

# Recurrent invasive pneumococcal disease in children - host factors and vaccination response

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The thesis is based on the following original papers:

1. Recurrent invasive pneumococcal disease in children: epidemiological, microbiological and clinical aspects from a Danish 33-year nationwide survey (1980–2013), Ingels H, Lambertsen L, Harboe Z, Marquart H, Konradsen HB, Christensen JJ, Heilmann C, Scand J Infect Dis. 2014 Apr;46(4):265-71.

2. Immunodeficiency among children with recurrent invasive pneumococcal disease, Ingels H, Schejbel L, Lundstedt AC, Jensen L, I Laursen I.A, Ryder LP, Heegaard N, Konradsen H, Christensen JJ, Heilmann C., Marquart HV, Ped Inf Dis J. 2015 Jun;34(6):644-51.

3. Impact of pneumococcal vaccination in Denmark during the first 3 years after PCV introduction in the childhood immunization programme, Ingels H, Rasmussen J, Andersen PH, Harboe ZB, Glismann S, Konradsen H et al, Hoffmann S, Valentiner-Branth P, Lambertsen L, On behalf of Danish Pneumococcal Surveillance Collaboration Group 2009–2010, Vaccine. 2012 Jun 6;30(26):3944-50.

## PREFACE

During the first year of my specialization in paediatrics, my way crossed a patient which kept on puzzling me. It was a case of a 8-month old boy with repetitive bacteremic epi-

sodes always culturing *Streptococcus pneumoniae*. This boy without comorbidity had no focus of infection and was apparently immunological competent. We (my colleagues and I) never succeeded in finding the reason for this boy's enhanced susceptibility to infections, in spite of a thorough search for an underlying immunological or structural explanation. However, the case made me curious to know more about the interaction between microbe and host and was the kick off for the idea behind this Ph.D thesis. The work presented in this thesis was carried out during my employment as a Ph.D student at Statens Serum Institut, Denmark 2009–2012.

## ABBREVIATIONS

CVID	Common variable immune deficiency
HSCT	Haematopoietic stem cell transplantation
IRAK-4	Interleukin-1-receptor associated kinase-4
IgA1	Immunoglobulin A1
IRR	Incidence rate ratio
IPD	Invasive pneumococcal disease
MBL	Mannose binding lectine
MLST	Multi locus sequence typing
MYD88	Myeloid differentiation factor 88
NEMO	Nuclear factor-kB essential modulator deficiency
PCV	Pneumococcal conjugate vaccine
PCV7	7-valent pneumococcal conjugated vaccine
PCV10	10-valent pneumococcal conjugate vaccine
PCV13	13-valent pneumococcal conjugate vaccine
PFGE	Pulse-field gel electrophoresis
PID	Primary immune deficiency
PPV	Polysaccharide based pneumococcal vaccine
PPV23	23-valent polysaccharide based pneumococcal vaccine
rIPD	Recurrent invasive pneumococcal disease
TLR	Toll-like receptor

## 1.0 BACKGROUND

### 1.1. Introduction

*Streptococcus pneumoniae* is still a major cause of morbidity and mortality in children worldwide with over a half million children dying annually from pneumococcal disease, mostly in developing countries (5). Some children are prone to repetitive invasive pneumococcal disease (rIPD) either because of an underlying predisposing disease or due to unknown causes.

The aim of this PhD thesis was to examine laboratory-confirmed cases of paediatric rIPD during a 33-year nationwide study, to determine risk factors and study aspects of the immunological background for rIPD.

In October 2007, a pneumococcal conjugate vaccine (PCV) was implemented in the Danish infant immunization programme (6,7). An additional aim of this thesis was to examine the vaccination impact on a population level, following the first three years of general PCV vaccination in Denmark. Below follows a short review of aspects of microbial virulence factors as well as a description of host factors of importance for the outcome in the meeting between the host and the pneumococcus. Furthermore, a review is presented of the current knowledge on paediatric rIPD.

## 1.2 STREPTOCOCCUS PNEUMONIAE

*S. pneumoniae* is a Gram-positive bacterium. It is a part of the normal nasopharyngeal flora, especially in young children attending daycare, where up to 40% are carriers (8,9). *S. pneumoniae* is a frequent human pathogen in non-invasive (sinusitis, otitis media, pneumonia) and in invasive infections (bacteraemia/septicaemia and meningitis). The pneumococcus consists of cytoplasmic DNA and RNA surrounded by a cell membrane, cell wall and, importantly, a polysaccharide capsule (Figure 1). This capsule allows typing of the bacteria and due to differences in polysaccharide

### 1.2.1. Serotyping

Classical serotyping of pneumococci is performed by using Neufelds quellung reaction and specific pneumococcal rabbit antisera (11,13). Denmark has a long tradition, going back to 1938, of laboratory-based nationwide surveillance of IPD, including serotyping (14). Serotyping of pneumococci is important for epidemiological reasons, to guide vaccine development, and to monitor impact of pneumococcal vaccination (7). Pneumococcal serotyping is also useful in rare clinical situations in relation to cases of rIPD. Here, serotyping may help to clarify whether the recurrence is a) a relapse caused by the same strain or b) a novel infection with a new serotype

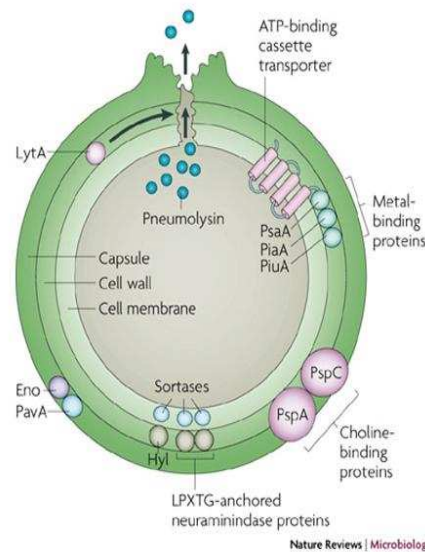
### 1.2.2 Molecular typing methods

Apart from the classical pneumococcal phenotyping, described above, molecular typing methods may be used to characterize the pneumococcus. Pulse-field gel electrophoresis (PFGE) is separation of large DNA molecules in a gel (15). PFGE provides a genetic fingerprint of an organism and is used to determine the relatedness between pathogens. Multi locus sequence typing (MLST) characterizes isolates of microbial species by using the DNA sequences of multiple housekeeping genes. Due to sequence conservation in housekeeping genes, MLST is useful in determining clonal relationships between strains, but capsule switching event and vaccine escape variants of pneumococci can also be explored.

### 1.2.3 Virulence factors

*S. pneumoniae* virulence is multi-faceted. Besides the polysaccharide capsule, which has long been recognized as an important virulence factor, the importance of proteins has recently become clear. Research in this area has been

**Figure 1 Virulence Factors the *Streptococcus Pneumoniae***



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composition, more than 90 capsular serotypes have been defined (10,12)

stimulated by the realization that pneumococcal proteins common to all pneumococcal serotypes, represent promising candidates for the development of vaccines. (Figure 1) (17). Beneath follows a description of a few of the most important virulence factors.

The polysaccharide capsule is indispensable for pneumococcal virulence and is strongly anti-phagocytic in non-immune hosts (17). The antibodies raised against the polysaccharide capsule are protective only against that particular serotype (although cross immunity between serotypes has been observed). Virulence appears to be associated with the capsular serotype. As an example, certain serotypes (3,6B,9V,19F) are considered to be associated with otitis media (18) and others (3, 6A, 6B, 9N, 19F) may be associated with an increased risk of death when causing pneumonia (19). Pneumococci without a capsule can cause conjunctivitis, but, apart from this, they are non-virulent(20).

Peptidoglycan and teichoic acid are released upon lyses of the pneumococcus and are potent mediators of inflammation in the host through stimulation of toll-like receptors (TLR) 2. In Paper 2, we used synthetic peptidoglycan as a ligand to test TLR function in children with unexplained episodes of rIPD.

Pneumolysin is released upon lyses of the bacteria (Figure 1). It is lytic for host cells and stimulates production of TNF and IL-6 in macrophages. It has been suggested in animal models, that pneumolysin plays an important role for hearing loss following pneumococcal meningitis (21,22).

Zinc metalloprotease is a protein produced by the *S. pneumoniae* to degrade immunoglobulin A1 (IgA1), thereby facilitating colonization.

Pneumococcal surface protein antigen (PspA) is needed for the pneumococcus to be fully virulent (23). The protein is an

important candidate for next generation pneumococcal vaccines (protein vaccines) (17).

