Research Paper

# Genetic Variation in *NFKBIE* Is Associated With Increased Risk of Pneumococcal Meningitis in Children

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## ARTICLE INFO

## ABSTRACT

*Background:* Streptococcus pneumoniae and Neisseria meningitidis are frequent pathogens in life-threatening infections. Genetic variation in the immune system may predispose to these infections. Nuclear factor-κB is a key component of the TLR-pathway, controlled by inhibitors, encoded by the genes *NFKBIA*, *NFKBIE* and *NFKBIZ*. We aimed to replicate previous findings of genetic variation associated with invasive pneumococcal disease (IPD), and to assess whether similar associations could be found in invasive meningococcal disease (IMD).
*Methods:* Cases with IPD and IMD and controls were identified by linking Danish national registries. DNA was obtained from the Danish Neonatal Screening Biobank. The association between SNPs and susceptibility to IPD and IMD, mortality and pneumococcal serotypes was investigated.
*Results:* 372 children with pneumococcal meningitis, 907 with pneumococcal bacteremia and 1273 controls were included. We included 406 cases with meningococcal meningitis, 272 with meningococcal bacteremia, and 672 controls.
The *NFKBIE* SNP was associated with increased risk of pneumococcal meningitis (aOR 1.68; 95% CI: 1.20–2.36), but not bacteremia (aOR 1.08; 95% CI: 0.86–1.35). The remaining SNPs were not associated with susceptibility to invasive disease. None of the SNPs were associated with risk of IMD or mortality.
*Conclusions:* A *NFKBIE* polymorphism was associated with increased risk of pneumococcal meningitis.

## 1. Introduction

Invasive pneumococcal disease (IPD) and invasive meningococcal disease (IMD) are major causes of morbidity and mortality (WHO|Pneumococcal disease; WHO|Meningococcal disease). Pneumococcal disease remains a frequent infection in children, and in 2005 WHO estimated that 0.7–1 million children aged

<5 years die of this infection every year worldwide (http://www.who.int/wer/2007/wer8212.pdf?ua=1). For meningococcal disease the incidence is highest in children younger than 1 year (MacNeil et al., 2015), and WHO has estimated that the infection was the cause of 171,000 deaths in all age groups worldwide in 2000 (Pinkbook|Meningococcal|Epidemiology of Vaccine Preventable Diseases|CDC).

The most severe presentations of infection with *S. pneumoniae* and *N. meningitidis* are meningitis and sepsis (Brouwer et al., 2009).

IPD is often preceded by an asymptomatic carrier state (Bogaert et al., 2004). Nasopharyngeal carriage of pneumococci is highest in children aged 1–2 years, and IPD primarily affects children under 5 years and the elderly (Harboe et al., 2012; Sleeman et al., 2001). Carriage of *N. meningitidis* is low in the first years of age, increases in teenagers and peaks in young adults aged 20–24 (Caugant et al., 2007). Multiple factors such as age, immunization status, ethnicity, immunosuppression, socioeconomic factors, exposure to respiratory viral diseases and

the host's genetic profile affect individual risk of IPD and IMD (Harboe et al., 2012; Chapman et al., 2007). Genetic variation in the innate immune system most likely predisposes individuals to these infections. The genetics of the complicated pathways have been elucidated only partially.

Activation of nuclear factor (NF)-κB through Toll-like receptor binding is considered to be the central initiating event of host responses to invasion of microbial pathogens, including encapsulated bacteria (Rahman and McFadden, 2011; Clausen et al., 2013). Innate and adaptive immune responses are dependent on activation of the NF-κB pathway (Janssen et al., 2004). The degradation of NF-κB inhibitors including IκB-α, IκB-ε and IκB-ζ (encoded by the genes *NFKBIA*, *NFKBIE*, and *NFKBIZ*) (Chapman et al., 2010a) leads to NF-κB translocation to the nucleus and gene transcription (Chapman and Hill, 2012). Single nucleotide polymorphisms (SNPs) in *NFKBIA*, *NFKBIE*, *NFKBIZ* and related genes have been associated with susceptibility to IPD in adults (Chapman et al., 2006, 2007, 2010a, 2010b; Khor et al., 2007).

Studies exploring already described associations between certain SNPs and the susceptibility to diseases are important in order to verify those associations in new, independent studies (Tabor et al., 2002).

This study evaluates the association of SNPs in NF-kB inhibitors, and susceptibility to IPD and pneumococcal serotypes in a pediatric population (Chapman et al., 2006, 2007, 2010a, 2010b).

