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NFKBIZ polymorphisms and susceptibility to pneumococcal disease in European and African populations

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Abstract

The proinflammatory transcription factor nuclear factor-kappaB (NF-κB) plays a central role in host defence against pneumococcal disease. Both rare mutations and common polymorphisms in the *NFKBIA* gene encoding the NF-κB inhibitor IκB-α associate with susceptibility to bacterial disease, but the possible role of polymorphisms within the related IκB-ζ gene *NFKBIZ* in the development of invasive pneumococcal disease has not previously been reported. To investigate this further, we examined the frequencies of 22 single-nucleotide polymorphisms spanning *NFKBIZ* in two case-control studies, comprising UK Caucasian (n=1008) and Kenyan (n=723) individuals. Nine polymorphisms within a single UK linkage disequilibrium block and all four polymorphisms within the equivalent, shorter Kenyan linkage disequilibrium block displayed either significant association with invasive pneumococcal disease or a trend towards association. For each polymorphism, heterozygosity was associated with protection from invasive pneumococcal disease when compared to the combined homozygous states (e.g. for rs600718, Mantel-Haenszel 2×2 χ^2=7.576, P=0.006, OR=0.67, 95% CI for OR: 0.51-0.88; for rs616597, Mantel-Haenszel 2×2 χ^2=8.715, P=0.003, OR=0.65, 95% CI: 0.49-0.86). We conclude that

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multiple *NFKBIZ* polymorphisms associate with susceptibility to invasive pneumococcal disease in humans. The study of multiple populations may aid fine-mapping of associations within extensive regions of strong linkage disequilibrium ('transethnic mapping').

Keywords

genetic polymorphism; pneumococcal disease; nuclear factor-kappaB; IkappaB-zeta; NFKBIZ

INTRODUCTION

Streptococcus pneumoniae is a major global health problem and a leading cause of death in children world-wide (1). Invasive pneumococcal disease (IPD) occurs when *Streptococcus pneumoniae* invades a normally sterile site, most commonly blood (bacteraemia), and is associated with a mortality of at least 20% even in developed countries (1-3). Although asymptomatic nasopharyngeal carriage of the pneumococcus is common in the general population, invasive disease develops in only a minority of individuals and the factors that determine invasion remain poorly understood (4). Increasing evidence from mouse models and rare human primary immunodeficiency states supports a central role for the proinflammatory transcription factor nuclear factor-kappaB (NF-κB) in host defence against pneumococcal disease (5-10).

Activation of NF-κB occurs following stimulation of a variety of immune receptors, including toll-like receptors (TLRs), members of the interleukin-1 (IL-1) and tumour necrosis factor (TNF) receptor superfamilies, and the interleukin-17 (IL-17) receptor. Control of NF-κB is mediated through direct interactions with a family of IκB inhibitors. The best-studied IκB members are the proteins IκB-α, IκB-β and IκB-ε, which trap NF-κB in the cytoplasm of unstimulated cells (11). The most recently described member of the IκB family is the protein IκB-ζ, encoded by the gene *NFKBIZ*. IκB-ζ differs from the major IκBs -α, -β and -ε in that it is localised in the nucleus rather than the cytoplasm (12, 13). Unlike the cytoplasmic IκBs, very little IκB-ζ protein is detected in resting macrophages or lung primary cells; IκBζ mRNA is however rapidly induced following TLR/IL-1 signalling (12, 14-16). Two variants of IκB-ζ mRNA appear to be generated as a result of alternative splicing: long IκB-ζ(L) and short IκB-ζ(S). The short form lacks the exon 3 which contains the initiation codon of IκB-ζ(L), and as a result encodes a shorter protein from a downstream initiation site, lacking the amino-terminal 99 amino acids of IκB-ζ(L) (13, 14). The IκBζ promoter region contains NF-κB binding sites, and indeed NF-κB has been shown to be essential for the induction of IκBζ (14). IκB-ζ in turn acts as an inhibitor of NF-κB and is likely to be regulated by NF-κB in a fast negative feedback loop, as has been demonstrated for the inhibitor IκB-α (16).

