Original Article

Innate Immunity

Innate Immunity
2018, Vol. 24(3) 163–170

DOI: 10.1177/1753425918762038
journals.sagepub.com/home/ini
SAGE

How many single-nucleotide polymorphisms (SNPs) must be tested in order to prove susceptibility to bacterial meningitis in children? Analysis of 11 SNPs in seven genes involved in the immune response and their effect on the susceptibility to bacterial meningitis in children

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Abstract

The aim of this study is to describe the prevalence of single single-nucleotide polymorphisms (SNPs) as well as their combinations in genes encoding proteins involved in the immune response in children with bacterial meningitis. The prospective study group consisted of 39 children with bacterial meningitis and 49 family members surveyed between 2012 and 2016. Eleven SNPs in seven genes involved in immune response were analysed. The mean number of minor frequency alleles (MAF) of studied SNPs was lowest in the control group and highest in patients with pneumococcal meningitis. We found that carrying ≥6 MAF of studied SNPs was associated with an increased risk of pneumococcal meningitis. The prevalence of risky variants was noted to be higher in patients with pneumococcal meningitis as compared to the control group. In conclusion, genetic factors are a relevant factor in determining the susceptibility to bacterial meningitis. A statistically significant cumulative effect of mutated variants on increasing the risk of bacterial meningitis was detected. Combining all three SNPs in *MBL2* improves the prediction of susceptibility to pneumococcal meningitis. Analysis of risky alleles can help indicate people prone to the disease who are 'gene-immunocompromised'.

Keywords

Complement, innate immunity, meningitis, meningococci, pneumococci

Date received: 30 October 2017; revised: 30 January 2018; accepted: 7 February 2018

Introduction

Streptococcus pneumoniae and *Neisseria meningitidis* are two of the main bacterial pathogens responsible for meningitis in children.[1–4] Colonisation with *S. pneumoniae* is common in the general population and reaches up to 50% in pre-school children, while *N. meningitidis* is detected in nasopharyngeal swabs only in around 10% of the general population.[3,5] Prevalence of bacterial meningitis in children aged

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1 mo to 2 yr is estimated to occur in 20 per 100,000 cases.[2,3] A clinically important question is why some patients develop devastating infections, while others become only asymptomatic carriers. In the general population, it is difficult to find one defined risk factor affecting the susceptibility to an invasive bacterial disease. Analysis of one single-nucleotide polymorphism (SNP) does not give an answer to this question. Keeping in mind the complexity of the immune system, in our study we have analysed 11 SNPs in seven different genes involved in the immune response.

The complement pathway represents an important part of innate immunity. It is widely known that complement factor C5–C9 deficiency predisposes to invasive bacterial diseases.[6–8] Complement factor H-related protein (CFHR) is responsible for the down-regulation of complement activation. Polymorphisms in CFH are independently associated with predisposition to meningococcal disease.[9] Individuals with SNPs in the CFH region, leading to higher factor H levels, are more prone to invasive meningococcal disease.[9,10] Mannose-binding lectin (MBL) is an opsonin which recognises pathogen polysaccharides, and activates an additional (lectin) complement pathway.[11–13]

The polymorphisms in the *MBL* gene are attributed to an increased susceptibility to pneumococcal and meningococcal infections.[12–14] Three polymorphisms in the *MBL2* gene have structural variants resulting in lowered levels of MBL.[14] MBL deficiency leads to reduced opsonisation in the early phase of infection, inducing longer initial survival of *S. pneumoniae*, thus enhancing the possibility of invasion and subsequent bacterial meningitis.[14]

TLRs belong to PRRs, which are crucial for initiating the immune response. SNPs in the *TLR2* gene has a substantial effect on the susceptibility to and severity of invasive bacterial infections.[15,16] Mutated variants of *TLR4* result in hyporesponsiveness to LPS and thus enhance the susceptibility to invasive meningococcal and pneumococcal infections.[14–16] Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) is a protein which binds to the intracellular domain of TLR2 and TLR4, and initiates the MyD88-dependent response leading to TNF-α, IL-12 and other cytokine production.[17,18] Khor et al. showed a protective effect of the heterozygotic variant of the *TIRAP* gene against pneumococcal infection, bacteraemia, malaria and tuberculosis.[18,19]

This study aims to describe the prevalence of single SNPs, as well as the combinations of SNPs in genes coding proteins involved in the immune response in children with bacterial meningitis.