To assess pathogen-specificity, we genotyped the same SNPs in a meningococcal population. We hypothesized that the association between susceptibility of pneumococcal meningitis and the chosen SNPs may be replicated in a population with IMD.

## 2. Patients and Methods

### 2.1. Study Population

The collection of IPD and IMD data has been described previously (Lundbo et al., 2014, 2015).

Individuals with IPD and IMD were identified by linking the Danish National Neisseria and Streptococcus Reference Center, Statens Serum Institute, the Danish National Patient Register (DNPR) (Lynge et al., 2011) and the Danish Civil Registration System (CRS) (Schmidt et al., 2014).

All IPD and IMD cases and controls included in the study were born in the 1982–2006 period, and the data was pulled in May 2009.

IPD cases were defined as children under the age of five, from whom a positive culture of *S. pneumoniae* from cerebrospinal fluid (CSF) or blood was obtained between July 1982 and April 2008. Only unvaccinated children were included. When CSF and blood isolates were recovered simultaneously, cases were categorized as meningitis. Bacteremia refers to patients with or without a known focus. Information on foci is not available through the Danish National Neisseria and Streptococcus Reference Center or the DNPR. Isolates were serotyped as previously described (Lundbo et al., 2014; Harboe et al., 2010). Recurrent (≥two) IPD episodes were defined as isolation of *S. pneumoniae* from blood or CSF ≥ 30 days after the primary positive culture or ≤30 days in cases of infection with another serotype.

IMD cases were children below the age of five years who had invasive meningococcal disease in the period 1982–2006.

The first date of the meningococcal bacteremia or meningitis diagnosis for each case was extracted from the Danish National Patient Registry (DNPR) using International Classification of Diseases, 8th Revision [ICD-8] (036 Meningococcal infection; 036.0 Meningococcal meningitis; 036.1 Meningococcemia without mention of meningitis) and 10th Revision [ICD-10] codes (A39.0 + Meningococcal meningitis (G01); A39.2 Acute meningococcemia; A39.3 Chronic meningococcemia; A39.4 Meningococcemia, unspecified) (Lynge et al., 2011).

We aimed to include one control per case of IPD and IMD. Controls could only be included in the analysis once.

For IPD and IMD cases with meningitis, we identified controls through the CRS.

For cases with bacteremia, controls were obtained from the Danish Neonatal Screening Biobank (DNSB) (Nørgaard-Pedersen and Hougaard, 2007) by selecting the same-sex dried blood spot card stored nearest to that of the case. Cases and controls with a prior hospitalization for any cause were excluded. Only cases and controls who themselves and whose parents were born in Scandinavia or Germany were included. We used the risk-set sampling technique to select controls (i.e., eligible control subjects had to be alive and at risk of a first hospitalization with IPD on the date that the corresponding case was hospitalized) (Navidi and Weinhandl, 2002).

The 7-valent pneumococcal conjugate vaccine was introduced into the Danish Childhood Immunization Program in October 2007. Meningococcal vaccines are not part of the Danish childhood immunization program (Childhood vaccination programme – Statens Serum Institut).

### 2.2. DNA Extraction and Genotyping

All samples from the Danish neonatal screening program are stored in the DNSB (Nørgaard-Pedersen and Hougaard, 2007).

DNA extraction and genome amplification were performed by the Department of Clinical Biochemistry, Immunology and Genetics at SSI, as previously described (Hollegaard et al., 2011).

Candidate SNPs (Tables 3 and 4) were chosen through a literature review based on known host genetic variation of susceptibility for IPD (Chapman et al., 2006, 2007, 2010a, 2010b; Khor et al., 2007).

All SNP genotyping was performed by LGC Genomics (LGC Ltd., Teddington, Middlesex, United Kingdom) using competitive allele-specific PCR (Nijman et al., 2008).

### 2.3. Statistical Analysis

Median and interquartile ranges (IQRs) were calculated for quantitative variables. Genotypes in each group were compared using $\chi^2$ and Fisher's exact test. Logistic regression, adjusted for sex, was performed to examine a possible association between genotypes and IPD risk, outcome and serotype distribution. The results are presented as adjusted odds ratios (aORs) with 95% confidence intervals (CIs). Genotype equilibrium was tested using the Hardy–Weinberg method (Rodriguez et al., 2009). The significance level was set at $p < 0.008$ (0.05/6) due to multiple comparisons (seven SNPs of which two were in linkage disequilibrium (LD)).

