hermannii*, *Streptococcus pneumoniae*, *Aerococcus viridans*, *Arcanobacterium haemolyticum*, *Dermabacter hominis*, *Kocuria* spp., *Micrococcus* spp., *Weeksella virosa*, *Raoultella ornithinolytica*, *Serratia marcescens*, *Lactococcus lactis*, *Weisella confusa*, *Neisseria meningitidis*

[2] Included pathogens listed from most to least prevalent: *Klebsiella* spp., *Aerococcus viridans*, *Lactococcus lactis*, *Leuconostoc* spp., *Macrococcus caseolyticus*, *Moraxella nonliquefaciens*, *Raoultella ornithinolytica*, *Streptococcus* spp., *Citerobacter koseri*, *Morganella morganii*, *Serratia marcescens*, *Dietzia maris*, *Lactobacillus salivarius*, *Shewanella putrefaciens*, *Stenotrophomonas maltophilia*

Most pathogenic bacteria in CSOM are susceptible to fluoroquinolones

Susceptibility patterns for gram-negative and gram-positive bacteria are presented in Table 4 and Table 5, respectively. The majority of tested isolates were susceptible to quinolones (i.e., ciprofloxacin and norfloxacin), and resistance rates in bacteria relevant to CSOM ranged from 6.3 to 30.8% for *P. aeruginosa* and *S. aureus*, respectively. Several isolates within *Enterobacteriaceae* were resistant against aminoglycosides (range 6.9–25.8%), slightly more increased resistance against gentamicin as compared to tobramycin. In contrast, resistance against aminoglycosides was lower for *P. aeruginosa* and higher in *S. aureus*. Almost half of isolated *Enterobacteriaceae* (42.6%), and all *P. aeruginosa*, were resistant against chloramphenicol. A high resistance against trimethoprim-sulfamethoxazol was observed in *Enterobacteriaceae* while more than 90% were susceptible to cefotaxime. We also found that a high proportion of *S. aureus* (53.8%) were methicillin-resistant (MRSA). Furthermore, most pneumococci were resistant against benzylpenicillin (53.1%) and trimethoprim-sulfamethoxazol (78.1%).

Discussion

It is a well known fact that infection of the middle ear in CSOM is usually polymicrobial, and the present study further confirms this as single isolates were identified in very few ear discharge samples [2]. In fact, a wide range of different species was identified. The high number of bacterial strains and degree of co-colonization reported, in both ear discharge and nasopharyngeal samples, can possibly be attributed to a higher specificity of MALDI-TOF MS as compared to conventional microbiological identification methods [15]. The slight difference in number of males and females in the current study is unclear, but may be due to differences in care-seeking

Table 3 Pneumococccal serotypes identified in 34 isolates

Serotype	*N*	(%)
19F*	4	(11.8)
6A*	3	(8.8)
17F	3	(8.8)
16	2	(5.9)
21	2	(5.9)
38	2	(5.9)
11A	2	(5.9)
23B	2	(5.9)
NT	2	(5.9)
4*	1	(2.9)
18C*	1	(2.9)
19A*	1	(2.9)
23F*	1	(2.9)
6B*	1	(2.9)
15A	1	(2.9)
15C	1	(2.9)
19B	1	(2.9)
12	1	(2.9)
13	1	(2.9)
20	1	(2.9)
34	1	(2.9)
Total	34	(100)
Total PCV13-serotypes	12	(35.3)

Asterisks indicate serotypes included in the 13-valent pneumococcal conjugate vaccine (PCV13). NT = non-typeable

behaviour, as CSOM has been observed to affect gender equally [2]. However, a predominance of males was shown in an earlier report from the same clinic [18].

The nasopharyngeal microbiome has been proposed as a reservoir for pathogens involved in CSOM, and culture from this locus may grant important clinical information during the disease [19]. However, we found a low degree of correspondence between organisms present in the nasopharynx and middle ear discharge in the same patients. On the other hand, > 25% of the isolates from ear samples in patients with bilateral disease corresponded between the ears. It is possible that this is due to spread via the nasopharynx as the Eustachian tube is dysfunctional in CSOM, but may also be due to invasion from the outer ear canal by the same species in both ears [4]. The role of the nasopharyngeal flora in the pathogenesis and sustaining of CSOM is a field that needs to be further explored.