Pneumococcal disease begins with colonization and creation of a carrier state. Once acquired, an individual strain can be carried for weeks to months before its eventual clearance

#### 1.2.4 Epidemiology

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**TABLE 1 Historical Milestones in Pneumococcal Research:**

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- 1881 Sternberg and Pasteur isolated the bacterium now known as *Streptococcus pneumoniae*. *S. pneumoniae* (119) was one of the first bacteria to be isolated and characterized and has played an important role in defining classical microbiology.
- 1884 HC.Gram used *S. pneumonia* in the development of Gram staining (120)
- 1902 F. Neufelds first attempt to test capsular swelling test (11)
- 1911 Wrights first attempts to produce pneumococcal (whole-cell) vaccine, trialed among Southafrican mineworkers (without success) (121)
- 1930 The Immunogenicity of capsule polysaccharide was proven (Francis og Tillet (122))
- 1940ies a 4- and 6-valent pneumococcal polysaccharide vaccines were registered in the US. However, because of the succes of use of penicillin, they were withdrawn again.
- 1964 R. Austrian published a paper about the need to continue development of polysach.based vacc.(123)
- 1967 a new programme for the development of a pneumococcal vaccine was initiated (124)
- 1978 a 14-valent polysaccharide based vaccine was licensed (124)
- 1983 blev en 23-valent polysaccharide based vaccine was licensed (124)
- 2000 a 7-valent pneumococcal conjugate vaccine was licensed (124)
- 2010 10-valent, 13 valent pneumococcal conjugate vaccines licensed

Ongoing research in trying to develop proteinbased vaccines

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(17, 23). Person-to-person spread occurs through direct contact with the secretions from colonized individuals. Some serotypes dominate in the carrier population, others are more prone to cause invasive disease (25). Carriage is generally shorter for serotypes with high invasive potential (25). Colonization is most common in early childhood among children up to 4 years old (24), who are thus an important reservoir of pneumococci in a population. The serotype distribution of pneumococcal isolates causing IPD varies between age groups and different geographical regions; however, 11 serotypes accounted for more than 75 % of all IPD-cases (12) prior to the introduction of general vaccination. This has been the basis for construction of pneumococcal vaccines over the years (Table 1). Adult IPD is caused by a broader spectrum of serotypes than is childhood IPD (27). The epidemiology of pneumococcal disease and serotypes is complex and highly dynamic. Temporal changes due to natural fluctuations, use of antibiotics and vaccinations, as well as comorbidity may influence the serotype epidemiology in a population (27, 12) and must be taken into account when evaluating changes in IPD incidence over time. Important geographical differences in serotype distribution have been reported between developing countries (5,26), who suffer the highest burden of disease, and that of

developed countries, and also between countries more alike (27,28). This makes it difficult to extrapolate experiences from one population to another when, for example, evaluating the impact of pneumococcal vaccination.

#### 1.2.5 Childhood risk factors for IPD

Children under the age of two years account for the highest risk of IPD(1). An underlying predisposing condition is found in 20–25% (29,30) of all children aged 0–15 years experiencing IPD. Current vaccination guidelines emphasize which groups of children are considered at high risk of IPD and who may benefit from a combination of pneumococcal vaccines in order to broaden serotype coverage (31,32) (Table 2). Environmental risk factors Important environmental risk factors for IPD are crowding, such as daycare attendance, the presence of older siblings, season of the year and passive smoking (33–35). A Danish study reported that the risk of IPD in Danish children was the highest the first 2 months after start of daycare attendance (34). Moreover, low socioeconomic status is associated with increased risk of IPD. As an example, Alaskan native children have a very increased risk of IPD both due to crowding factors and low socioeconomic status (36); however, genetic factors may also play a role in this population.

**Table 2 Danish 2012 guidelines for pneumococcal vaccination of high-risk children**

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Children at high risk of acquiring invasive pneumococcal disease

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Splenectomy (planned/performed)  
Splenic dysfunction (e.g. following radiation)  
Organ transplant  
Cochlear transplant  
Leakage of cerebrospinal fluid  
Previous invasive pneumococcal disease  
Immunosuppression (e.g. due to HIV infection or lymphoma)  
Heart disease (cyanotic heart disease, cardiac failure, palliative surgery for cardiac condition)  
Chronic pulmonary condition (e.g. cystic fibrosis)  
Hypodynamic respiratory insufficiency  
Nephrotic syndrome  
Immunodeficiencies, excluding agammaglobulinaemia and SCID

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Children 3–11 months age: 3 x PCV + booster PCV + booster PPV23 when the child is >24 months of age  
Children 12 months–6 years: 2 x PCV + booster PPV23 when the child is >24 months of age  
Children >6 years: 1 x PCV + booster PPV23 8 weeks after

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Any condition associated with poor synthesis of immunoglobulins, such as primary hypogammaglobulinaemia or acquired hypogammaglobulinaemia, may increase the risk of pneumococcal disease (37).

Asplenic children have a highly increased risk of acquiring IPD due to the specific role of the spleen in clearing opsonized bacteria from the circulation. HIV infected children are very susceptible to IPD due to defective antibody production and reduced mucosal clearance (38). Moreover, children with lung disease, neurological disease and chronic heart disease are at high risk of IPD, partly because of their reduced mucosal clearance (38) and also for reasons not completely understood. Among haematopoietic stem cell transplanted (HSCT) children, *S. pneumoniae* is an important pathogen. Early onset pneumococcal disease is seen during the first month after HSCT in both allogenic and autologous HSCT. Late onset disease occurs mostly in allogenic transplanted individuals (39). In these patients, functional asplenia due to former irradiation, chronic graft-versus-host disease and decreased antibody production all contribute to the high risk of IPD (38). Some solid organ transplant recipients have lifelong risk of IPD due to immunosuppressive treatment.

Anatomical abnormalities, such as cerebrospinal fluid leakage or congenital inner ear malformation, are risk factors for acquiring pneumococcal meningitis because these abnormalities facilitate the entrance of the bacteria to the meninges. In a study among children born in Denmark 1995–2004, hearing loss was associated with an odds ratio for acquiring meningitis (all causes) of 5.0 [95%CI 2.0–12.0] compared with the background population (40). In most countries, children with cochlear implants are advised to receive supplementary pneumococcal vaccination with first

Modified after EPINEWS week 51 2012 (32)

the PCV and then the 23-valent polysaccharide based pneumococcal vaccine (PPV23) (Table 2) (31,32).

#### **1.2.6 Recurrent IPD**

A small group of children are at particularly high risk of experiencing IPD more than once. Predispositions to IPD, such as acquired immunodeficiency or anatomical abnormalities, have been reported in 40–92% of the children with rIPD (42–48) (Table 3). Nevertheless, in some children with rIPD, the recurrence of pneumococcal infection is unexplained and therefore raises suspicion of underlying primary immunodeficiency (PID). More than 150 different primary immunodeficiencies (PIDs) have been defined. The risk of IPD is reported to be especially high in patients with B-cell dysfunction (37, 49), complement deficiencies (51,52) and defects in molecules involved in the TLR signalling pathway (53–55).

It is of great clinical importance to be able to predict which child is at a high risk of experiencing a subsequent case of IPD, because of the prophylactic measures that can be commenced. Such measures could be an optimized status of pneumococcal vaccination, treatment with replacement immunoglobulin as well as prophylactic antibiotics, and information to the parents and the child about how to handle signs of infection.

We hypothesized that in a population-based study of children with rIPD, there might be children with undetected primary immune deficiencies; thus undertook Study 2 to test this.

#### **1.3 HOST DEFENCE**

The exact way by which the pneumococcus is recognized by the immune system is partly unknown. However, both innate and adaptive immunity seem to be important in the host defence. In early childhood, at the first encounter with

**TABLE 3 Summary Of Published Studies On Paediatric Recurrent Invasive Pneumococcal Disease (rIPD)**

Reference	Design Geography	Observation period	Children (n) (single IPD/ rIPD)	Recurrence rate	Underlying disease	Systematic, immunological follow up
Lee et al 1994 (42)	Single centre, US Retrospective	15 y	— /5	—	60% (severe failure to thrive) sickle cell	no
Ortizek et al 1997 (43)	Single center Retrospective, US	2.5y	394/16	4.1%	40% (sickle cell)	no
Venez 1998 (44)	Nationwide, Retrospective, Switzerland	10y	393/14	3.6%	14% (CSF leak)	no
King et al 2003 (45)	Single and multicenter* Prospective	1.5-4y	— /23	2.3%**	52% (sickle cell, HIV)	no
Einarsdottir et al 2005 (46)	Nationwide, Island Retrospective	30y	— /12	4.4% **	92% (Immunoglobulin disorders anatomical defect)	no
Mason et al 2007 (47)	Multicenter study, US Retrospective	12y	4067/90	2.6%	80% (HIV, Renal disease)	no
Ingels et al (48) (included in thesis)	Nationwide, Denmark Retrospective Follow up	33y (follow up 29y)	2482/59	2.4%	87% (Transplantation, CSF leak, Complement deficiency)	yes

Y:years, CSF: cerebrospinal fluid

Included studies: all studies of rIPD in paediatric populations with 5 or more children.

\*Epidemiological and microbiological data were assessed from 8 centres, clinical data on underlying disease from a single centre.