Patients and methods

Our prospective study group consisted of 39 children with bacterial meningitis. All children were hospitalised at the St. Joseph Children's Hospital in Poznań, Poland. It is one of the two infectious disease departments serving the population of children of the Greater Poland Voivodeship. We collected blood samples from all of the patients hospitalised with meningitis, which was caused by either *S. pneumoniae* or *N. meningitides,* from 2012 to 2016. The bacterial aetiology was confirmed either by a cerebrospinal fluid microbiological culture or an identification of the pathogen's DNA using PCR. All children were previously healthy and not diagnosed with any immunodeficiencies. All group individuals were vaccinated according to the Polish vaccination schedule – which did not include pneumococcal and meningococcal vaccines. Clinical data were collected from patients' medical records.

The control group consisted of 49 healthy family members, with no history of meningitis during childhood. We collected data from 32 different families. In seven patients, we were unable to collect material from their family members, due to parental refusal to give blood, and/or the absence of parents during hospitalisation.

We have evaluated 11 SNPs in seven genes that are involved in the inflammatory response. The number of minor frequency alleles (MAF) of studied SNPs was determined for the studied individuals. Alleles with a higher prevalence in patients than in healthy controls were called 'risky alleles'. Synergism between the SNPs was investigated to identify possible combinations of alleles that could affect the occurrence of the disease. Data on the frequency of the SNPs in the Caucasian population were taken from the 1000 Genomes Project (http://www.ensembl.org).[20]

DNA extraction

Genomic DNA was extracted from 1.2 ml of peripheral blood according to the manufacturer's specifications, and using a Gentra Puregene Blood Kit (Qiagen, Germany). The quality and quantity of isolated DNA was assessed spectrophotometrically. The venipuncture was performed using a standard procedure commonly used for routine blood tests.

SNP genotyping

The PCR was performed in 25 µl of a reaction mixture containing 2 µl of isolated DNA, 10 pmol of each primer, 4 nmol of each deoxynucleotide, 1.5 U of Taq DNA Polymerase (Sigma-Aldrich, USA), 1 × PCR reaction buffer (containing 15 mM MgCl2; Sigma-Aldrich, USA) and additionally 25 mmol $MgCl_2$. PCR products were further purified with thermosensitive Exonuclease I and FastAP Alkaline Phosphatase (Fermentas, Thermo Fisher Scientific, USA) and sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit on an ABI Prism 3130XL Analyzer

(Applied Biosystems, Foster City, CA, USA) according to the manufacturers' protocols.

The sequences were compared between cases and healthy family members and the general population separately for *N. meningitidis* and *S. pneumoniae* cases.

The study was performed with the approval of the Poznan Medical University Ethical Committee and a written informed consent was obtained from all of the parents.

Statistics

Statistical association between disease status and cluster membership was performed to identify genes associated with bacterial meningitis. Hardy–Weinberg analysis was used to check the genotype distribution in patients and in the control group. In this analysis the χ^2 test and software available on the website: https://ihg.gsf.de/cgi-bin/hw/hwa1.pl have been applied. Haploview v4.2 software was used for linkage disequilibrium (LD) analysis. Comparisons between groups were performed using the Pearson χ^2 test of the Fisher exact test as appropriate, and odds ratio (OR) and 95% confidence intervals (95% CIs) were calculated. The Bonferroni correction was applied in the case of analysis of the effect of multiple alleles. All calculations were performed using GraphPad Prism software v6.04. Differences were considered statistically significant when $P < 0.05$. The Bonferroni correction was applied in the case of analysis of the effect of multiple alleles.

Results

Single allele analysis

Eleven SNPs in seven genes involved in the immune response were evaluated in all of the patients and their healthy family members. Details are presented in Table 1.

Polymorphic variants of the *MBL2* gene were analysed together – details are presented in Table 2. For research purposes the three *MBL2* genetic variants (rs1800450 variant B, rs1800451variant C and rs5030737 variant D) were grouped together as the dominant allele O, whereas the three wild type alleles were grouped as allele A, so wild type haplotypes combined with an SNP in B, C, or D is denoted A/O. This is in accordance with the literature.[11,12] In the analysis of the effects of individual alleles, in nine SNPs alleles more common in patients (risky alleles) were synonymous with MAF (exceptions: *TLR9* rs352140 and *TLR4* rs4986790). For all 11 analysed SNPs the prevalence of risky variants was higher in patients with pneumococcal meningitis when compared to the control group. In patients with meningococcal meningitis, this was true in nine out of 11 examined SNPs (except for *MBL2*

Table 1. Genotype distribution in patients with meningitis, family members and general population.