The dominant model used in several of our calculations describes a comparison of variant allele carriers (variant homozygotes and heterozygotes) with non-carriers (wild type homozygotes) (Clarke et al., 2011).

Analyses were performed using Statistical Analysis Systems (SAS version 9.3, SAS institute, Cary, NC, USA).

## 3. Results

### 3.1. Subjects

Characteristics of cases and controls are shown in Tables 1 and 2.

Children with IPD were significantly younger than children with IMD (median 13 vs 19 months, $p < 0.0001$).

We genotyped seven SNPs, previously described to be associated with susceptibility to IPD (Table 3). Genotype call rates were >95%, except for rs760477 (call rate of 94% for IPD and call rate of 91% for IMD).

All SNPs in the control subjects were in the Hardy–Weinberg Equilibrium (HWE) at the adjusted significance level ($p > 0.01$) (Rodriguez et al., 2009). However, in the IPD population a few of the control groups were not in HWE at the $p < 0.05$ level.

**Table 1**
Characteristics of invasive pneumococcal disease cases and their controls.

| Group | Status, n (%) | Male sex, n (%) | Age at infection (months), median (IQR) | Birth year, median (IQR) |
|---|---|---|---|---|
| Meningitis | Control (362, 49) | 204 (56) | – | 1994 (1989–2000) |
| | Case (372, 51) | 209 (56) | 10 (6–15)[a] | 1995 (1990–2000) |
| Bacteremia | Control (901, 50) | 533 (59) | – | 1997 (1992–2002) |
| | Case (907, 50) | 537 (59) | 14 (10–21)[a] | 1997 (1992–2002) |
| Combined | Control (1263, 50) | 737 (58) | – | 1996 (1991–2002) |
| | Case (1279, 50) | 746 (58) | 13 (8–19) | 1996 (1991–2002) |

[a] Kruskal–Wallis test for difference in age p < 0.0001.

### 3.2. Primary IPD

Before Bonferroni correction, genotypic and allelic tests of the *NFKBIE* SNP showed an association with increased susceptibility to meningitis (but not bacteremia) when children carried variant alleles (p = 0.0001 and p = 0.036, respectively).

Results for the dominant model are shown in Table 3.

As well, heterozygosity for *NFKBIE* in the meningitis group was associated with increased risk of IPD compared to homozygosity (wild type GG combined with the variant AA) (OR 1.96; 95% CI 1.38–2.78). Testing of the heterozygous state vs. the homozygous variant (OR 0.23; 95% CI 0.05–1.07) and heterozygosity vs. wild type homozygosity (OR 1.90; 95% CI 1.34–2.71) indicated an increased risk of pneumococcal meningitis among children carrying the variant genotype.

None of the SNPs in NFKBIA, NFKBIZ, PTPN22, TIRAP, or TONSL reached the p < 0.008 significance level (Table 3).

Pneumococcal capsular serotypes were available for 1276 of the IPD cases (99·8%). In the meningitis group the most frequent serotypes were 6B (n = 84, 23%), 14 (n = 47, 13%), 7F (n = 39, 10%), 19F (n = 33, 9%), 18C (n = 30, 8%) and correspondingly in the bacteremia group 14 (n = 179, 20%), 6B (n = 135, 15%), 1 (n = 85, 9%), 18C (n = 73, 8%), 19F (n = 63, 7%). Serotype distribution has previously been described (Lundbo et al., 2014).

Among cases the serotype distribution did not differ significantly according to genotypes. Serotype 23F was relatively more frequent in individuals, who were heterozygous for both *NFKBIA* SNPs (p = 0.03 respectively 0.03) in the combined group. In both the meningitis group and in the combined group serotype 6A was more frequent in individuals carrying variant alleles (for rs3138053: p = 0.01 respectively 0.01 and for rs2233406 p = 0.04 and p = 0.048). These effects were not statistically significant after Bonferroni correction.

### 3.3. Recurrent IPD

Twelve individuals had multiple episodes of bacteremia. For *NFKBIA*, rs2233406, subjects carrying at least one mutant allele vs. wild type homozygous state had higher odds for multiple infections compared to single cases of IPD (OR 5.2; 95% CI, 1.1–24.0, p = 0.03); for rs3138053 (OR 5.1; 95% CI, 1.1–23.4, p = 0·04). When comparing multiple cases to all controls, the corresponding ORs were 5.5 (95% CI 1.2–25, p = 0.03) for rs2233406 and 5.5 (95% CI 1.2–25, p = 0.03) for rs3138053. When corrected according to the Bonferroni method, the p-values became insignificant.