\*\* The calculated recurrence rate includes adults with rIPD

M dash: information not available

the microbe, innate mechanisms, such as complement activation, TLR signalling and phagocytosis in the spleen, are crucial for the defence. Later, the adaptive immune system is important, especially the B cells' production of specific antibodies and clearing of the microorganism in the spleen (37). Primary or secondary defects in any of the above-mentioned parts of the immune system may lead to an enhanced susceptibility to invasive infections with *S. pneumoniae* (37).

### 1.3.1 Mucosal barrier and antigen presentation

The transition from nasopharyngeal colonization to invasive disease reflects the relationship between *S. pneumoniae* and its host and, on the pathogen side, involves the previously mentioned virulence factors, and, on the host side, various immunological mechanisms. The hydrophilic polysaccharide capsule prevents direct contact between non-opsonized pneumococci and the phagocytes. Consequently, opsonization of pneumococci by complement and/or serotype specific antibody is crucial to allow phagocytosis and bacterial killing (37, 56, 17). In the naïve host, without specific antibody, TLR 2 on host cells, which binds peptidoglycan from the surface of gram positive bacteria, is thought to play an important role as initiator of the inflammatory response in mucosa (17). In addition, murine studies have shown that T cells contribute to the initial protection against *S. pneumoniae* disease and carriage though the mechanism is not fully understood (17, 57).

Once the bacterium passes through the mucosa, it is presented to local immature B lymphocytes and T lymphocytes. Activated IgA determined B cells spread with the systemic circulation to other parts of the immune system (58). When the same antigen is next introduced, the specific memory B cells quickly proliferate in to IgA producing plasma cells (58). In general, B cell responses are classified as T-dependent or T-independent based on the requirement for T cell help in the production of antibodies. Polysaccharides, such as *S. Pneumoniae* capsule material, induce a T-independent response (41) and production of IgG2 antibodies. Protein antigens induce a T-dependent B cell response and the production of IgG1 antibodies. (37)

The immunological response of the mucosa consists mainly of IgA antibodies, produced by local plasma cells, but because of the pneumococcal secretion of IgA1 protease, inflammation is partly prevented. Full antibody-mediated mucosal clearance will occur only after sufficient amounts of other classes of specific antibody have been generated (17). This is one reason why specific IgG (acquired by induction from the pneumococcal conjugate vaccine), which is not degraded by the IgA proteases, decreases pneumococcal colonization (17).

Nasopharyngeal carriage rate of pneumococci is high in young children. The mucosa is normally highly resistant to penetration of bacteria; however, if injured by viral infection or exposure to irritants such as cigarette smoke, it may be unable to withstand bacterial invasion (37,59). The temporal association between viral respiratory infections and pneumococcal disease (pneumonia as well as IPD) illustrates this

and has been demonstrated in a number of epidemiological studies (60,61). As an example, it was reported that the incidence of IPD cases increased in the US following the A/H1N1 influenza pandemic in 2009 (62). Additionally, Respiratory Syncytial Virus in children has been related to enhanced risk of subsequently experiencing IPD (63).

### **1.3.2 The Spleen**

The spleen plays a crucial role in the clearance of opsonized bacteria from the blood and as the site for T-cell-independent antibody responses to bacteria in marginal zones (64,65). Therefore, the spleen has an important role in the defence against pneumococci and other encapsulated organisms such as *Haemophilus influenzae* and *Neisseria meningitidis*. This relation is illustrated by the finding that splenectomy is accompanied by a life-long risk of overwhelming postsplenectomy infection (OPSI), mainly caused by *S. pneumoniae*, and is the reason why splenectomy is an absolute indication for vaccination (31,32,66). Young asplenic children, who have not yet developed specific antibodies by previously encountering the microbe, are at especially high risk of OPSI. This risk declines with age (67). Most splenectomized persons respond well to the PPV23 (68); however, some (mainly patients with haematological background conditions) have an impaired response (69). In these patients immunization with PCVs may result in a sufficient antibody response (70).

### **1.3.3 Adaptive immune defence and opsonization**

The production of specific antibodies and complement activation are cornerstones in the host defence against pneumococci. When invading the host the pneumococcus becomes coated with appropriate antibodies. Complexes of immobilized antibodies and complement proteins (C3) cover the bacterium. This leads to opsonisation and facilitates the phagocytes to eliminate the microbe. The level of naturally occurring pneumococcal antibodies is low before the age of two years, increases with age and reaches a plateau during adulthood (37, 56). The important role of specific antibodies in the host defence is demonstrated indirectly by the number of serious pneumococcal infections in patients with primary and acquired antibody defects (e.g. renal disease, transplanted children, common variable immunodeficiency (CVID) (30,49,50) and other B-cell disorders). Delayed diagnosis of a primary antibody deficiency may lead to serious, recurrent infections mainly in the lungs, causing permanent damage (bronchiectasis) (71,72). Substitution with immunoglobulin can reduce breakthrough bacterial infections and thereby optimize clinical outcome (71,72).

### **1.3.4 Complement**

The complement system is an ancient innate host defence system, primarily guarding the host's intravascular space with the main goal to destruct microbes. Additionally, complement in some respects acts as a bridge between innate and adaptive immunity and has a role in both B- and T-cell differentiation and activation (51,52). The complement system consists of plasma proteins and membrane receptors. The plasma proteins interact via three major cascades: the classical, alternative, and lectin pathways. The complement proteins in plasma are synthesized in the liver while

the components at other sites, such as tissues, represent a combination of local synthesis (many cell types) and filtration from plasma, (51,52). Opsonization, refers to the coating of targets with complement ligands (mainly C3b and cleavage products) and/or IgG to promote their elimination through phagocytosis by cells bearing complement receptors. Opsonization facilitates the adaptive immune response, including antigen presentation as well as immunologic memory and co-stimulation of B lymphocytes through the complement receptors (73).

Genetically determined deficiencies of the complement system are associated with an increased susceptibility to some bacterial infections, reflecting the biologic functions of the missing component. Deficiencies of the classical pathway (C1,C2,C4), such as homozygous complement C2 deficiency, are associated with autoimmune disease and susceptibility to recurrent invasive infections with encapsulated bacteria, for which opsonization through C3 is the primary defence, such as *S. pneumoniae*, and *H. influenzae*. Deficiencies of the terminal components of complement (C5–C9) or properdin are associated with a high risk of contracting meningococcal disease. Studies show that a family history of meningococcal disease, recurrent meningococcal disease or meningococcal disease with an unusual subgroup of *N. meningitidis* should lead to a suspicion of complement deficiency (51,52,74–78). Inborn complement disorders are rare and publications are based on case reports or on highly selected cohorts of patients. Although many studies of complement deficient persons report a predisposition to infections with *S. pneumoniae*, information is sparse on the prevalence of complement disorders in clinically defined groups of patients with, for example, recurrent pneumococcal pneumonia or recurrent invasive disease (78).

### **1.3.5 MBL**

Mannose-binding lectin (MBL) is a mediator of innate host immunity activating the complement pathway and directly opsonizing pathogens. About 5% of North Europeans and North Americans are homozygotes for MBL codon variants and most of them go through life without symptoms (79). Several studies have shown that MBL deficiency may predispose to infections in combination with a chronic disease, young age or other immunological defects (80–82). It is plausible that MBL plays a role in the host defence against IPD as well. A study in the UK assessed the association between MBL mutant genotypes and IPD in a case-control study of 229 patients and 353 controls and found an odds ratio of 2.59 ([95% CI 1.39–4.83],  $p=0.002$ ) for experiencing IPD and being homozygotes for MBL codon variants; a result which the authors confirmed in a control study of 787 additional subjects (83). However, two other studies, from Belgium (84) and Denmark (85), could not confirm this finding. Notably, the two latter studies comprised fewer patients and might have reached similar results if more patients had been included.