	Patients with meningitis		Control group	
	S. pneumoniae $n = 14$	*N. meningitidis* $n = 25$	Family $n = 49$	1000 Genomes Project[20]
MBL2 rs5030737				
CC	11 (79%)	21 (84%)	43 (88%)	86%
CT	3 (21%)	4 (16%)	6 (12%)	1%
TT	0	0	0	0
MBL2 rs1800450				
GG	10 (71%)	21(84%)	33 (67%)	72%
AG	3 (21%)	4 (16%)	16 (33%)	26%
AA	1 (7%)	0	0	2%
MBL2 rs1800451				
GG	12 (86%)	22 (88%)	47 (96%)	96%
AG	2 (14%)	3 (12%)	2 (4%)	3.5%
AA	0	0	0	0.5%
CFH rs1065489				
GG	9 (63%)	18 (72%)	35 (71%)	72%
GT	5 (27%)	6 (24%)	14 (29%)	23%
TT	0	1 (4%)	0	15%
CFHR3 rs3753396				
AA	9 (63%)	17 (68%)	35 (71%)	73%
AG	5(27%)	7 (28%)	14 (29%)	22%
GG	0	1 (4%)	0	5%
TIRAP rs8177374				
CC	10 (71%)	21 (84%)	45 (92%)	68%
CT	4 (29%)	4 (16%)	4 (8%)	30%
TT	0	0	0	2%
TLR2 rs4696480				
TT	5 (36%)	7 (28%)	17 (35%)	23%
TA	3 (21%)	12 (48%)	19 (39%)	48%
AA	6 (43%)	6 (24%)	13 (26%)	28%
TLR 2 rs5743708				
GG	12 (86%)	22 (88%)	46 (94%)	92%
GA	2 (14%)	3 (12%)	3 (6%)	8%
AA	0	0	0	0
TLR4 rs4986790				
AA	12 (86%)	22 (88%)	41 (84%)	94%
AG	2 (14%)	3 (12%)	8 (16%)	6%
GG	0	0		
TLR9 rs5743836				
TT	10 (72%)	20 (80%)	34 (69%)	75%
CC	1 (7%)	0	1 (2%)	2%
TC	3 (21%)	5 (20%)	14 (29%)	23%
TLR9 rs352140				
CC	2 (14%)	2 (8%)	7 (14%)	20%
CT	5 (36%)	15 (60%)	22 (45%)	50%
TT	7 (50%)	8 (32%)	20 (41%)	30%

CFH: complement factor H-related protein; *MLB*: mannose-binding lectin; *TIRAP*: Toll-interleukin 1 receptor domain containing adaptor protein.

Table 2. Distribution of *MBL* genotypes.

MBL	*S. pneumoniae* $n=14$	*N. meningitidis* $n=25$	Family $n=49$
AA	6	15	27
A0	6	9	21
AB	2	4	14
AC	2	2	1
AD	2	3	6
00	2	1	1
BC	0	1	1
BB	1	0	0
BD	1	0	0
ALLELE			
A	18	39	75
0	10	11	23
B	5	5	15
C	2	3	2
D	3	3	6

AA: wild type; A0: heterozygotes; 00: homozygotes. Allele: A: wild type; 0: mutated variant of *MBL2*; rs1800450 variant B, rs1800451 variant C, rs5030737 variant D. *MLB*: mannose-binding lectin.

Table 3. Comparison of allele frequency between patients with pneumococcal meningitis and family members.

Allele	Patients $n=14$	Family $n=49$	*P* value	OR 95%CI
MBL2				
A	18 (64%)	75 (76%)	0.1937	OR = 0.55
0	10 (36%)	23 (24%)		95% CI (0.21–1.54)
CFH rs1065489				
G	23 (82%)	84 (86%)	0.6414	OR = 0.77
T	5 (18%)	14 (14%)		95% CI (0.23–3.01)
CFHR3 rs3753396				
A	23 (82%)	84 (86%)	0.6414	OR = 0.77
G	5 (18%)	14 (14%)		95% CI (0.23–3.01)
TIRAP rs8177374				
C	24 (86%)	94 (96%)	0.0508	OR = 0.26
T	4 (14%)	4 (4%)		95% CI (0.05–1.50)
TLR2 rs4696480				
T	13 (46%)	53 (54%)	0.4745	OR = 0.74
A	15 (54%)	45 (46%)		95% CI (0.29–1.86)
TLR2 rs5743708				
G	26 (93%)	95 (97%)	0.3292	OR = 0.41
A	2 (7%)	3 (3%)		95% CI (0.04–5.20)
TLR4 rs4986790				
A	26 (93%)	90 (94%)	0.8602	OR = 1.16
G	2 (7%)	8 (6%)		95% CI (0.21–11.81)
TLR9 rs5743836				
T	23 (82%)	82 (84%)	0.8480	OR = 0.90
C	5 (18%)	16 (16%)		95% CI (0.27–3.47)
TLR9 rs352140				
C	19 (68%)	62 (63%)	0.6547	OR = 1.23
T	9 (32%)	36 (17%)		95% CI (0.47–3.41)

CFH: complement factor H-related protein; *MLB*: mannose-binding lectin; *TIRAP*: Toll-interleukin 1 receptor domain containing adaptor protein; OR: odds ratio; 95% CI: 95% confidence interval.