A further difference between IκB-ζ and the cytoplasmic IκBs is that, in addition to exerting an inhibitory effect on NF-κB, IκB-ζ also demonstrates intrinsic transcriptional activity and appears to be essential for the activation of a subset of inflammatory genes including interleukin-6 (IL-6) (12, 13, 15, 17). IκB-ζ additionally appears to act as a negative regulator of TNF-α signalling in mice (15, 18), and may therefore have a role in specific signalling in response to subgroups of pro-inflammatory cytokines. The functional consequences of IκB-ζ activation remain incompletely understood, and are further complicated by apparent differences in IκB-ζ signalling between cell lines, mice and humans (16, 18).

Recent work has demonstrated that both rare mutations and common polymorphisms in the *NFKBIA* gene encoding the NF-κB inhibitor IκB-α are associated with susceptibility to

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bacterial disease in humans (5, 6, 19). The possible role of polymorphism within the related IκB-ζ gene *NFKBIZ* in the development of human disease has not previously been studied, however. To investigate this further we studied the frequencies of polymorphisms in the *NFKBIZ* gene in European and African individuals with IPD and controls.

SUBJECTS AND METHODS

Sample information

The UK Caucasian IPD sample collection has been previously described (20). Blood samples were collected on diagnosis from all hospitalised patients with microbiologically-proven IPD (defined by the isolation of *Streptococcus pneumoniae* from a normally sterile site, most commonly blood) as part of an enhanced active surveillance programme in three hospitals in Oxfordshire, UK (John Radcliffe, Horton General, and Wycombe General Hospitals). Consecutive cases of IPD were enrolled between June 1995 and May 2001. There were no exclusion criteria for the study. DNA samples were available from 275 patients. Clinical details, including age, gender, clinical presentation and the presence of underlying risk factors, were recorded on a standardized proforma. During the study, Oxfordshire was a region of very low HIV prevalence and HIV testing was not routinely performed. Frequencies of initial clinical presentation were as follows: pneumonia 69%, isolated bacteraemia 15%, meningitis 11%, and other presentations 5%. The mean age of the patients was 58 years, ranging from 0 to 94 years; 50% were male. Pneumococcal serotypes were identified using polyclonal rabbit antisera (Statens Seruminstitut, Copenhagen, Denmark). The distribution of serotypes was very similar to that of previous UK studies, with serotype 14 being the commonest.

The control group comprised a combination of 163 UK healthy adult blood donors and 570 cord blood samples. For the cord samples, blood was collected anonymously from the discarded umbilical cords of healthy neonates born at the John Radcliffe Hospital, Oxford, UK, as previously described (20). Examination of microsatellite markers excluded contamination with maternal DNA. The study of cord blood samples is intended to reveal background population allele frequencies; recent extensive use of a UK birth cohort control group for association studies of multiple disease phenotypes has confirmed the validity of such an approach (21). The mean age of the adult blood donors was 38 years, and 50% were male; 54% of the cord blood donors were male. Individuals of non-European ancestry were excluded from cases and controls. The study was approved by the research ethics committees of the participating hospitals.

The Kenyan bacteraemia case-control collection has also been previously described (22). Kenyan children (less than 13 years old) with bacteraemia were recruited from Kilifi District hospital between 1998 and 2002. The 687 bacteraemic cases comprised patients with isolated Gram-positive and Gram-negative infections, diagnosed using standard blood culture techniques. The most frequent organisms isolated were *Streptococcus pneumoniae* (25%), non-Typhi Salmonella species (16%), *Haemophilus influenzae* (14%), and *Escherichia coli* (8%). The 550 community controls were individually matched to a subset of the cases on the basis of time (recruited within 14 days), location of homestead, age, and sex. Only children with complete data for HIV, malnutrition, and malaria status were included in the analysis. Ethical approval for the study was given by the Kenya Medical Research Institute (KEMRI) National Scientific Steering and Research Committees.