### **1.3.6 Toll-like receptor signalling**

TLR signalling is critically important in the first unspecific meeting between host and microbe. TLRs belong to a class of membrane-bound surface molecule known as pattern recognition receptors. The ligands for these receptors are

**Table 4 Toll like receptor ligands and associated microorganisms**

Toll-like receptor	Patogen associated molecule	Microorganism
TLR 2	Peptidoglycan	Gram-positive bacterias ( <i>S.aureus</i> , <i>S.pneumoniae</i> ) Herpes simplex vira
TLR2/TLR1	Trisylic polysaccharides	Bacterial surface antigens
TLR2/TLR6	Disylic polysaccharides	Mycoplasma surface antigens
TLR3	Double-stranded RNA	Herpes simplex vira
TLR4	Lipopolysaccharide	Gram-negative bacterias
TLR5	Flagellin	Bacterias with flagellas, e.g. <i>L.pneumophila</i> , <i>S. typhimurium</i>
TLR7	Single-stranded RNA	Influenza virus
TLR8	Single-stranded RNA	HIV-1
TLR9	Double-stranded DNA	Vira (HSV1,HSV2), Bacterias,

components of pathogenic microbes are called "pathogen-associated molecular patterns" (PAMPs) (Table 4). TLRs are expressed on macrophages and dendritic cells and when stimulated initiate a wide range of cytokine responses, thereby inducing inflammation and antigen presentation. Apart from being an important part of the innate immune defence, they also serve as modulators of adaptive immunity. Specific defects of molecules in the TLR signalling pathway: interleukin-1-receptor associated kinase-4 deficiency (IRAK4), myeloid differentiation factor 88 (MYD88), IKBA and nuclear

factor- $\kappa$ B essential modulator deficiency (NEMO) (53–55,86–88) have recently been defined. These TLR signalling defects may result in a narrow phenotype of patients experiencing repetitive invasive pyogenic infections and can be a diagnostic challenge because the affected patients have normal results in routine immunological evaluation (86). A TLR defect will be revealed only if specifically tested in a functional stimulation assay (87,88). The susceptibility of IRAK-4-deficient patients to pneumococcal infections is high and many experience rIPD in early childhood. High mortality (40%) is reported before the age of 8 years; however, among survivors, patients with IRAK-4 and MyD88 deficiencies present no further invasive bacterial infections after their teens (86). Children with NEMO-related defects are severely ill and may have other developmental abnormalities, such as ectodermal dysplasia (55, 86). Children with these disorders may fail to show clinical and laboratorial signs of inflammation, such as fever, leukocytosis and high C-reactive protein levels in serum, even during ongoing systemic infections (49,86). Mutations in the NEMO gene cause impaired NF- $\kappa$ B-mediated cellular responses to multiple receptors; therefore, these patients confer a broader predisposition to infections, including infections with atypical mycobacteria and herpes virus (55, 86).

#### 1.4 VACCINATION

##### 1.4.1. Pneumococcal vaccination

The polysaccharide pneumococcal vaccines (PPV23 and formerly PPV14) have been available since the late 1970s (Table 1). Polysaccharide-based vaccines are T-cell independent antigens and therefore not immunogenic in children under the age of 2 years, who suffer the highest burden of disease. The reason for this is that young children mainly have naïve B cells and consequently need stimuli from CD4+ T cells to respond to a polysaccharide antigen. A PPV vaccine cannot stimulate naïve B-cells and does not recruit CD4+ T cells, thus resulting in no or little antibody formation (37,56). PPVs do not elicit a memory response and have no documented effect on nasopharyngeal carriage, but they have an effect on invasive disease and the vaccines have been widely used (and still are) among paediatric and adult risk groups and in elderly individuals (89).

Vaccination with recently developed highly efficacious pneumococcal conjugate vaccines (PCVs) is now possible. The conjugation of polysaccharides to a carrier protein results in a T-cell dependent immune response, giving a good response to immunization of infants including induction of memory cells and the possibility for a booster response upon subsequent polysaccharide antigen exposure (90, 91). Additionally, the PCVs have an effect upon nasopharyngeal carriage because the vaccine-induced IgG may spread to lymphoid tissue in the mucosa. The introduction of conjugate vaccines and their widespread implementation has had a significant impact on the morbidity (28).

In Denmark, a 7-valent protein conjugate vaccine was introduced in the infant immunization programme in 2007 (6,7). From April 2010, PCV7 was replaced by the 13-valent protein conjugate vaccine (92). In Paper 3 we evaluated the impact of PCV in the Danish population after the first three years of pneumococcal vaccination.

Schedules combining PCVs with PPV23 have been proposed and studied in order to expand disease protection against serotypes not included in the PCVs (93-95). This is of great importance for high risk children (Table 2), who will need to be protected after the early childhood as well and adults responding insufficiently to PPV23. When combining the PCV with PPV23 vaccination, it is generally recommended to vaccinate with the PCV before PPV23. This is firstly because of the observed booster effect of PPV23 upon PCV priming (96-98) and secondly because of the theoretical and observed possibility of hyporesponsiveness following PPV23 vaccination (90).

#### 1.4.2 Vaccination response

The main purpose of measuring pneumococcal antibodies is to evaluate the ability to respond to pneumococcal vaccine and to give the best recommendations for revaccination. The use of antibody concentration is a surrogate marker for clinical efficacy. Exact serological correlates of protection requires an established protective antibody concentration. Such protective levels exist for some but not for all vaccine-preventable diseases (89,91). The importance of the cell mediated immunity varies among diseases; therefore, defining protection from antibody concentrations alone is not always possible. In some high risk groups, it may be very individual which antibody level correlates with protection (91).

Since the implementation of PCV, it has been of high priority to establish serum-antibody levels that correlates of protection to facilitate evaluation of new PCVs. Large randomized studies among children have been undertaken, and it is now internationally accepted that 0.35ug/ml- is the threshold antibody concentration, which is believed to correlate with protection against invasive disease in children (90). Regarding the antibody response to the PPV23, no exactly correlates of protection against pneumococcal disease has been defined (89).

#### OBJECTIVES

The aim of this PhD thesis was to describe and examine cases of rIPD in children. Additionally, we sought to analyse the impact of general PCV vaccination on a population level following the first three years of PCV vaccination in the childhood immunization programme.

The specific aims were:

- Paper I To examine epidemiological, microbiological and clinical aspects of all Danish children with rIPD during a 33-year period 1980–2013
- Paper II To evaluate immunological parameters including complement function, B-cell function and TLR signalling in a nationwide cohort of apparently healthy children experiencing rIPD.
- Paper III To assess the impact of pneumococcal vaccination in Denmark during the first 3 years of PCV introduction in the childhood immunization programme by analysing: 1) direct and indirect effects on incidence of IPD 2) changes in pneumococcal serotype distribution 3) changes in IPD related mortality.

#### 3.0 MATERIAL AND METHODS

The thesis consists of three papers, which are all directly or indirectly based on data retrieved from the National Streptococcus Pneumoniae Registry. This registry is nationwide and contains data from all laboratory-confirmed cases of IPD in Denmark and is continually updated for national surveillance. In Paper 2, the participating children were tested immunologically. Some of the immunological methods are considered standard methods and some, such as the test for Somatic hyper mutation and the two Toll like receptor signalling assays are performed in more specialized laboratories.

During my PhD study I was a guest 6 months at the Department of Clinical Immunology, Section 7631, Rigshospitalet, Copenhagen University Hospital, where I performed many of the Toll-like receptor stimulations myself.

Below follows a short description of the used data sources and methods.

#### 3.1 DATA SOURCES

##### Study population

Paper 1 and 2: the study cohort comprises all Danish children aged 0–15 years with IPD in the period 1980–2013. Paper 3: This cohort comprises the population of Denmark in the years 2000–2010 with a population of approximately 5,500,000 inhabitants (varying from 5,330,020 to 5,534,738 in the study years) (99).

##### The Streptococcus pneumoniae Registry and national surveillance of IPD

This registry contains nationwide laboratory data from IPD cases in Denmark, including cases since 1938, and was the primary data source for this thesis and the source from where children with rIPD were identified (Paper 1 and 2). Denmark has a long tradition of pneumococcal surveillance and pneumococcal isolates are submitted routinely on a voluntary basis from all departments of clinical microbiology in Denmark to the Neisseria and Streptococcus Reference Center (NSR), Statens Serum Institut (SSI). It has been estimated that more than 90% of all IPD isolates are submitted to the NSR (100, 101). Since the implementation of the PCV7 in the childhood immunization programme, October 2007 in Denmark, it has become mandatory to submit all pneumococcal isolates causing IPD to the NSR (102) and to note paediatric cases of IPD at the Department of Epidemiology, SSI.

The Streptococcus pneumoniae registry includes information on the submitting laboratory, the patients civil registration number (CRS number), date of sampling, clinical site from where isolates were obtained, serotype and antibiotic resistance patterns of the isolates.

##### The Danish Civil Registration System (CRS)

In this registry, established in 1968, all citizens in Denmark are registered with a unique identification number, name, address, marital status, offspring, and vital status. This registry was used in Study 1 and 3 to determine vital status of persons experiencing IPD. Moreover, the registry was used (Paper 1 and 2) to localize patients with rIPD. Finally, in Study 3 the patient's CRS number was used to link the vaccination registry with cases of pediatric IPD.



TABLE 5 Immunological Evaluation (study 2)

Basic immunological parameters:

- T-, B-, NK cell
- Plasma concentrations of immunoglobulin classes and -subclasses
- Complement function (C3, C4, CH50)
- MBL genotype

Functional B cell tests:

- Tests for somatic hypermutation in the rearranged immunoglobulin genes of B-cells (117)
- Specific IgG response to Pneumococcal polysaccharides (vaccination response) (104,105)

Toll like receptor function

- Functional stimulation CD62 cleavage assay (118)
- Functional stimulation Cytokine based assay (117)

### The National Patient Registry (NPR)

This registry was established in 1977 and contains information on all hospital discharges in Denmark (103). The registry includes date of admission, date of discharge, an international classification of diseases (ICD8 and ICD10) and the patient's CRS number. This registry was used in Study 1 to determine background disease of the children when the hospital records were not accessible.