In accordance with our results, Taipale et al. [12] previously found *Proteus* and *P. aeruginosa* to be the most prevalent bacteria in 18 patients with CSOM in Luanda. In a Kenyan study, enterococci (28%) were reported as a common pathogen in CSOM together with *Proteus*, *S. aureus* and *P. aeruginosa*, representing 32%, 12% and 11% of isolates, respectively [20]. Orji et al. [21] and Afolabi et al. [22] both found that *P. aeruginosa* is the dominating species in Nigeria followed by *S. aureus* and *Klebsiella,* respectively. On the other hand, Chirwa et al. [23] defined *Proteus* spp. as dominating followed by *P. aeruginosa* in Malawi. Thus, our results on the most prevalent pathogens in CSOM are in concordance with other recent studies from sub-Saharan Africa, although there seem to be geographical differences in the proportions between the species present.

An interesting finding was 19 isolates of *Alcaligenes faecalis,* which may be due to the occasional custom of filling the external meatus of the ear with bird droppings to prevent discharge, that we previously reported [24]. Furthermore, 12 isolates of *Kerstersia gyiorum* was detected. This gram-negative species belongs to the *Alcaligenaceae* family and has previously been found in CSOM with treatment failure due to antimicrobial resistance [25]. We did not have the possibility to study anaerobes in the present work due to methods used for sampling, transportation and microbiological diagnostics, although there is support for the presence of these bacteria in a majority of CSOM infections [6]. The high prevalence of CoNS and *Corynebacterium* spp., that both belong to the skin microbiome of the external auditory channel, was most likely contamination during collection [26]. It is possible that other isolated species also represent contamination from adjacent anatomical sites, and thus more studies are needed to elucidate the rich bacterial spectrum found in CSOM applying methods that enable specific species identification.

Aural toilet combined with empirical antibiotic therapy with topical quinolone antibiotics is the recommended first-hand treatment for uncomplicated CSOM although topical aminoglycosides, polymyxins or chloramphenicol are also used. Moreover, antiseptic topical agents such as aluminium acetate, acetic acid or boric acid may be effective and more feasible in resource-limited conditions due to their lower cost and availability [27–29]. The susceptibility patterns of the most frequently isolated species in this study suggest that quinolones or aminoglycosides may be more successful in clearing infection than colistin or chloramphenicol, the latter being the currently most used topical antibiotic for CSOM in Angola. Quinolones have previously been shown to be more effective than aminoglycosides, and would also be the preferred choice of the two drugs due to the potential ototoxicity of aminoglycosides [2]. However, resistance rates over 10% were observed for quinolones, which highlights the need to continuously determine antimicrobial susceptibility patterns. Other findings of interest regarding the general occurrence of antimicrobial resistance are high rates of

Table 4 Susceptibility of gram-negative bacterial species to selected antimicrobial agents