Genotyping techniques

DNA extraction from blood was performed using Nucleon II kits (Scotlab Bioscience, Buckingham, UK). Polymorphisms within *NFKBIZ* and neighbouring chromosomal regions

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were selected from the dbSNP (www.ncbi.nlm.nih.gov) and ensembl (www.ensembl.org) databases. Polymorphisms were chosen on the basis of their likely functionality, as well as to provide an overview of the extent of linkage disequilibrium across the gene and flanking regions. Genotyping was performed using the Sequenom Mass-Array® MALDI-TOF primer extension assay (23). A touchdown PCR protocol was used, with cycling conditions as follows: 95°C for 15 minutes; 94°C for 20 seconds; 65°C for 30 seconds; 72°C for 30 seconds; steps 2 to 4 repeated for 5 cycles; 94°C for 20 seconds; 58°C for 30 seconds; 72°C for 30 seconds; steps 5 to 7 repeated for 5 cycles; 94°C for 20 seconds; 53°C for 30 seconds; 72°C for 30 seconds; steps 8 to 10 repeated for 38 cycles; final extension at 72°C for 3 minutes. Primer sequences are listed in table E1 (online data supplement). Each genotyping plate contained a mixture of case and control samples.

General PCR conditions for amplifying products prior to sequencing were as follows: 95°C for 15 minutes, and then 40 cycles of 95°C for 30 seconds, 55-65°C for 30 seconds, and 72°C for 60 seconds, followed by 72°C for five minutes. Direct sequencing was performed using BigDye v3.1 terminator mix (ABI) followed by ethanol precipitation. Plates were run on an ABI 3700 capillary sequencer and sequence analysis was performed with the Lasergene DNAstar package (Lasergene), using SeqMan software. Primer sequences are listed in table E2 (online data supplement).

Statistical analysis

Statistical analysis of genotype associations and logistic regression was performed using the program SPSS v16.0. Analysis of linkage disequilibrium (LD) was performed using the Haploview v4.1 program (24). Haplotype blocks were defined as regions demonstrating strong evidence of historical recombination between less than 5% of SNP-pair comparisons (25). All control genotype distributions were in Hardy-Weinberg equilibrium.

RESULTS

A total of twenty-two single-nucleotide polymorphisms (SNPs) were genotyped in the UK Caucasian IPD cases and controls, of which seven SNPs were non-polymorphic or very rare (table 1). Of the remaining fifteen SNPs, seven appeared to be associated with susceptibility to IPD at the 0.05 significance level (table 1). The seven associated SNPs span a distance of 20kb across the gene, from intron 2 (rs685666) to the 3′ untranslated region (rs601225). Flanking polymorphisms on either side of *NFKBIZ* were also genotyped to determine the extent of LD and to exclude the possibility that the associated SNPs were simply markers linked to a true association in a neighbouring gene. The disease-associated SNPs were all found to be located within a single block of strong LD which involves *NFKBIZ* and extends in a 3′ direction (figure 1a). No other genes are predicted to lie within this region, and the lack of associated SNPs outside this LD block suggests that the observed association is indeed localised to *NFKBIZ*.

In an attempt to fine map the pneumococcal disease association within such a high-LD region, the *NFKBIZ* polymorphisms were next genotyped in the Kenyan bacteraemia case and control study (table 1). As expected, the extent of LD appeared to be shorter in this African population, with evidence of prior recombination breaking up the extensive 60kb LD block which was observed in Europeans (figure 1b). A smaller LD block was predicted in the Kenyan population, containing only the SNPs rs685666, rs6441627, rs616597 and rs600718 (figure 1b). Each of these four linked SNPs displayed either a trend towards susceptibility or a significant association with pneumococcal bacteraemia in the Kenyan study (table 1), although no association with overall bacteraemia was observed. Of these SNPs, rs600718 and rs616597 displayed the most statistically significant associations with IPD in the Kenyan and UK Caucasian populations; their genotypic distributions are

presented in table 2. Inclusion of patient covariates (age, co-morbid conditions, and gender) within a conditional logistic regression framework demonstrated no effect of these factors on genotype in the UK or Kenyan studies, and additionally no effect of HIV infection and protein-energy malnutrition in the Kenyan study. Neither of the SNPs was associated with outcome from IPD in these groups (data not shown).