### Vaccination Registry

Immunizations administered through the Danish Childhood Vaccination Programme have been registered in the Danish National Vaccination Registry since 1990. Vaccine uptake (Paper 3) was calculated on the basis of person-identifiable data from this vaccination registry by using the administrative service codes indicated by general practitioners when administering the first, second and third PCV dose of vaccine (7).

### Hospital records and other records

Hospital records of children experiencing rIPD were retrieved at the local paediatric departments. Serological databases at the SSI were used to obtain or to confirm information on a child's serotype-specific response to pneumococcal vaccination (Paper 2).

## 3.2 STUDY DESIGN

### Study 1, Formation of cohort of children with rIPD

A retrospective analysis was conducted using the National Streptococcus Pneumoniae Registry at the National Neisseria and Streptococcus Reference Center. Children aged 0–15 years with laboratory-confirmed cases of rIPD during Jan 1980–Dec 2013 were identified. Clinical data were obtained from hospital records and from the National Registry of Patients. The following data were collected: sex, age, background disease, localization of infection and outcome.

### Study 2, Follow-up of children with rIPD

In Study 2, a follow-up of the cohort of children described in Paper 1 was carried out. Of this unselected cohort of rIPD, all children without an obvious underlying disease predisposing to pneumococcal disease (such as acquired immunodeficiency or anatomical abnormalities) were invited to partici-

pate in an investigation study by having a blood sample taken and being screened for abnormalities in standard immunological parameters, including complement-, B-cell- and TLR signalling function (Table 5). In cases with positive family history of infections, all close relatives (parents, siblings and grandparents) were also invited to be tested for abnormalities in standard immunological parameters and DNA was extracted and stored. We offered to visit the families in their homes, to obtain the bloodsamples.

**Study 3** By comparing age-specific disease incidences of IPD in the pre-PCV7 (years 2000–2007) and the PCV7 periods (years 2008–2010), we sought to assess direct and indirect effects of general PCV vaccination on incidence of IPD. IPD incidence rates were estimated based on surveillance data on IPD and population data from Statistics Denmark using yearly population numbers. Age-specific IPD incidences were determined and by using the pre-PCV period (2000–2007) as a baseline, changes in IPD incidence rates were calculated as incidence rate ratios (IRRs).

## 3.3 LABORATORY INVESTIGATIONS

### Pneumococcal serotyping

All IPD isolates received at the NSR are routinely serotyped by using pneumotest latex and/or Quellung reaction using type-specific pneumococcal rabbit antisera from SSI (SSI-Diagnostica, Copenhagen, Denmark). In this serotyping assay the swelling (=quellung reaction) of the capsule of the pneumococcus is observed through a microscope after mixing the bacteria with different serotype specific antisera. The antisera are produced in rabbits immunized with killed pneumococci (11,13). Cases of serotype 6C have been distinguished from serotype 6A cases since 2007, but before 2007, 6A included both serotypes 6A and 6C.

### Pneumococcal serology

#### Response to the 23-valent polysaccharide pneumococcal vaccine

In children vaccinated with the PPV23 (Paper 1 and 2) data on the serotype-specific IgG response were accessed retrospectively. A standardized ELISA assay, including absorption with polysaccharide 22F and cell wall polysaccharide, was used to determine antibody concentrations. Concentrations of antibodies against six serotypes: 1, 4, 7F, 14, 18C, and 19F, and a total geometric mean for all six serotypes combined were determined. Based on these measures patients were categorized as having a normal response or an impaired response to the vaccine as described in detail previously (104–106). This ELISA assay is carried out at the Pneumococcal Serology Laboratory (SSI), which is the only laboratory in Denmark performing pneumococcal antibody testing.

#### Response to 7-valent conjugate vaccine

In children vaccinated with the PCV7, serum samples were assayed for individual antibodies included in the vaccine. The samples were analyzed by ELISA after absorption with polysaccharide 22F and cell wall polysaccharide and results were expressed as concentrations of antibody against each PCV serotype. A serotype specific antibody value of 0.35 µg/mL was considered protective. ELISA assays were carried

out at the Pneumococcal Serology Laboratory (SSI) as previously described (107, 108)

#### **Standard immunological parameters**

Plasma concentrations of immunoglobulin classes and – subclasses were determined at Statens Serum Institut, Denmark. B-, T- and NK-cell counts were performed in a single-platform assay on EDTA anticoagulated blood using anti-CD19, –CD20, –CD3, –CD4, CD8, –CD56 and –CD16 and TRUCount beads (BD Biosciences) and analyzed on a Beckman Coulter-FC500 flow cytometer at Department of Clinical Immunology, Rigshospitalet, Denmark. Age-matched controls were used as previously described (109).

#### **Complement analysis**

Screening of the classical, alternative and lectin complement pathways was performed using a commercially available solid-phase assay (Wielisa) as recommended by the manufacturer (Wieslab, Malmö, Sweden). In short, the wells of microtiter strips for classical pathway evaluation were pre-coated with IgM, strips for alternative pathway determination were coated with lipo-polysaccharide and Mannan-Binding-Lectin (MBL) pathway strips were coated with mannan. To ensure that only the intended pathway was activated on each microtiter strip, sera were diluted in buffers containing specific blockers of the other complement-activating pathways. Complement activation was detected by measuring the amount of C5b–9 terminal complex (TCC), with an antibody against a neoepitope expressed during TCC formation (110). Detection of individual complement components was performed in patients with reduced complement activation, using rocket immunoelectrophoresis as previously described (111). Cases with suspected complement C2 deficiency were genetically confirmed by investigation for the 28bp genomic deletion (112).

**MBL genotypes:** were determined as previously described (113).

#### **Somatic hypermutation**

The introduction of somatic hypermutations into the rearranged immunoglobulin genes of B cells occurs with a frequency around 100,000 times higher than the spontaneous mutation rate of immunoglobulin genes in other cell types (114). Nucleotide substitutions are most prevalent, but deletions, insertions and duplications occur as well (115). Some motifs (hot spots) are especially targeted by mutations. To define the level of affinity maturation of antibodies, somatic hypermutation in kappa light-chain transcripts was assessed using a V<sub>kappa</sub>A27-specific restriction enzyme-based hot-spot mutation assay (IgkappaREHMA) as previously described (116).

#### **Toll-like receptor function**

Toll-like receptor function was assessed in two different assays. In both assays full blood or human peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with different TLR receptor ligands to assess the function of the specific TLR receptor responding to the ligand (Table 4).

#### **TLR signalling monitored by analysis of the CD62L shedding**

Whole blood was stimulated as previously described by von

Bernuth et al. 2006 (118), with little modification in relation to the used agonists. Pam3csk48 (Invivogen) (TLR1/2), Pam2csk4(Invivogen)(TLT2/6),LPS (Invivogen) (TLR4), FLA-ST(Invivogen) (TL5), Imiquimod-R837(Invivogen) (TLR7), ssRNA40(LyoVec) (TLR8) CL097 (TLR7/8) 3 µg/mL (Invivogene by Sigma Aldrich) were used as agonists and PMS(Sigma-Aldrich) and TNFα(R&DSystems) as positive controls. Briefly, 100 µl heparinized blood was stimulated with agonists for TLR 1-2 and 4-8 and incubated at 37°C in a 5% CO2 atmosphere for 1 hour. Stimulation with PMA or TNFα was used as positive control. All (except the stimulation with LPS, which was not incubated with polymyxin) stimulation was proceeded after 20 minutes' incubation with Polymyxin B, 10 µg/mL. After activation, the erythrocytes were lysed in EasyLyse (Dako) and the whole blood cells were washed twice in PBS. The cells were incubated with anti-human CD62L or isotype control (BD Bioscience Pharmingen 555544 resp. 555749) for 20 min in 4°C and thereafter the receptor expression before and after stimulation was analyzed by flow cytometry using BD FACS Calibur and CellQuest pro (BD Bioscience) or Kaluza 1.2 (Beckman Coulter). The results were shown as histograms of events gated on granulocytes in a sideward scatter/forward scatter diagram (Paper 2).

#### **TLR signalling monitored by analysis of cytokine production**

Human peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep™ density gradient centrifugation according to the manufacturer's instructions (Nycom A/S). PBMCs were stimulated in duplicates with TLR 1-2 and 4-8 agonists as described above, and incubated at 37°C in a 5% CO2 atmosphere for 24 hours. After incubation, the supernatant was analysed for the presence of IL-6, and TNFα. Cytometric bead array (Invitrogene) was used to simultaneously measure the concentration of IL-6 and TNFα based on Luminex technology (119).