Antimicrobial agent	Susceptibility[1]	*Proteus* spp. (*N* = 89)[2]		non-*Proteus Enterobacteriaceae* (*N* = 131)[3]		*P. aeruginosa* (*N* = 63)[4]		*H. influenzae* (*N* = 5)		*M. catarrhalis* (*N* = 9)	
		N	(%)	*N*	(%)	*N*	(%)	*N*	(%)	*N*	(%)
Amoxicillin	S							4	(80)		
	R							1	(20)		
Piperacillin-tazobactam	S	88	(98.9)	125	(95.4)	61	(96.8)				
	I			5	(3.8)						
	R	1	(1.1)	1	(0.8)	2	(3.2)				
Cefotaxime	S	88	(98.9)	121	(92.3)			5	(100)	9	(100)
	I			1	(0.8)						
	R	1	(1.1)	9	(6.9)						
Ceftazidime	S	88	(1.1)	122	(93.1)	62	(98.4)				
	I			2	(1.5)						
	R	1	(1.1)	7	(5.3)	1	(1.6)				
Imipenem	S	89	(100)	122	(93.1)	60	(95.2)				
	I			9	(6.9)	3	(4.8)				
Meropenem	S	89	(100)	131	(100)	52	(82.5)	5	(100)	9	(100)
	I					11	(17.5)				
Ciprofloxacin	S	76	(85.4)	105	(80.2)	58	(92.1)	5	(100)	9	(100)
	I	3	(3.4)	7	(5.3)	1	(1.6)				
	R	10	(11.2)	19	(14.5)	4	(6.3)				
Gentamicin	S	63	(70.8)	106	(80.9)	58	(92.1)				
	I	3	(3.4)	3	(2.3)	1	(1.6)				
	R	23	(25.8)	22	(16.8)	4	(6.3)				
Tobramycin	S	72	(80.9)	109	(83.2)	59	(93.7)				
	I	5	(5.6)	13	(9.9)						
	R	12	(13.5)	9	(6.9)	4	(6.3)				
Tetracycline	S							4	(80)	7	(77.8)
	R							1	(20)	2	(22.2)
Trimethoprim-sulfamethoxazole	S	31	(34.8)	71	(54.2)			2	(40)	1	(11.1)
	I	2	(2.2)	2	(1.5)					1	(11.1)
	R	56	(62.9)	58	(44.3)			3	(60)	7	(77.8)
Chloramphenicol	S			27	(57.4)						
	R			20	(42.6)	42	(100)				
Colistin	S			26[3]	(55.3)	40[4]	(95.2)				
	R	70[2]	(100)	21[3,5]	(44.7)	2[4,6]	(4.8)				

Isolates grouped as non-*Proteus Enterobacteriaceae* were 38 *Providencia* spp., 25 *Morganella morganii*, 18 *Klebsiella* spp., 18 *Citrobacter* spp., 13 *Enterobacter* spp., 13 *Escherichia* spp., 3 *Pantoea agglomerans*, 2 *Serratia marcescens* and 1 *Raoultella ornithinolytica*

[1] S, susceptible; I, intermediate; R, resistant

[2] Susceptibility testing for *Proteus* spp. against colistin was done with 70 randomly selected isolates

[3] Susceptibility testing for non-*Proteus Enterobacteriaceae* against chloramphenicol and colistin was done with 47 randomly selected isolates

[4] Susceptibility testing for *P. aeruginosa* against chloramphenicol and colistin was done with 42 randomly selected isolates

[5] All MIC ≥64 mg/L including *Providencia*, *Morganella*, *Enterobacter* and *Serratia* spp.

[6] All MIC = 4 mg/L

MRSA, aminoglycoside-resistance among gram-negatives and penicillin non-susceptible pneumococci. On the other hand, only a small number of *Enterobacteriaceae* were resistant to cefotaxime, i.e. probable carriage of extended-spectrum beta-lactamases, and carbapenems which has previously been reported as common in Luanda [14].

Considering the risk for antimicrobial resistance to all topical antibiotics tested, and the fact that these

Table 5 Susceptibility of gram-positive bacterial species to selected antimicrobial agents

Antimicrobial agent	Susceptibility[1]	*S. pneumoniae* (*N* = 32)		*S. pyogenes* (*N* = 9)		*S. aureus* (*N* = 13)	
		N	(%)	*N*	(%)	*N*	(%)
Benzylpenicillin	S	15	(46.9)	9	(100)		
	R	17	(53.1)				
Cefoxitin[2]	S					6	(46.2)
	R					7	(53.8)
Norfloxacin[3]	S	32	(100)			9	(69.2)
	R					4	(30.8)
Tobramycin	S					4	(30.8)
	R					9	(69.2)
Erythromycin	S	32	(100)	9	(100)	12	(92.3)
	R					1	(7.7)
Clindamycin	S	32	(100)	9	(100)	11	(84.6)
	I					1	(7.7)
	R					1	(7.7)
Tetracycline	S	24	(75)	3	(33.3)		
	I	1	(3.1)				
	R	7	(21.9)	6	(66.7)		
Fusidic acid	S					13	(100)
Rifampicin	S	27	(84.4)				
	R	5	(15.6)				
Trimethoprim-sulfamethoxazole	S	6	(18.8)			13	(100)
	I	1	(3.1)				
	R	25	(78.1)				