Table 4. Comparison of allele frequency between patients with meningococcal meningitis and family members.

Allele	Patients $n=25$	Family $n=49$	*P* value	OR 95% CI
MBL2				
A	39 (78%)	75 (76%)	0.8407	OR = 1.09
0	11 (22%)	23 (24%)		95% CI (0.45–2.74)
CFH rs1065489				
G	42 (84%)	84 (86%)	0.7816	OR = 0.88
T	8 (16%)	14 (14%)		95% CI (0.31–2.61)
CFHR3 rs3753396				
A	41 (82%)	84 (86%)	0.5553	OR = 0.76
G	9 (18%)	14 (14%)		95% CI (0.28–2.17)
TIRAP rs8177374				
C	46 (92%)	94 (96%)	0.3187	OR = 0.49
T	4 (8%)	4 (4%)		95% CI (0.08–2.77)
TLR2 rs4696480				
T	26 (52%)	53 (54%)	0.8103	OR = 0.92
A	24 (48%)	45 (46%)		95% CI (0.44–1.93)
TLR2 rs5743708				
G	47 (94%)	95 (97%)	0.3912	OR = 0.49
A	3 (6%)	3 (3%)		95% CI (0.06–3.86)
TLR4 rs4986790				
A	47 (94%)	90 (94%)	0.6351	OR = 1.39
G	3 (6%)	8 (6%)		95% CI (0.31–8.51)
TLR9 rs5743836				
T	45 (90%)	82 (84%)	0.2968	OR = 1.75
C	5 (10%)	16 (16%)		95% CI (0.56–6.52)
TLR9 rs352140				
C	31 (62%)	62 (63%)	0.8802	OR = 0.95
T	19 (38%)	36 (17%)		95% CI (0.44–2.05)

CFH: complement factor H-related protein; *MLB*: mannose-binding lectin; *TIRAP*: Toll-interleukin 1 receptor domain containing adaptor protein; OR: odds ratio; 95% CI: 95% confidence interval.

rs1800450 and *TLR9* rs5743836). Details are presented in Tables 3 and 4.

In analysis of a single SNP, we observed that the *TIRAP rs8177374* T allele was associated with pneumococcal meningitis at the level of statistical tendency ($P = 0.0508$).

Cumulative effect of studied polymorphisms

In the analysis of the effects of multiple alleles, the cumulative effect of MAF of studied SNPs was examined, regardless of the direction of their impact observed in the analysis of single SNP. Carrying two risky alleles of *TIRAP* rs8177374 and *MBL2* rs1800451 had a positive cumulative effect on the risk of developing meningitis (OR = 3.9 (95% CI 1.24–12.3); $P = 0.0277$) and in particular of pneumococcal aetiology (OR = 4.9 (95% CI 1.17–20.48); $P = 0.035$; Table 5). This relationship has been confirmed both in the analysis of the whole group of patients vs. the family member as well as separately for pneumococcal meningitis vs. the control group. Co-occurrence of any mutated variant of the *MBL2* gene with *TIRAP* rs8177374 MAF resulted in an increased risk of meningitis (OR = 6.25 (95% CI 1.40–27.97); $P = 0.0204$) (Table 5). The most important effect was observed when all studied SNPs were considered. Carrying ≥6 MAF of the studied SNPs was associated with an increased risk of developing pneumococcal meningitis (OR 8.4 (95% CI 1.9–36.7) $P = 0.0055$); Table 5; Supplementary Table a). This result was also statistically significant after an adjustment for the number of studied genes ($P = 0.049$). Altogether, the mean number of MAF of the studied SNPs was the lowest in the control group (family members) (3.35 ± 1.64), intermediate in patients with meningococcal meningitis (3.44 ± 1.66) and the highest in patients with pneumococcal meningitis (4.07 ± 2.13).

The mean number of MAF of the studied SNPs was lowest in the control group (family members) (3.35 ± 1.64), intermediate in patients with meningococcal meningitis (3.44 ± 1.66) and highest in patients with pneumococcal meningitis (4.07 ± 2.13). Carrying ≥ 6 MAF of the studied SNPs was associated with an increased risk of developing pneumococcal meningitis (OR 8.4 (95% CI 1.9–36.7); $P = 0.0055$); Table 5; Supplementary Table a). This result was also statistically significant after an adjustment for the number of studied genes ($P = 0.049$).