#### **TLR genetics**

Boys were screened for mutations in IKBKG and IKBA, and all included patients for mutations in IRAK4, and MYD88. Sequencing was done with BigDye technology and included the coding exons and consensus splice sites.

#### **3.4 STATISTICS**

In Paper 1, dichotomous variables were analysed using Fisher's exact test with 95% confidence intervals. (GraphPad Prism version 5 2007, San Diego, USA). Two-sided p-values <0.05 were considered statistically significant. Cytokine production between groups in Paper 2 was compared using Student's t-test or one way ANOVA, a P-value less than 0.05 was considered significant.

In Paper 3, age-specific IPD incidences were determined and by using the pre-PCV period (2000–2007) as a baseline and changes in IPD, incidence rates were calculated as incidence rate ratios (IRRs) with 95% confidence intervals (CIs). Two-sided p-values <0.05 were considered statistically significant.

#### **3.5 ETHICAL APPROVAL**

The Danish Data Protection Agency (Datatilsynet) approved the usage of the data in Study 1 and 2 (jr.n 2007-41-1407) and Study 3 (J.nr. 2011-41-6399). The local ethics committee

approved the protocol used in Study 2 (jr.n H-B-2008-094 and jr.n H-4-2010-004).

## RESULTS

### PAPER 1

In this paper we present results from a 33-year retrospective nationwide study of rIPD. We describe one of the largest known cohorts of children (n:59) with rIPD and cover epidemiological, microbiological, and clinical features of this clinical entity. During January 1980–June 2013 all children aged 0–15 years with laboratory-confirmed IPD were identified from the National Streptococcal Reference Center, Statens Serum Institut. Clinical data were obtained from hospital records and from the National Registry of Patients

**Results:** 2482 children were diagnosed with IPD with 75 episodes of rIPD observed in 59 (2.4%) children. Of all cases, 34 (58%) children experienced recurrent pneumococcal bacteraemia and 24 (41%) experienced one or more episodes of meningitis. In one patient the *S. pneumoniae* were isolated from the peritoneal cavity. Among the 59 children, 28 (47%) had a known predisposing underlying disease at the time of the rIPD, most common was immune deficiency due to transplantation. In 11 children (19%), the episode of rIPD was the clinical manifestation that subsequently led to a diagnosis of an underlying disease (complement deficiency n:4, dura deficiency n:4, congenital asplenia n:3). In 18 (31%) children, no underlying disease was detected. Vaccination data were available for 68% (n:40) of the cohort. Of the children eligible for pneumococcal vaccination, 14 (35%) received no vaccination. Among those vaccinated, one case of vaccine failure was documented. This child, with complement C2 deficiency and MBL deficiency, developed PCV-type IPD (serotype 14) despite being vaccinated twice with PCV7. In conclusion, an underlying disease was found in 66% of the children experiencing rIPD, supporting the notion that recurrent invasive infection in a child should prompt a thorough search for an underlying disease. Optimal pneumococcal vaccination of this group is essential and vaccination response of children with underlying conditions may be assessed on an individual basis.

### PAPER 2

Paper 2 covers data from a follow-up of the cohort of children with rIPD from Paper 1. Of this unselected cohort of rIPD, children without an obvious underlying disease predisposing to pneumococcal disease (such as malignancy, HIV or cerebrospinal-fluid leakage) were invited to participate in a follow-up study. The children and their families were screened for basic immunological parameters including activity of complement-pathways, T-, B-, NK-cell count. B-cell function including antibody response to polysaccharide-based pneumococcal vaccination and somatic hypermutation was evaluated. TLR function was screened using a panel of agonists.

**Results:** When children with classical risk factors for IPD were excluded, 15 were eligible. Of whom, 6 (40%) complement C2 deficient children were identified: 4 were diagnosed with C2 deficiency in the initial clinical investigation and 2 were diagnosed in our investigational follow-up. The age at diagnoses of the complement deficiency varied from 3 to 18 years and the mean duration from onset of symp-

toms to diagnosis was 5.5 years [range 1-13 years]. In addition, impaired vaccination response was found in 6 children: 3 with concurrent C2 deficiency and 3 with no other detectable immune abnormality. Two children had reduced SHM. One patient with a severe TLR signalling dysfunction was diagnosed. In 5 children, no underlying immunological condition was detected. In summary, in this nationwide study of rIPD, we found a surprisingly high prevalence of C2 deficient children. Moreover, three children with a selective antibody deficiency towards pneumococcal polysaccharides, and one patient with a severe TLR signalling dysfunction

**Figure 2**  
Somatic hypermutation vs. age in individuals experiencing repetitive IPD in childhood

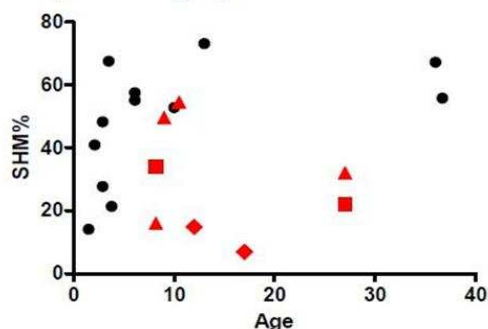


Figure 2 “Red dots”: persons with complement deficiency, of note; 2 complement deficient siblings without recurrent IPD are included (red square) diamond: persons with reduced SHM (pt 5 and pt 6, Table 2, Paper 2).

“Black dots”: the rest of the cohort of children with rIPD (all children enrolled in study 2 except complement deficient children)

Were identified. Our findings strengthen the assumption that two invasive episodes of infection, in the absence of background disease knowing to predispose to IPD, is a major pointer towards primary immune deficiency.

**Comment to SHM results:** At the moment, we have no age adjusted normal area for children. We are currently trying to establish such a normal area for young children in order to evaluate the children in this study and for future clinical use, when evaluating children under suspicion of B cell dysfunction. Among this rIPD cohort we found 3 children, all Complement C2 deficient, at the age of 8, 12 and 17 years with respectively 16%, 15% and 6.9% in SHM fraction, which are possibly reduced (Figure 2). Normally such patients are reexamined after 6 months of time to ensure that the SHM fraction has not stagnated. Re examination of SHM fraction was not possible in this project.

### PAPER 3

In Paper 3, we aimed to assess the impact of PCV7 in Denmark following the first three years of infant immunization. PCV7 was introduced in October 2007 in a 2+1 programme and including a catch-up programme for children up to 17 months of age. By comparing age-specific disease incidences of IPD in pre-PCV (years 2000–2007) and PCV periods (years 2008–2010) on a population level, we sought to assess direct and indirect effects of PCV7 vaccination. In addition, changes

in pneumococcal serotype distribution and IPD-related mortality were analysed. Data were retrieved from the National Streptococcus Pneumoniae Registry and the Danish National Vaccination Registry.

**Results:** We documented a marked decline in incidence of IPD in both vaccinated and non-vaccinated age groups. In children aged 0–5 years the overall incidence of IPD decreased from 26.7 to 16.3 cases per 100,000 (IRR 0.58; 95% Confidence Interval (CI) [0.48-0.69]) and case fatality declined from 1.8% (12 deaths) in the eight-year pre-PCV period to 0% (no deaths) in the three-year PCV period. In the whole population the overall incidence of IPD declined significantly from 19.5 to 17.7 and from 7.7 to 3.8 cases per 100,000 persons when comparing the two periods. A minor but statistically significant increase in incidence of IPD due to non-vaccine type IPD was observed in both vaccinated and non-vaccinated groups but with predominance of the serotypes 1,7F and 19A, which are covered by higher valence pneumococcal conjugate vaccines. In conclusion, after 3 years of PCV vaccination in the childhood immunization programme, we report a substantial effect of the 2 + 1 schedule used in Denmark, both as a direct effect in vaccinated children with a marked reduction in incidence of IPD and also as an indirect effect with an important decline in vaccine serotype IPD burden in unvaccinated age groups. A minor but significant increase in non-vaccine serotype IPD was observed.

## DISCUSSION

### *Underlying disease in children experiencing rIPD*

In our nationwide study of children with rIPD, we found that 87% of the children had a predisposing condition that could explain the recurrence. Most common was immunodeficiency related to transplantation. In 50% of the cases the child had a known underlying disease at the time of the recurrence (Paper 1), but in the rest of the cases (37%) the recurrent infection was the first serious manifestation of an underlying disease (cerebrospinal fluid (CSF) leakage, complement deficiency, asplenia congenitalis, specific antibody deficiency towards polysaccharides, TLR signalling deficiency (Paper 1 and 2)). This emphasizes the importance of searching for an underlying disease in an apparently healthy child experiencing rIPD.

Our findings on underlying conditions predisposing to rIPD are in line with those of other studies. Mason et al published a study of 90 children with rIPD, with 79% of the children having predisposing factors, of which the most common was HIV (18% of all children) followed by renal disease and central nervous system disease. Among other studies asplenia and CSF leakage are common (Table 3), but primary immune deficiencies (PID) also account for a substantial proportion of cases.