Polymorphisms in the remaining SNPs studied were not associated with increased risk of recurrent IPD.

### 3.4. IPD-associated Mortality

Twenty-four cases (2%) (15 cases of meningitis and nine of bacteremia) died within the first 30 days after their IPD diagnosis. None of the examined polymorphisms were associated with increased 30-day mortality in meningitis cases [for NFKBIE, rs529948 (in a dominant model): OR 0.94; 95% CI 0.28–3.14] or in bacteremia cases [for NFKBIE, rs529948 (in a dominant model): OR 0.34; 95% CI 0.05–3.21] for any SNPs.

### 3.5. IMD

No SNPs were associated with increased risk of IMD at the p < 0.008 significance level. Results for the dominant model are shown in Table 4.

During the first 30 days after their IMD diagnosis, 26 (6%) of the meningitis patients and 18 (7%) of the bacteremia patients died. In tests for association between mortality and SNPs, rs3138053 and rs2233406 (both for meningitis and the whole population) in a dominant model yielded p-values of 0.02–0.05. However, the associations became insignificant when adjusted for multiple testing. No other SNPs were associated with increased mortality.

## 4. Discussion

In this study of children with invasive bacterial disease, we found an association between a polymorphism in the *NFKBIE* gene and increased susceptibility to IPD. This association was specific for the risk of pneumococcal meningitis. A number of other previously described associations between IPD and the SNPs, mainly in adults, were not replicated in our population of children. We did not find an association between any of the SNPs and IMD, neither with an increased risk for 30-day mortality after IPD or IMD.

The pathogenesis and pathophysiology of bacterial meningitis involve a complex interplay between virulence factors characterizing the pathogens and the host immune response. The exact role of *NFKBIE* in this process is unknown, but our findings may suggest that IκB-ε is of greater importance in more disseminated infections, compared to the other IκB inhibitor proteins included in this study. Our inability to replicate the results in our bacteremia population may suggest that the host defense against pathogens crossing the blood–brain barrier is compromised in patients carrying a *NFKBIE* polymorphism.

**Table 2**
Characteristics of invasive meningococcal disease cases and their controls.

| Group | Status, n (%) | Male sex, n (%) | Age at infection (months), median (IQR) | Birth year, median, (IQR) |
|---|---|---|---|---|
| Meningitis | Controls: 397 (49) | 233 (59) | – | 1994 (1990–1998) |
| | Cases: 406 (51) | 237 (58) | 19 (8–32)[a] | 1994 (1990–1998) |
| Bacteremia | Controls: 275 (50) | 149 (54) | – | 1992 (1987–1996) |
| | Cases: 272 (50) | 145 (53) | 20 (9–32)[a] | 1991 (1987–1996) |
| Combined | Controls: 672 (50) | 381 (57) | – | 1993 (1989–1997) |
| | Cases: 678 (50) | 382 (56) | 19 (9–32) | 1993 (1989–1997) |

[a] Kruskal–Wallis test for difference in age p = 0.38.