Haplotype analysis

The genotyping of both *TLR9* SNPs allows all four rs352140–rs5743836 haplotypes to be distinguished: T-T (0.498), C-T (0.354), T-C (0.138), and C-C (0.010). Because of the small sample size and relatively rare frequency of the rs5743708 SNP, only the three common *TLR2* rs5743708-rs4696480 haplotypes were observed in the studied population: T-G (0.477), A-G (0.477), and T-A (0.045). For both genes we observed high values of D' (0.817 and 1.00, respectively), but relatively low values of r^2 (0.066 and 0.043, respectively), which is another measure of LD. Thus, we might therefore conclude that both variants should be tested in an analysis of susceptibility.

Discussion

Our analysis showed a cumulative effect of SNPs in genes involved in the immune response in increasing the risk of meningitis in children. In order to assess the risk of developing meningitis, it is essential to analyse SNPs in several genes involved in the immune response, for example, complement pathway and pattern-recognition particles, because the cumulative effect

Table 5. Cumulative effect of 11 studied polymorphisms.

Number of minor frequency alleles of studied SNPs	Controls (I)	Patients		Statistical analysis OR (95% CI), *P* value		
		Meningococcus meningitis (II)	Pneumococcus meningitis (III)	II + III vs I	II vs I	III vs I
TIRAP rs8177374 and *MBL2* rs1800451						
0	44 (89.8)	18 (72.0)	9 (64.3)	3.9 (1.2–12.3), 0.0277	3,4 (0.96–12.2), 0.092	4.9 (1.2–20.5), 0.035
≥1	5 (10.2)	7 (28.0)	5 (35.7)			
TIRAP rs8177374 and *MBL2* protein phenotype						
<2	45 (91.8)	24 (96.0)	9 (64.3)	2.0 (0.53–7.8), 0.328	0.47 (0.05–4.4), 0.657	6.3 (1.40–28.0), 0.020
≥2	4 (8.2)	1 (4.0)	5 (35.7)			
All studied SNPs						
<6	45 (91.8)	23 (92.0)	8 (57.1)	2.9 (0.80–10.5), 0.122	0.98 (1.2–5.7), 1.00	8.4 (1.9–36.7), 0.0055[a]
≥6	4 (8.2)	2 (8.0)	6 (42.9)			

[a]Bonferroni correction for the number of studied genes, $P = 0.049$. *CFH*: complement factor H-related protein; *MLB*: mannose-binding lectin; SNPs: single-nucleotide polymorphisms; *TIRAP*: Toll-interleukin 1 receptor domain containing adaptor protein; OR: odds ratio; 95% CI: 95% confidence interval.

of SNPs is of clinical importance. Although risky alleles of the examined SNPs were more prevalent in patients compared to controls, the difference was not statistically significant, but carrying more than six MAF of any of the examined SNPs increased the risk of meningitis. This effect was not previously described in the literature. It can be explained by the interactions and complexity of the immune response. We were able to test such associations because in contrast to many other studies, our control group was composed of family members. We could check the SNPs profile in any member of the control group, not only prevalence of the single MAF. Comparing MAF frequency with data from the general population proved representative of the control group.

In patients, risky alleles of 11 studied SNPs were more frequent than in the control group. Most of them alone did not have a statistically significant effect, but the cumulative effect of co-occurrence of different SNPs in patients was significant in comparison to the effect of a single SNP. Our results indicated that there is a cumulative effect of the co-occurrence of variants of *MBL2* rs1800451 and *TIRAP* rs8177374. This effect has not been previously described. Both proteins are involved in immune response at different levels. But a combination of defects could give a bigger immune deficit. Weakening of the immune response at several steps (with the participation of different genes) gives a cumulative effect and increases the risk of meningitis. A cumulative effect of different SNPs was described for *TLR2* rs5743708 and *TLR4* rs4986790.[21] In our previous study we did not detect the significance of a cumulative effect of those SNPs.[22] Only in the case of two polymorphisms – *TLR9* rs352140 and *TLR*4 rs4986790 – were MAF more common in the control group than in the patient group. This is in accordance with literature data. Yuan et al., based on the analysis of a group of 85 children with pneumococcal sepsis, proved that there was a protective effect of the *TLR4* rs4986790 polymorphism.[23] Van Well et al. arrived at the same conclusion, based on the analysis of 391 survivors of meningococcal meningitis.[21] In our group (patients and controls), we did not find allele A for *TLR9* rs352140, which was showed by Sanders et al. to protect against meningococcal meningitis.[24]