For the UK study, the direction of association best fits a model of heterozygote protection, with an excess of both wild-type and mutant homozygotes in the IPD groups (analysing heterozygotes against combined homozygotes for rs600718, 2×2 χ^2=4.70, *P*=0.03, odds ratio (OR)=0.71, 95% confidence interval (CI) for OR: 0.52-0.97; for rs616597, 2×2 χ^2=6.20, *P*=0.01, OR=0.68, 95% CI: 0.50-0.92). The Kenyan results are also consistent with a heterozygote protective effect against pneumococcal bacteraemia (for rs600718, 2×2 χ^2=3.82, *P*=0.05, OR=0.51, 95% CI for OR: 0.25-1.01; for rs616597, 2×2 χ^2=3.15, *P*=0.07, OR=0.54, 95% CI: 0.27-1.08), although this cannot be concluded with complete confidence owing to the rarity of the mutant allele for each SNP in this population. Comparison of odds ratios for rs600718 and rs616597 did not demonstrate any evidence of heterogeneity between the UK and Kenyan IPD-control groups for either SNP; after combining and stratifying the two study groups, heterozygosity was associated with protection from IPD when compared to the combined homozygous states (for rs600718, Mantel-Haenszel 2×2 χ^2=7.576, P=0.006, OR=0.67, 95% CI for OR: 0.51-0.88; for rs616597, Mantel-Haenszel 2×2 χ^2=8.715, P=0.003, OR=0.65, 95% CI: 0.49-0.86).

In the context of the shorter LD observed in the Kenyan population, these results localise the IPD disease association to a limited region within *NFKBIZ*. No further polymorphisms in the vicinity of rs600718 were listed on databases. In an attempt to identify novel polymorphisms, a 2.2 kb region surrounding SNPs rs600718 and rs616597 was sequenced directly in 96 UK Caucasian individuals. The sequenced region comprised the exon downstream of the two SNPs (exon 3 of IκB-ζ(S)) and the entire intron within which the SNPs were located. Only a single new variant was identified, located 21bp 3′ of exon 3 of IκB-ζ(S): one individual was found to be CT heterozygous at this position, the remaining individuals sequenced were TT homozygotes. The rarity of this intronic variant suggests that it is unlikely to be of any significance to the present study, however. The sequencing also confirmed the genotyping accuracy of SNPs rs600718 and rs616597 (100% concordance between sequencing and Sequenom genotyping).

DISCUSSION

This study has identified associations between multiple *NFKBIZ* polymorphisms and susceptibility to IPD. The finding that the same SNP (rs600718) independently associates with susceptibility to IPD in both UK and Kenyan populations suggests that this is likely to be a real effect. Although the LD architecture differs between these European and African populations, it is noteworthy that nine SNPs within a single UK LD block and all four SNPs within the Kenyan LD block displayed either significant association with IPD or a trend towards association. In this context, we believe that the results are unlikely to represent an artefact from multiple testing or population stratification. The absence of an association between *NFKBIZ* polymorphisms and susceptibility to bacteraemia overall suggests that the effect is likely to be specific to IPD.

Although the finding of multiple associated SNPs within a large LD block provides reasonably compelling evidence of an association with this genomic region, it also renders the localisation of the functional variant within this block extremely difficult (26). A theoretical approach to this problem is through the study of the same disease phenotype in another ethnic group. African populations are ‘older’ than European populations, and as a

result have undergone a greater number of recombination events, leading to less extensive LD (25, 27). The study of a second population may therefore aid fine-mapping of associations within extensive regions of strong LD ('transethnic mapping'). Furthermore, it has been argued that the demonstration of genetic loci that impact on disease susceptibility across different populations is perhaps of even more value than the identification of population-specific effects (28). Genetic studies incorporating more than one ethnic group remain relatively unusual however, reflecting the challenge in assembling adequate sample collections from individuals with the same phenotype in different populations.

The differing frequency of the rs600718 and rs616597 mutant alleles between European and African populations is noteworthy: frequencies of approximately 22% in the UK Caucasians and nearly 6% in the Kenyans were observed. The UK genotype distributions suggest that mutant homozygotes are particularly vulnerable to IPD, and in the setting of a developing country with a very high burden of pneumococcal disease mortality, such genotypes are likely to be subject to significant negative selective pressure. The mutant allele frequency noted in the Kenyan population may therefore reflect a balance between a deleterious effect of the mutant homozygous state versus a possible evolutionary benefit from heterozygosity. Heterozygote protection against major infectious disease phenotypes is well-described (29-32), and may also result from additional selective pressures acting at this locus – investigation of *NFKBIZ* in the setting of other major infectious and inflammatory disease phenotypes may therefore be of interest.