[1] S, susceptible; I, intermediate; R, resistant
[2] Screening substance for methicillin resistance
[3] Screening substance for quinolone resistance

antibiotics may not be readily available in resource-limited settings, the use of topical antiseptics should be explored. Treatment with one-off application of boric acid powder has been reported to be as effective as topical ciprofloxacin while treatment with aluminium acetate has showed similar results compared to topical aminoglycosides [2, 29]. In a randomized controlled trial in Tanzania the treatment choice for CSOM in children was daily aural toilet and topical boric acid in alcohol solution [30]. Furthermore, Youn et al. [31] showed a high bactericidal effect of aluminium acetate and acetic acid against MRSA and quinolone-resistant *P. aeruginosa*.

Within the framework of the current project at HJM, a film has been produced informing about the condition to improve care of CSOM. Here ear care by "dry mopping" and protecting the ear from contamination is described in detail. It is available online and is planned to be presented in social media and television in Angola [32]. Such information and improved treatment in health care centers and hospitals might reduce the burden of CSOM and its complications.

As CSOM is generally preceded by an episode of AOM, which is predominantly caused by pneumococci, non-typeable *Haemophilus influenzae* or *Moraxella catarrhalis* [33], an important aspect of CSOM prevention is the reduction of AOM. In fact, incidence rates of AOM in western sub-Saharan Africa has been estimated to be over 40%, with a majority of cases occurring in children under 5 years of age [3]. Some studies suggest that the use of PCV may have a positive effect on the all-cause AOM incidence, which may lead to a subsequently reduced CSOM incidence [34, 35]. PCV13 was introduced in Angola in 2013 and WHO estimates that the proportion of newborns who received three vaccine doses has risen from 9% in 2013 to 58% in 2015 and 2016 [36]. Although a small number of pneumococci were isolated, our results show that vaccine serotypes are present in Angola indicating that any obvious serotype replacement, which is a well-documented effect of

PCV in many areas of the world, has not occurred, at least not in the mixed age group studied [37, 38]. Considering these numbers and the possible effects on otitis media, a higher degree of vaccine coverage is desirable in Angola. However, further studies are required on this topic.

Conclusions

The results of the current study largely agree with other studies concerning the dominating pathogens found in CSOM although a greater number of individual species were identified. *Proteus* spp., *P. aeruginosa* and enterococci were the most frequently identified bacteria in ear discharge. Based on the susceptibility testing performed the best choices for topical antibiotic treatment of CSOM in the current outpatients setting would be quinolones. However, considering the risk for unsuccessful treatment due to antimicrobial resistance, topical antiseptic agents should be considered as the first-hand choice for treatment of CSOM in Angola.

Additional files

Additional file 1: Multilingual abstracts in the six official working languages of the United Nations. (PDF 675 kb)

Additional file 2: Primer list and PCR cycling conditions. (PDF 126 kb)

Additional file 3: Full patient list. (PDF 258 kb)

Abbreviations

AOM: Acute otitis media; CoNS: Coagulase-negative staphylococci; CSOM: Chronic suppurative otitis media; ENT: Ear, nose and throat; HJM: Hospital Josina Machel; MALDI-TOF MS: Matrix-assisted laser desorption/ionization – time of flight mass spectrometry; mPCR: Multiplex polymerase chain reaction; MRSA: Methicillin-resistant *S. aureus*; PCV: Pneumococcal conjugate vaccine; WHO: World Health Organization

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MF initiated the study, collected samples and epidemiological data. KR, MF, ÅR and TP designed the study and wrote the manuscript together with FU. EM did susceptibility testing and wrote the manuscript. MP and FU typed bacteria and did susceptibility testing. JT and SH contributed to the microbiological diagnostics. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Authorization for the study was obtained from the Ethical committee at the Medical National Council of Angola, Scientific Council of the Faculty of Medicine of Agostinho Neto University and the General Director of Hospital Josina Machel. Before enrollment, the patients or their guardians gave informed consent.

Competing interests

The authors declare that they have no competing interests.

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