Our analysis has shown the important role of SNPs in the *MBL2* gene. MBL deficiency is common in the general population. It leads to the reduction of opsonisation in the early phase of infection, leading to longer survival of *S. pneumoniae*, thus enhancing the possibility of invasion.[25,26] Around 5% of the general population has polymorphic variants.[27] Because of its high frequency, *MBL* polymorphisms may be present in around 32% of patients with meningococcal disease.[28] Our results are in line with some recent studies of MBL deficiency and infections. However, the importance of MBL deficiency in susceptibility to infections is still being discussed, and previous studies have yielded conflicting results. A study by Faber et al. suggested that *MBL2* variants are significantly associated with the susceptibility to invasive childhood meningococcal disease in an age-dependent manner and that low serum levels of MBL have been associated with a five-fold increased risk of death due to pneumococcal disease.[28] In this group of patients, the overall frequency of *MBL* variants was 31.8% versus 8.2% in the general population. Roy et al. reported that individuals with homozygous mutations for *MBL* codon variants are at an increased risk of invasive pneumococcal disease.[13] Lundbo et al. in their study of 451 Danish children did not show any increased incidence of invasive pneumococcal disease, but this analysis was performed only on patients with pneumococcal meningitis.[25]

A crucial role of TLR in invasive bacterial infections was previously confirmed by a number of studies.[29–31] *S. pneumoniae* and *N. meningitidis* have the potential to activate immune cells through TLR2, TLR4 and TLR9. In meningococcal infections, TLR2 recognises porin B and TLR4 recognises LOS.[32–35] In our patients we have not found statistically important results when compared to the control group.

We have shown that components of signal transduction routes of TLRs, such as TIRAP, are important in the pathogenesis of bacterial meningitis. We also studied the *TIRAP* variant, which was associated with bacterial meningitis in this study, at the level of tendency in analysis of single SNP, and significantly in the analysis of multiple alleles. Some previous studies suggest that the variation in *TIRAP* is potentially important in determining the susceptibility to infectious diseases, such as tuberculosis.[8] Ladhani et al. showed that polymorphic variants of *TIRAP* were associated with an increased risk of invasive *Haemophilus influenzae* disease.[17] Homozygous variants of *TIRAP* rs8177374 are rare in developing countries.[17] This fact supports speculation that the homologous recessive *TIRAP* variant may increase the susceptibility to specific infectious diseases to such an extent that it may have selected itself out of population.[19]

The previously described association between SNPs in *CFH* and meningococcal meningitis was not found in our population.[9,10,36] Davila et al. showed in two studies a protective effect of minor alleles of *CFH* rs1065489. The prevalence of mutated variants in patients was 12% and 17% in the control group. Bradley et al. had similar results in the patient group, that is, 11.5% (both studies used the same control group).[9,36] In our patients, the prevalence of *CFH*-mutated variants was similar to the control group population. We do not have data for the Polish population, but the data for the control group were similar to the Central European Group (CEU) from the 1000 Genome Project. MAF frequency in *CFH* differs between populations: high frequency in East Asia

(49.7%), moderate frequency in America (19.1%) and Europe (18.3%), and low in Africa (3.7%).[20] The prevalence of meningococcal disease is the opposite, namely highest in Sub-Saharan Africans, for whom the frequency of MAF is the lowest.

A weak point of this study was the relatively small group of patients; for example, we did not have enough genotype data to study the role of the *TLR2* and *TLR9* haplotypes. Thanks to vaccination, the number of bacterial meningitis cases in children is decreasing, thus it is difficult to collect a large study group. On the other hand, we collected and studied a standardised group; all our patients were of Caucasian origin and were vaccinated according to the same vaccination schedule, not including pneumococcal and meningococcal vaccines. Our analysis was based on the co-occurrence of several SNPs, so we couldn't use data from the general population to increase the control group. Prevalence of SNPs is different in each population, while our group was representative of the Polish population. Additionally, we examined the usefulness of available data from the CEU population for the analysis of haplotypes in this study. Unfortunately, the CEU population is underrepresented in the 1000 Genomes Project database ($n = 99$), and there were too few data to calculate LD for the studied *TLR2* SNPs. In the case of *TLR9,* results were comparable to ours, showing high values of *D'* (0.894) and low r^2 (0.133; data not presented), which confirms the need to determine both variants for risk assessment.

From another point of view, the strength of this study was that we focused exclusively on meningitis patients in order to find the host genetic determinants of the disease. Our cohort of patients with meningitis was relatively small; however, the results that we found in this group were quite significant, especially for pneumococcal meningitis. Using healthy family members as a control group is another big advantage of our study. Family members share the same environmental risk factors and have similar exposure to pathogens, especially in the youngest group. This enabled the comparison, not only of single SNPs, but the co-occurrence of 11 SNPs in patients and their family members. It increased the chances of finding people with the same set of polymorphisms.