To our knowledge this is the first reported disease association with *NFKBIZ*, and the identity of the IPD-associated polymorphism which exerts a functional effect on *NFKBIZ* remains unclear. Study of the Kenyan population suggests that the true functional variant is likely to be either rs600718 itself, or a polymorphism within this LD block tagged by rs600718. The SNP rs600718 is located in the intron between exon 1 of IκB-ζ(L) and exon3 of IκB-ζ(S); it does not itself appear to disrupt a splice site and its possible effects on IκB-ζ gene function are unknown. Stabilisation of IκBζ mRNA has recently been shown to be required for stimulus-specific IκBζ induction (14), and it is possible that a *NFKBIZ* polymorphism may exert a functional effect through modulation of the rate of mRNA degradation.

IκB-ζ is known to be highly expressed in the lungs and on mucosal surfaces (16, 33), consistent with a role in host defence against the pneumococcus. The mechanism by which IκB-ζ variation influences the development of IPD is likely to be complex, given the apparent dual actions of IκB-ζ on NF-κB signalling. The requirement for IκB-ζ in the expression of a subset of inflammatory genes, in particular IL-6, may account for its role in susceptibility to IPD. A further possibility is that IκB-ζ variation modulates IL-17A signaling in response to pneumococcal infection. Interleukin-17A has been shown to mediate immunity to pneumococcal colonisation, perhaps in part reflecting the induction of human β-defensin 2 (*hBD-2*), which exerts a potent inhibitory effect on pneumococcal growth (34-36). Indeed, IκB-ζ has recently been reported to play a key role in the induction of *hBD-2* expression in airway epithelium in response to IL-17A (36). There is increasing evidence that IL-17A is central to the control of inflammatory airway responses (37), and the possible role of *NFKBIZ* polymorphisms in the regulation of pneumococcal clearance following IL-17A-induced *hBD-2* expression is worthy of further investigation. IκB-ζ is itself the subject of considerable interest and ongoing research is likely to clarify its precise signalling roles. This study adds to the increasing evidence that control of NF-κB is central to host defence against pneumococcal disease in humans, and for the first time highlights a role for IκB-ζ in this process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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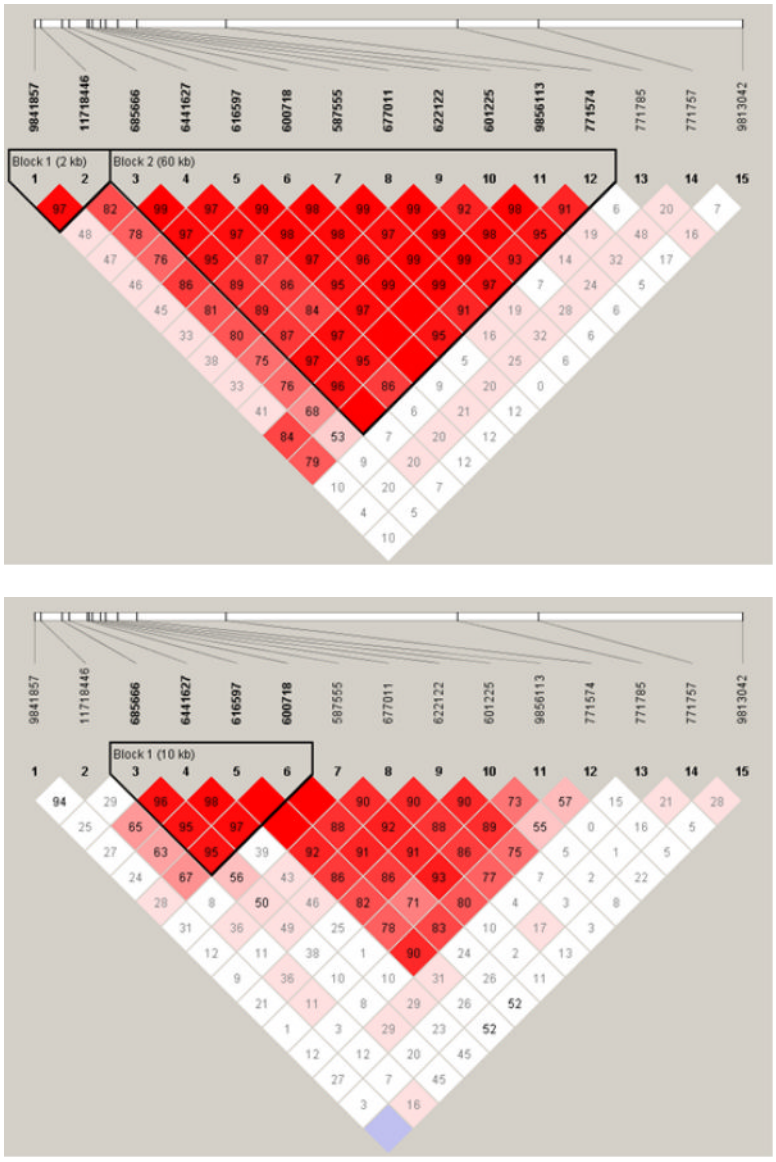