**Table 3**
Single nucleotide polymorphism frequencies in invasive pneumococcal disease cases and their controls.

| SNP | Status | AA[a] | AB[a] | BB[a] | Total | HWE (controls) p-value | Genotypic p-value[b] | Odds ratio (95% CI)[c] |
|---|---|---|---|---|---|---|---|---|
| NFKBIE | Meningitis | | | | | | | |
| rs529948 | Control | 284 (79) | 66 (18) | 10 (3) | 360 | 0.01 < p < 0.02[d] | 0.0001 | 1.68 (1.20–2.36) |
| G → A | Case | 245 (69) | 108 (30) | 2 (1) | 355 | | | |
| | Bacteremia | | | | | | | |
| | Control | 687 (78) | 184 (21) | 15 (2) | 886 | p > 0.05 | 0.78 | 1.08 (0.86–1.35) |
| | Case | 646 (76) | 188 (22) | 14 (2) | 848 | | | |
| | Combined | | | | | | | |
| | Control | 971 (78) | 250 (20) | 25 (2) | 1246 | p > 0.05 | 0.01 | 1.24 (1.03–1.49) |
| | Case | 891 (74) | 296 (24) | 16 (1) | 1203 | | | |
| NFKBIA | Meningitis | | | | | | | |
| rs3138053 | Control | 183 (52) | 146 (41) | 25 (7) | 354 | p > 0.05 | 0.93 | 1.04 (0.78–1.40) |
| T → C | Case | 177 (51) | 146 (42) | 27 (8) | 350 | | | |
| | Bacteremia | | | | | | | |
| | Control | 468 (53) | 349 (39) | 68 (8) | 885 | p > 0.05 | 0.43 | 1.10 (0.91–1.33) |
| | Case | 425 (50) | 358 (42) | 60 (7) | 843 | | | |
| | Combined | | | | | | | |
| | Control | 651 (53) | 495 (40) | 93 (8) | 1239 | p > 0.05 | 0.51 | 1.09 (0.93–1.28) |
| | Case | 602 (50) | 504 (42) | 87 (7) | 1193 | | | |
| NFKBIA | Meningitis | | | | | | | |
| rs2233406 | Control | 183 (51) | 148 (41) | 27 (8) | 358 | p > 0.05 | 0.98 | 1.0 (0.74–1.34) |
| G → A | Case | 179 (51) | 146 (42) | 25 (7) | 350 | | | |
| | Bacteremia | | | | | | | |
| | Control | 468 (53) | 347 (39) | 72 (8) | 887 | p > 0.05 | 0.46 | 1.07 (0.88–1.29) |
| | Case | 434 (51) | 354 (42) | 60 (7) | 848 | | | |
| | Combined | | | | | | | |
| | Control | 651 (52) | 495 (40) | 99 (8) | 1245 | p > 0.05 | 0.51 | 1.05 (0.89–1.23) |
| | Case | 613 (51) | 500 (42) | 85 (7) | 1198 | | | |
| Mal/TIRAP | Meningitis | | | | | | | |
| rs8177374 | Control | 261 (73) | 92 (26) | 4 (1) | 357 | p > 0.05 | 0.53 | 0.94 (0.67–1.31) |
| C → T | Case | 259 (74) | 82 (24) | 7 (2) | 348 | | | |
| | Bacteremia | | | | | | | |
| | Control | 651 (74) | 220 (25) | 14 (2) | 885 | p > 0.05 | 0.70 | 1.03 (0.83–1.27) |
| | Case | 619 (73) | 211 (25) | 18 (2) | 848 | | | |
| | Combined | | | | | | | |
| | Control | 912 (73) | 312 (25) | 18 (1) | 1242 | p > 0.05 | 0.47 | 1.00 (0.84–1.20) |
| | Case | 878 (73) | 293 (25) | 25 (2) | 1196 | | | |
| TONSL | Meningitis | | | | | | | |
| rs760477 | Control | 81 (25) | 151 (47) | 87 (27) | 319 | p > 0.05 | 0.11 | 0.75 (0.53–1.05) |
| G → A | Case | 106 (31) | 161 (47) | 72 (21) | 339 | | | |
| | Bacteremia | | | | | | | |
| | Control | 277 (31) | 428 (49) | 177 (20) | 882 | p > 0.05 | 0.83 | 1.05 (0.85–1.28) |
| | Case | 257 (30) | 422 (50) | 165 (20) | 844 | | | |
| | Combined | | | | | | | |
| | Control | 358 (30) | 579 (48) | 264 (22) | 1201 | p > 0.05 | 0.50 | 0.96 (0.81–1.14) |
| | Case | 363 (31) | 583 (49) | 237 (20) | 1183 | | | |
| NFKBIZ | Meningitis | | | | | | | |
| rs616597 | Control | 207 (59) | 129 (37) | 17 (5) | 353 | p > 0.05 | 0.42 | 0.82 (0.61–1.11) |
| C → A | Case | 226 (63) | 114 (32) | 17 (5) | 357 | | | |
| | Bacteremia | | | | | | | |
| | Control | 527 (59) | 334 (37) | 33 (4) | 894 | 0.02 < p < 0.05[§] | 0.32 | 0.95 (0.79–1.15) |
| | Case | 514 (60) | 299 (35) | 42 (5) | 855 | | | |
| | Combined | | | | | | | |
| | Control | 734 (59) | 463 (37) | 50 (4) | 1247 | 0.01 < p < 0.02[§] | 0.21 | 0.91 (0.78–1.07) |
| | Case | 740 (61) | 413 (34) | 59 (5) | 1212 | | | |
| PTPN22 | Meningitis | | | | | | | |
| rs2476601 | Control | 291 (81) | 63 (18) | 4 (1) | 358 | p > 0.05 | 0.70 | 0.93 (0.64–1.36) |
| G → A | Case | 294 (82) | 61 (17) | 2 (1) | 357 | | | |
| | Bacteremia | | | | | | | |
| | Control | 730 (82) | 152 (17) | 7 (1) | 889 | p > 0.05 | 0.87 | 0.94 (0.73–1.20) |
| | Case | 705 (83) | 138 (16) | 6 (1) | 849 | | | |
| | Combined | | | | | | | |
| | Control | 1021 (82) | 215 (17) | 11 (1) | 1247 | p > 0.05 | 0.72 | 0.94 (0.76–1.15) |
| | Case | 999 (83) | 199 (17) | 8 (1) | 1206 | | | |