Our analysis showed that genetic factors are important in determining the susceptibility to bacterial meningitis. In order to assess the risk of developing bacterial meningitis, it is essential to analyse SNPs in several genes involved in the immune response, for example, complement pathway and pattern recognition particles. Not a single SNP carriership but the cumulative effect of several SNPs is of clinical importance. Different results were found for *S. pneumoniae* and *N. meningitidis*. Analysis of risky alleles can indicate people prone to the disease who are somehow 'gene-immunocompromised'.

Conclusions

1. Genetic factors are important in determining the susceptibility to bacterial meningitis.
2. We have detected a statistically significant cumulative effect of mutated variants on increasing the risk of bacterial meningitis in children.
3. Carriers of at least six risky alleles have an 8.4-times higher risk of developing pneumococcal meningitis.
4. Combining all three SNPs in the *MBL2* gene improves the prediction of susceptibility to pneumococcal meningitis.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Financial support was obtained from the Poznan University of Medical Sciences.

References

1. World Health Organization. Surveillance for vaccine preventable diseases (VPDs), http://www.who.int/immunization/monitoring_surveillance/burden/VPDs/en/ (accessed 22 December 2016).
2. Centers for Disease Control and Prevention. Active bacterial core surveillance report, emerging infections program network, *Neisseria meningitidis*, https://www.cdc.gov/abcs/reports-findings/survreports/mening14.pdf, (accessed 22 December 2016).
3. Borrow R, Alarcón P, Carlos J, et al. The Global Meningococcal Initiative: Global epidemiology, the impact of vaccines on meningococcal disease and the importance of herd protection. *Expert Rev Vaccines* 2017; 16: 313–328.
4. McIntyre PB, O'Brien KL, Greenwood B, et al. Effect of vaccines on bacterial meningitis worldwide. *Lancet* 2012; 380: 1703–1711.
5. Christensen H, May M, Bowen L, et al. Meningococcal carriage by age: A systematic review and meta-analysis. *Lancet Infect Dis* 2010; 10: 853–861.
6. Wright V, Hibberd M and Levin M. Genetic polymorphisms in host response to meningococcal infection: The role of susceptibility and severity genes. *Vaccine* 2009; 27: 90–102.
7. van Well GT, Sanders MS, Ouburg S, et al. Single nucleotide polymorphisms in pathogen recognition receptor genes are associated with susceptibility to meningococcal meningitis in a pediatric cohort. *PLoS One* 2013; 8: e64252.
8. Brouwer MC, Read RC and van de Beek D. Host genetics and outcome in meningococcal disease: A systematic review and meta-analysis. *Lancet Infect Dis* 2010; 10: 262–274.
9. Davila S, Wright VJ, Khor CC, et al. International Meningococcal Genetics Consortium Genome-wide association study identifies variants in the CFH region associated with host susceptibility to meningococcal disease. *Nat Genet* 2010; 42: 772–776.
10. Haralambous E, Dolly SO, Hibberd ML, et al. Factor H, a regulator of complement activity, is a major determinant of meningococcal disease susceptibility in UK Caucasian patients. *Scand J Infect Dis* 2006; 38: 764–771.
11. Hibberd ML, Sumiya M, Summerfield JA, et al. Association of variants of the gene for mannose-binding lectin with