Around one third of the published cases on rIPD (Table 3) occur in apparently healthy children. As discussed in Paper 2 our study was the only one aiming to investigate these cases systematically by inviting the children to a subsequent immunologically follow-up. It seems plausible that cases of subtle immune deficiencies could have been missed in other studies.

It has been shown that single gene mutations in key molecules (IRAK4, MYD88, IKBA, and NEMO) of the TLR signaling pathways play an important role as predisposing factor in children with rIPD. We investigated an unselected population predicted to be enriched for TLR defects and found no mutations, suggesting that these mutations are rare in a Danish setting. Nevertheless, one patient in our cohort, who experiencing 5 episodes of IPD (Paper 2), had a severe TLR signalling dysfunction. Further genetic evaluation of this child and his relatives is ongoing.

Infectious diseases in childhood are extremely frequent, and in a clinical setting it is a challenge to determine which child should be investigated for rare conditions such as impaired complement function and other inborn defects of the immune system. Our findings reported in Paper 2 in addition to publications by others (49, 51–54) strengthen the assumption that two invasive episodes of infection, in the absence of background disease knowing to predispose to IPD, is a major pointer towards primary immune deficiency. This is in line with guidelines put forward by various specialist committees (125, 126). On the basis of findings in this PhD thesis and other studies, we formulated a proposal of how children with repetitive invasive infections could be examined (Fig 3).

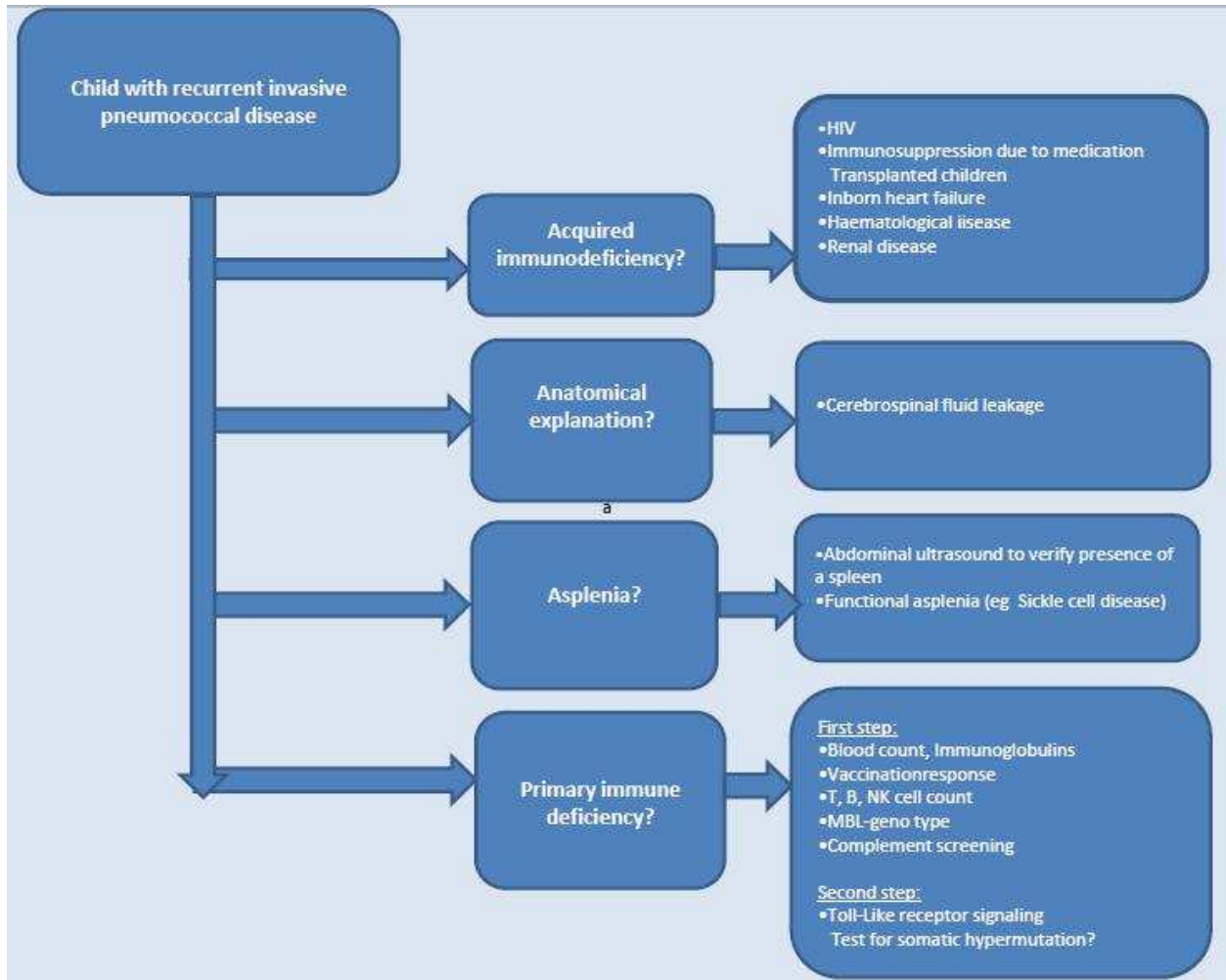
### *Pneumococcal vaccination response*

An important factor for the pathogenesis of pediatric IPD is the high prevalence of nasopharyngeal colonization with *S. pneumoniae*. Furthermore antibody response to polysaccharide antigens is impaired in young children less than two years of age, which also explain why these young patients are predisposed to episodes of IPD. If this non-responsiveness to polysaccharides persist over the year of two it becomes an abnormality. We identified three children with rIPD, who all had this disturbance including a family with three brothers (Paper 2).

Nonresponsiveness to pneumococcal polysaccharides may be the only detectable immune abnormality or it may be associated with IgG2 subclass deficiency or other B-cell disturbances (127, 128). The prevalence of this selective immunodeficiency is not known, but it is common finding in some groups of children (127, 128). A defective immune response to polysaccharide antigens in patients requires long-term follow-up to distinguish transient maturational delay from a persistent selective impaired response to polysaccharide antigens, which on occasion may precede the development of humoral immunodeficiency disease, such as Common Variable Immunodeficiency (129).

In addition to the above mentioned children with impaired vaccination response, we observed that three out of six C2 deficient patients had an impaired response to pneumococcal polysaccharides, which might be an additional explanation for these particular C2 deficient patients to experience rIPD. Moreover, one C2 deficient experienced a case of PCV7-VT-IPD (bacteraemia due to serotype 14) in spite of being vaccinated with two doses of PCV7 (paper 2). This child had antibodies in normal ranges to all PCV7 serotypes including type 14 which were also found to be functional in the OPA assay (data not presented). This finding illustrates that serotype-specific correlates of protection cannot always predict the level of protection at the individual level especially not in groups of patients with immunodeficiency.

Figure 3 A proposal of how to handle children with recurrent invasive pneumococcal disease



### STRENGTHS AND LIMITATIONS

Our cohort of children with rIPD used in Paper 1 and 2 was established on the basis of high quality data from nationwide registration of IPD, which made it possible for us to collect information on a cohort of rIPD that was much larger than in most other similar studies (Table 3). Secondly, it was a nationwide study of an unselected population, and thirdly our follow-up period was long (33 years), making it possible to include children with recurrences separated by many years. To our knowledge, our study of prevalence of immune defects in an unselected population of children with rIPD has not been done before (Paper 2). The retrospective design made it impossible to document transient immunological abnormalities at the time of the IPD, which could have further improved our understanding of the pathogenesis in young, apparently healthy children experiencing rIPD (Paper 2). Our population size is small, which is a limitation. It would be interesting to confirm our results on complement

deficiency and B-cell deficiency in a larger population from another country. A selection bias may be present in all three

studies because we used a highly specific case definition including only culture confirmed IPD cases in a setting where blood- and CSF-cultures are almost exclusively obtained from hospitalized patients. Furthermore, we were not able to investigate the immunological status of all children experiencing rIPD (Paper 2), which could have contributed further to the understanding of the underlying mechanisms. All cases were laboratory-confirmed and isolates were serotyped according to international standards; nevertheless, owing to the retrospective design, we were not able to characterize our isolates molecularly with MLST or PFGE, which might have shown interesting strain differences among single and recurrent episodes (Paper 1). The Danish population is small, which makes it difficult to analyse changes in IPD incidence on a serotype specific level (Paper 3) following PCV implementation. However, an important

strength of Study 3 is the comprehensive data on IPD in Denmark from the Pre-PCV period.