[a] Number of individuals (%); AA: wild type homozygote; AB: heterozygote; and BB mutant homozygote.
[b] $\chi^2$ or Fisher's exact test applied as appropriate.
[c] Comparison of variant allele carriers (BB + AB) vs. wild type homozygotes (AA).
[d] Controls in the Hardy–Weinberg disequilibrium at the p > 0.05 level. When adjusted for multiple comparisons (p > 0.008), all controls are in HWE.

Also, our meningitis cases were significantly younger than the bacteremia cases, and this may contribute to some of the difference.

We chose to include children <5 years in order to create a homogeneous population. It has been suggested that severe primary infections in childhood are more likely a result of single-gene variations compared to more complex genetics in adults (Alcaïs et al., 2010), which also makes genetic predisposition to infectious diseases particularly interesting in pediatric populations.

In a Caucasian population aged 0–94 years (mean 59 years) Chapman et al. demonstrated that polymorphisms in the *NFKBIE* SNP were associated with protection from IPD but not pneumococcal empyema (Chapman et al., 2007). Heterozygotes tended to have increased

**Table 4**
Single nucleotide polymorphism frequencies in invasive meningococcal disease cases and their controls.

| SNP | Status | AA[a] | AB[a] | BB[a] | Total | HWE (controls), p-value | Geno-typic p-value[b] | Odds ratio (95% CI)[c] |
|---|---|---|---|---|---|---|---|---|
| NFKBIE | Meningitis | | | | | | | |
| rs529948 | Control | 311 (79) | 76 (19) | 9 (2) | 396 | p > 0.05 | 0.37 | 1.24 |
| G → A | Case | 292 (75) | 91 (23) | 8 (2) | 391 | | | (0.89–1.73) |
| | Bacteremia | | | | | | | |
| | Control | 205 (76) | 57 (21) | 6 (2) | 270 | p > 0.05 | 0.71 | 1.06 |
| | Case | 199 (75) | 56 (21) | 9 (3) | 264 | | | (0.72–1.58) |
| | Combined | | | | | | | |
| | Control | 516 (78) | 133 (20) | 15 (2) | 664 | p > 0.05 | 0.50 | 1.16 |
| | Case | 491 (75) | 147 (22) | 17 (3) | 655 | | | (0.90–1.50) |
| NFKBIA | Meningitis | | | | | | | |
| rs3138053 | Control | 194 (50) | 166 (43) | 30 (8) | 390 | p > 0.05 | 0.97 | 0.98 |
| T → C | Case | 193 (50) | 165 (43) | 28 (7) | 386 | | | (0.74–1.30) |
| | Bacteremia | | | | | | | |
| | Control | 142 (53) | 106 (40) | 20 (7) | 270 | p > 0.05 | 0.92 | 0.90 |
| | Case | 147 (56) | 99 (38) | 18 (7) | 264 | | | (0.64–1.27) |
| | Combined | | | | | | | |
| | Control | 336 (51) | 272 (43) | 50 (8) | 658 | p > 0.05 | 0.88 | 0.95 |
| | Case | 340 (52) | 264 (41) | 46 (7) | 650 | | | (0.76–1.18) |
| NFKBIA | Meningitis | | | | | | | |
| rs2233406 | Control | 193 (49) | 165 (42) | 32 (8) | 390 | p > 0.05 | 0.89 | 0.96 |
| G → A | Case | 193 (50) | 163 (42) | 28 (7) | 384 | | | (0.73–1.28) |
| | Bacteremia | | | | | | | |
| | Control | 147 (54) | 105 (39) | 20 (7) | 272 | p > 0.05 | 0.89 | 0.93 |