susceptibility to meningococcal disease. Meningococcal Research Group. *Lancet* 1999; 353: 1049–1053.
12. Bradley DT, Bourke TW, Fairley DJ, et al. Genetic susceptibility to invasive meningococcal disease: *MBL2* structural polymorphisms revisited in a large case-control study and a systematic review. *Int J Immunogenet* 2012; 39: 328–337.
13. Roy S, Knox K, Segal S, et al. *MBL* genotype and risk of invasive pneumococcal disease: A case-control study. *Lancet* 2002; 359: 1569–1573.
14. Darton TC, Jack DL, Johnson M, et al. *MBL2* deficiency is associated with higher genomic bacterial loads during meningococcemia in young children. *Clin Microbiol Infect* 2014; 20: 1337–1342.
15. Echchannaoui H, Frei K, Schnell C, et al. Toll-like receptor 2-deficient mice are highly susceptible to *Streptococcus pneumoniae* meningitis because of reduced bacterial clearing and enhanced inflammation. *J Infect Dis* 2002; 186: 798–806.
16. Mogensen TH, Paludan SR, Kilian M, et al. Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J Leukoc Biol* 2006; 80: 267–277.
17. Ladhani SN, Davila S, Hibberd ML, et al. Association between single-nucleotide polymorphisms in Mal/TIRAP and interleukin-10 genes and susceptibility to invasive *Haemophilus influenza* serotype B infection in immunized children. *Clin Infect Dis* 2010; 51: 761–767.
18. Khor CC, Chapman SJ, Vannberg FO, et al. A functional variant in *TIRAP*, also known as *MAL*, and protection against invasive pneumococcal disease, bacteraemia, malaria and tuberculosis. *Nat Genet* 2007; 39: 523–528.
19. Hawn TR, Dunstan SJ, Thwaites GE, et al. A polymorphism in Toll-interleukin 1 receptor domain containing adaptor protein is associated with susceptibility to meningeal tuberculosis. *J Infect Dis* 2006; 194: 1127–1134.
20. 1000 Genomes Project (http://www.ensembl.org) (accessed 1 October 2017).
21. van Well GT, Sanders MS, Ouburg S, et al. Polymorphisms in Toll-like receptors 2, 4, and 9 are highly associated with hearing loss in survivors of bacterial meningitis. *PLoS One* 2012; 7: e35837.
22. Gowin E, Świątek-Kościelna B, Kałużna E, et al. Analysis of *TLR2*, *TLR4*, *TLR9* single nucleotide polymorphisms in children with bacterial meningitis and their healthy family members. *Int J Infect Dis* 2017; 60: 23–28.
23. Yuan FF, Marks K, Wong M, et al. Clinical relevance of *TLR2*, *TLR4, CD14* and *FcgammaRIIA* gene polymorphisms in *Streptococcus pneumoniae* infection. *Immunol Cell Biol* 2008; 86: 268–270.
24. Sanders MS, van Well GT, Ouburg S, et al. Single nucleotide polymorphisms in *TLR9* are highly associated with susceptibility to bacterial meningitis in children. *Clin Infect Dis* 2011; 52: 475–480.
25. Lundbo LF, Harboe ZB, Clausen LN, et al. Mannose-binding lectin gene, *MBL2*, polymorphisms are not associated with susceptibility to invasive pneumococcal disease in children. *Clin Infect Dis* 2014; 59: e66–e71.
26. Kronborg G, Weis N, Madsen HO, et al. Variant mannose-binding lectin alleles are not associated with susceptibility to or outcome of invasive pneumococcal infection in randomly included patients. *J Infect Dis* 2002; 185: 1517–1520.
27. Turner MW. Mannose-binding lectin (*MBL*) in health and disease. *Immunobiology* 1998; 199: 327–339.
28. Faber J, Schuessler T, Finn A, et al. Age-dependent association of human mannose-binding lectin mutations with susceptibility to invasive meningococcal disease in childhood. *Pediatr Infect Dis J* 2007; 26: 243–246.
29. Ribes S, Ebert S, Regen T, et al. Toll-like receptor stimulation enhances phagocytosis and intracellular killing of nonencapsulated and encapsulated. *Streptococcus pneumoniae* by murine microglia. *Infect Immun* 2010; 78: 865–887.
30. Allen A, Obaro S, Bojang K, et al. Variation in Toll-like receptor 4 and susceptibility to group A meningococcal meningitis in Gambian children. *Pediatr Infect Dis J* 2003; 22: 1018–1019.
31. Smirnova I, Mann N, Dols A, et al. Assay of locus-specific genetic load implicates rare Toll-like receptor 4 mutations in meningococcal susceptibility. *Proc Natl Acad Sci U S A* 2003; 100: 6075–6080.
32. Faber J, Henninger N, Finn A, et al. A Toll-like receptor 4 variant is associated with fatal outcome in children with invasive meningococcal disease. *Acta Paediatr* 2009; 98: 548–552.
33. Sjölinder H, Mogensen TH, Kilian M, et al. Important role for Toll-like receptor 9 in host defense against meningococcal sepsis. *Infect Immun* 2008; 76: 5421–5428.
34. Biebl A, Muendlein A, Kazakbaeva Z, et al. CD14 C-159T and toll-like receptor 4 Asp299Gly polymorphisms in surviving meningococcal disease patients. *PLoS One* 2009; 4: e7374.
35. Read RC, J, Gregory S, Borrow R, et al. A functional polymorphism of Toll-like receptor 4 is not associated with likelihood or severity of meningococcal disease. *J Infect Dis* 2001; 184: 640–642.
36. Bradley DT, Bourke TW, Fairley DJ, et al. Susceptibility to invasive meningococcal disease: Polymorphism of complement system genes and *Neisseria meningitidis* factor H binding protein. *PLoS One* 2015; 10: e0120757.