## 7. CONTRIBUTION AND PERSPECTIVES

### 7.1 Contribution

In the present PhD thesis we contributed with the following:

- Confirming the existing knowledge on which background diseases in children predispose to IPD.
- Finding a surprisingly high frequency of complement deficient children in our follow-up of children with rIPD, highly underlining the importance of screening for complement deficiencies in patients with more than one episode of IPD.
- Documenting that impaired vaccination response to pneumococcal polysaccharides is a frequent phenomenon in selected groups of children experiencing repetitive infections. This implies that vaccination and vaccination response of frail children and children with underlying conditions should be assessed on an individual basis.
- Documenting that TLR function can be easily tested in a cytokine-based assay as supplement or in place of the CD62L shedding assay.
- Reporting findings from the first three years of PCV7 vaccination in the Danish immunization programme, suggesting that the vaccine is effective against all serotypes included in the vaccine when administered in a 2+1 schedule.

### 7.2 Future studies

It is well established that certain groups of children run a very high risk of IPD, and our findings confirm previous reports on background diseases predisposing to IPD. We did, however, find a surprisingly high prevalence of complement C2 deficient children in our cohort. It would be of great interest to confirm our findings of complement deficiencies in another population of children with rIPD (for example in one of our Scandinavian neighbour countries, where similar registry facilities exist) or in other clinically defined groups of patients (children experiencing repetitive pneumonia or repetitive invasive episodes with other encapsulated microbes).

Additionally, to our knowledge, our observational finding on impaired somatic hypermutation in complement patients (Paper 2, Figure 2) has not been reported before. We are currently planning to address this in more detail in a larger cohort of complement deficient patients.

Although primary immune deficiencies seem to be rare, the prevalence of many of these conditions is unknown. Denmark has a unique tradition of central registration of diseases and the personal identification number, which is linked uniquely to every inhabitant, makes it possible to follow-up persons over a long time. It would be interesting to use these pathogen-specific registries to determine the prevalence of immune variations in other clinically defined groups of patients, such as patients with atypical mycobacteria or repetitive herpes simplex viral infections.

### 7.3 Views on preventing IPD in children

The implementation of highly effective conjugate vaccines in childhood immunization programmes has had a marked effect on child morbidity in many countries (28). Continued surveillance is important to evaluate the benefits of vaccination and also to monitor changes in non-vaccine pneumococcal serotypes (NVT). The PCV induced removal of vaccine-type (VT)-pneumococci from the nasopharynx opens a niche for NVT-pneumococci and other bacteria in general (130, 131). These emerging non-vaccine serotypes can cause IPD and have been observed in a number of countries following PCV introduction, partly neutralizing the benefits of PCV implementation (132-133). Some vaccine-escape serotypes may have altered virulence profiles and reduced susceptibility to standard antibiotics. This could have clinical implications in both normal and frail children in the future, illustrating the needs for continual development in the vaccine field. Moreover, it underlines why a clinician should always be extra cautious in handling patients with known predispositions to IPD, also in the era of PCV vaccination.

Optimal pneumococcal vaccination of risk groups with combination regimes of vaccines to broaden serotype coverage seems rational. Consequently, it will be of importance to evaluate the benefits of combination vaccines in different subpopulations of immune deficient children and adults.

### DEFINITIONS

**IPD**, A case of IPD was defined as a positive culture of *S. pneumoniae* from cerebrospinal fluid (CSF), blood or another normally sterile clinical sample site. If both CSF and blood isolates were received for a case, the case was categorized as pneumococcal meningitis.

**rIPD**, A recurrent episode of IPD was defined as isolation of any *S. pneumoniae* from any normally sterile site  $\geq 30$  days after initial positive culture or  $\leq 30$  days if the recurrent infection was with a new pneumococcal serotype.

**Case fatality**, Death was considered to be related to IPD when the date of death was within 30-days after the clinical sample date from the patient. Vital status of patients with IPD was obtained through the Danish Civil Registration System (CRS) (Paper 1 and 3) and verified in the clinical journals (Paper 1).

**Vaccine type-IPD (VT-IPD)**, IPD caused by one of the pneumococcal serotypes included in PCV7: 4, 6B, 9V, 14, 18C, 19F, and 23F

**Non-Vaccine type-IPD (NVT-IPD)**, IPD caused by one of the pneumococcal serotypes not included in PCV7. See details above.

**PCV-Failure**, A case of PCV vaccine failure was defined as a case of VT-IPD in a vaccinated child, with IPD onset more than two weeks after complete primary immunization with two vaccine doses (1).

**PPV23 non-responder**, Of all 23 serotypes included in the PPV23, the concentrations of antibodies against six serotypes: 1, 4, 7F, 14, 18C, and 19F were determined (more details laboratory investigations page 24). Children with an impaired response to more than half of the six tested serotypes were defined as being non-responders (2-4).

**PPV23 responder**, A child with a response over threshold (1.3 mcg/ml) to half or more of the six tested serotypes was defined as being a PPV23 responder (2-4).

**SUMMARY**

*Streptococcus pneumoniae* is still a leading cause of septicaemia, pneumonia and meningitis in young children worldwide with over half a million children dying annually from pneumococcal disease. Some children are prone to repeated episodes of invasive pneumococcal disease (IPD) because of an underlying predisposing disease. Recurrent IPD (rIPD) is a rarity and published reports on rIPD are limited by having few children included, selected groups of patients or short follow-up periods. Deficiencies in the innate or adaptive immune system have been described in children with rIPD, but the frequency of immunodeficiency among such patients is unknown.

The aim of this PhD thesis was to examine paediatric cases of laboratory-confirmed rIPD, over a 33-year period in Denmark, to determine risk factors and study aspects of the immunological background for this problem in children. In October 2007, a seven-valent pneumococcal conjugate vaccine (PCV7) was implemented in the Danish infant immunization programme. An additional aim of the thesis was to examine the impact of vaccination on a population level, following the first three years of general PCV7 vaccination in Denmark.

The thesis consists of three papers, which are all directly or indirectly based on data retrieved from the National *Streptococcus Pneumoniae* Registry. This registry is nationwide and dates back to 1938. The registry contains data from all laboratory-confirmed cases of IPD in Denmark and is continually updated for national surveillance.

In Paper 1, we conducted a 33-year retrospective nationwide study of paediatric rIPD. By using data from the National *Streptococcus Pneumoniae* Registry combined with clinical data from hospital records, we could describe one of the largest known cohorts of children (n:59) with rIPD. We covered epidemiological, microbiological, and clinical features of this clinical entity. Of all children experiencing rIPD, 47% had a known predisposing underlying disease at the time of the rIPD. Most common was immune deficiency due to transplantation. In 19% the episode of rIPD was the clinical manifestation that subsequently led to a diagnosis of an underlying disease. Finally, in 31% of the children no underlying disease was detected.

Paper 2 covers data from a follow-up of the cohort of children described in Paper 1. Of this unselected cohort of rIPD, all children without an obvious underlying disease predisposing to pneumococcal disease (such as malignancy, HIV or

cerebrospinal-fluid leakage) were invited to participate in the study by undergoing a thorough immunological evaluation. Basic immunological parameters including activity of complement-pathways and T-, B-, NK-cell count were examined in the children and their families. Furthermore, B-cell function including antibody response to polysaccharide-based pneumococcal vaccination and somatic hypermutation was evaluated. Toll like receptor (TLR) signalling function was evaluated in a functional assay. When children with classical risk factors for IPD were excluded, 15 individuals were eligible. Of whom, 6 (40%) children with complement C2 deficiency were identified. Moreover, impaired vaccination response was found in 6 children: 3 with concurrent C2 deficiency and 3 with no other immune abnormality. One patient with a severe TLR signalling dysfunction was diagnosed.

In Paper 3, we aimed to assess the impact of PCV7 in Denmark following the first three years of infant immunization. By comparing age-specific disease incidences of IPD in the pre-PCV7 (years 2000–2007) and the PCV7 periods (years 2008–2010) we sought to assess direct and indirect effects on incidence of IPD. In addition, changes in pneumococcal serotype distribution and IPD-related mortality were assessed. We documented a marked decline in the incidence of IPD in both vaccinated and non-vaccinated age groups. The overall incidence of IPD among children aged 0–5 years declined from 26.7 to 16.3 cases per 100,000 (IRR 0.58; 95% Confidence Interval (CI) [0.48-0.69]). A minor but statistically significant increase in the incidence of IPD due to non-vaccine type IPD was observed in both vaccinated and non-vaccinated groups, but with predominance of serotypes covered by the higher valence pneumococcal conjugate vaccines.

This thesis confirms the existing knowledge on underlying diseases predisposing children to IPD, such as cerebrospinal fluid leakage, congenital heart disease and malignant diseases. Our findings support the notion that rIPD in a child should prompt a thorough search for an underlying disease. Moreover, our results underline that rIPD in a child without a known predisposing disease is a major pointer towards primary immune deficiency, such as complement deficiency and B cell dysfunction. This is in line with the guidelines put forward by various specialist committees. Finally, we reported data from the first three years of PCV7 vaccination in the Danish immunization programme, suggesting that the vaccine is effective against all serotypes included in the vaccine when administered in a 2+1 schedule.

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