| | Case | 150 (56) | 100 (37) | 18 (7) | 268 | | | (0.66–1.30) |
| | Combined | | | | | | | |
| | Control | 340 (51) | 270 (41) | 52 (8) | 662 | p > 0.05 | 0.82 | 0.95 |
| | Case | 343 (53) | 263 (40) | 46 (7) | 652 | | | (0.76–1.18) |
| Mal/TIRAP | Meningitis | | | | | | | |
| rs8177374 | Control | 285 (73) | 102 (26) | 5 (1) | 392 | p > 0.05 | 0.93 | 1.03 |
| C → T | Case | 276 (72) | 101 (26) | 6 (2) | 383 | | | (0.75–1.41) |
| | Bacteremia | | | | | | | |
| | Control | 193 (71) | 72 (27) | 5 (2) | 272 | p > 0.05 | 0.18 | 0.98 |
| | Case | 192 (72) | 63 (24) | 12 (4) | 267 | | | (0.67–1.43) |
| | Combined | | | | | | | |
| | Control | 478 (72) | 174 (26) | 10 (2) | 662 | p > 0.05 | 0.28 | 1.01 |
| | Case | 468 (72) | 164 (25) | 18 (3) | 650 | | | (0.79–1.28) |
| TONSL | Meningitis | | | | | | | |
| rs760477 | Control | 87 (24) | 183 (51) | 88 (25) | 358 | p > 0.05 | 0.12 | 0.72 |
| G → A | Case | 116 (31) | 179 (48) | 79 (21) | 374 | | | (0.52–0.99) |
| | Bacteremia | | | | | | | |
| | Control | 66 (26) | 129 (51) | 59 (23) | 254 | p > 0.05 | 0.69 | 0.86 |
| | Case | 70 (29) | 114 (47) | 57 (24) | 241 | | | (0.58–1.27) |
| | Combined | | | | | | | |
| | Control | 153 (25) | 312 (51) | 147 (24) | 612 | p > 0.05 | 0.12 | 0.77 |
| | Case | 186 (39) | 293 (48) | 136 (22) | 615 | | | (0.60–0.99) |
| NFKBIZ | Meningitis | | | | | | | |
| rs616597 | Control | 236 (61) | 133 (34) | 21 (5) | 390 | p > 0.05 | 0.81 | 0.93 |
| C → A | Case | 244 (62) | 129 (33) | 18 (5) | 391 | | | (0.70–1.24) |
| | Bacteremia | | | | | | | |
| | Control | 181 (67) | 80 (30) | 9 (3) | 270 | p > 0.05 | 0.23 | 1.36 |
| | Case | 162 (60) | 98 (36) | 10 (4) | 270 | | | (0.96–1.93) |
| | Combined | | | | | | | |
| | Control | 417 (63) | 213 (32) | 30 (5) | 660 | p > 0.05 | 0.72 | 1.08 |
| | Case | 406 (61) | 227 (34) | 28 (4) | 661 | | | (0.87–1.53) |
| PTPN22 | Meningitis | | | | | | | |
| rs2476601 | Control | 319 (81) | 71 (18) | 3 (1) | 393 | p > 0.05 | 0.23 | 1.27 |
| G → A | Case | 301 (77) | 82 (21) | 7 (2) | 390 | | | (0.90–1.80) |
| | Bacteremia | | | | | | | |
| | Control | 227 (83) | 42 (15) | 4 (1) | 273 | p > 0.05 | 0.65 | 1.22 |
| | Case | 214 (80) | 49 (18) | 4 (2) | 267 | | | (0.79–1.89) |
| | Combined | | | | | | | |
| | Control | 546 (82) | 113 (17) | 7 (1) | 666 | p > 0.05 | 0.22 | 1.25 |
| | Case | 515 (78) | 131 (78) | 11 (2) | 657 | | | (0.95–1.64) |

[a] Number of individuals (%); AA: wild type homozygote; AB: heterozygote; and BB mutant homozygote.
[b] $\chi^2$ or Fisher's exact test applied as appropriate.
[c] Comparison of variant allele carriers (BB + AB) vs. wild type homozygotes (AA).

susceptibility to pneumococcal empyema. However, this association was not significant when analyzed with logistic regression using a dominant model (Chapman et al., 2007).

In our population we were not able to replicate the finding that mutant allele carriers were protected from IPD. However, our results are similar to Chapman's findings in the sense that empyema is more invasive than bacteremia. This may support our hypothesis, that *NFKBIE* is important in invasive disease. Furthermore, bacteremia and meningitis are two different manifestations of pneumococcal disease, and the frequency of each serotype varies in these two patient groups. This might also be reflected in our results of the *NFKBIE* SNP.

Differences in study results could be partly explained by differences in the study populations, such as age and number of patients when our population is compared to Chapman et al.'s.

Although there was no significant association with IPD for the two adjacent *NFKBIA* polymorphisms, a possible trend towards increased susceptibility for mutant allele carriers appeared in the small subgroup of children with multiple IPD episodes. The *NFKBIA* polymorphisms were associated significantly with recurrent IPD at the 0.05 significance level, but lost significance when adjusted for multiple comparisons according to the Bonferroni method. This finding sets the stage for further investigation in a larger study population. However, since recurrent IPD is a rare condition, it may be challenging to include a sufficient number of patients in a future study.

The interaction between *NFKBIA* and *NFKBIE* may be important, because the two genes probably have similar functions in determining susceptibility to IPD (Chapman et al., 2007, 2010a). In contrast to mutations in monogenic diseases most disease-associated polymorphisms in complex diseases have moderate effects on disease susceptibility, and hundreds or thousands of loci may contribute to subjects' increased risk (Gibson, 2011). This could be one reason why we did not find an association between *NFKBIA* SNPs and IPD. We cannot rule out that an even larger study could demonstrate a relatively small effect of a single SNP. A potential limitation in this study would be that we assume that cases were healthy prior to invasive disease. As described by Gaschignard et al. some of these children might have unidentified immunodeficiencies or other chronic diseases that have not been diagnosed at the time of IPD (Gaschignard et al., 2014), and the same might occur for the IMD cases.

It is unlikely that our study's results are significantly influenced by population stratification, because we included Northern European children only. Controls were in HWE at the Bonferroni adjusted significance level, suggesting a lowered risk of selection bias and genotyping errors in our study.

Due to the young age of our subjects there might be a risk for misclassification regarding the results on recurrent IPD, because it cannot be ruled out that some of the children will develop a new episode of IPD later in life.

Although our results suggest an association between *NFKBIE*, and possibly *NFKBIA*, and susceptibility to IPD, we cannot exclude that the SNPs are in LD with disease-associated polymorphisms in nearby causative genes (Gabriel et al., 2002). This makes replication in independent populations important.

The lack of replication of the associations in the IMD population was not unexpected. No similar previous studies have been conducted, and these results contribute with new knowledge.

Polymorphisms were not associated with increased 30-day mortality in cases for any of the SNPs. However, very few children in our population died and this may obviously affect the statistics. No previous studies have examined the association between mortality and genotypes.

The Bonferroni method was applied in order to adjust for multiple comparisons. However, this correction is known to be conservative, when a number of SNPs are evaluated for association with traits (Gao et al., 2008). This may increase the risk of making type II errors in our study.

To our best knowledge we conducted the largest study of its kind on selected SNPs previously associated with pneumococcal disease. We found that a polymorphism in *NFKBIE* gene, which is essential for NF-κB in the innate immune response/TLR pathways, predisposed Danish children under the age of five to pneumococcal meningitis.

## Author Contributions

T.B. provided the study concept. Z.B.H., M.N., M.V.H., H.T.S., and T.B. designed the study. M.V.H., D.M.H. and T.B. performed the experiments. L.F.L., L.N.C. and T.B. analyzed the data. H.B.K. coordinated the national surveillance system. Z.B.H., M.V.H., and H.B.K. identified and collected the samples. L.F.L. and T.B. wrote the first draft of the manuscript. All authors participated in writing the final manuscript.

## Conflict of Interests

Prof. Benfield reports grants from Lundbeck Foundation, grants from Novo Nordisk Foundation, grants from Hindsgaul Fund, and grants from Preben & Anna Simonsen Foundation, during the conduct of the study; personal fees from GSK, personal fees from Bristol Myers Squibb, personal fees from Gilead, grants from Pfizer, personal fees from Bristol Myers Squibb, personal fees from GSK, personal fees from Bristol Myers Squibb, personal fees from GSK, non-financial support from Bristol Myers Squibb, non-financial support from Gilead, non-financial support from Janssen, non-financial support from MSD, and personal fees from Abbvie, outside the submitted work.

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None of the other authors report any conflict of interests.

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