Figure 1. Location of SNPs and linkage disequilibrium (LD) map for *NFKBIZ* in the (a) UK and (b) Kenyan populations studied

Polymorphisms are identified by their dbSNP rs numbers, and their relative positions are marked by vertical lines. Unfilled squares indicate a high degree of LD (LD coefficient D'=1) between pairs of markers. Numbers indicate the D' value expressed as a percentile. Red squares indicate pairs in strong LD with LOD scores for LD≥2, pink squares D'<1 with LOD≥2, blue squares D'=1 with LOD<2, white squares D'<1.0 and LOD<2.

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Table 1

NFKBIZ and flanking regions polymorphism allele frequencies and *P* values in the UK Caucasian IPD and Kenyan pneumococcal bacteraemia case-control studies.

Polymorphism[e]/ Location[f]	UK Caucasian study		Kenyan study	
	Minor allele frequency (%)	*P* value[a]	Minor allele frequency (%)	*P* value[a]
rs9841857 −20918	23.2	0.517	1.0	0.272 [b]
rs11718446 −18387	25.6	0.782	20.5	0.055
rs685666 −10717	23.9	***0.036***	8.2	0.135
rs6441627 [c] −7960	24.2	***0.011***	7.8	0.171 [b]
rs616597 −1214	22.1	***0.001***	5.7	0.159[b]
rs600718 −565	22.4	***0.010***	5.8	***0.022*** [b]
rs587555 +611	33.7	***0.050***	35.8	0.969
rs677011 +3564	34.0	***0.042***	46.8	0.169
rs622122 +5465	37.4	0.077	34.5	0.813
rs601225 +9711	35.7	***0.049***	32.4	0.715
rs9856113 [d] +16854	43.9	0.086	20.7	0.158
rs771574 +49743	31.6	0.941	16.2	0.545
rs771785 +134870	47.3	0.237	39.7	0.989
rs771757 +164708	19.6	0.858	31.3	0.389
rs9813042 +239780	37.1	0.247	14.1	0.936

[a] *P* values are derived from 3×2 Chi-squared comparisons of genotypes, except where indicated. *P* values are uncorrected for multiple comparisons. Significant *P* values at 0.05 level are highlighted in bold italics. Derived from sample sizes: UK study 275 IPD cases and 733 controls; Kenyan study 173 pneumococcal bacteraemia cases with 550 controls.

[b] Two-sided Fisher's exact test.

[c] Duplicate nomenclature, also named rs645781.

[d] Duplicate nomenclature, also named rs694936.

[e] The following *NFKBIZ* SNPs were either non-polymorphic or very rare (minor allele frequency <0.01) in the UK population studied: rs3821727; rs1043339; rs7644329; rs11710599; rs13077110; rs7645725; rs7628891.

[f] SNP positions are relative to start of translation in exon 3 of IκB-ζ(S).

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Table 2

NFKBIZ polymorphism rs616597 and rs600718 genotype frequencies in UK Caucasian individuals with IPD and controls and in Kenyan individuals with bacteraemia (overall, Gram-positive, and pneumococcal) and controls.

Polymorphism /location [major/minor allele]	Population	Status	Genotype distribution[a]			Total	Chi-square[b] (*P* value)
			AA	Aa	aa		
rs616597 Intron 2 -1214 [C/A]	UK	Control	423 (60.0%)	254 (36.0%)	28 (4.0%)	705	12.677 (**0.001**)
		IPD	169 (63.8%)	73 (27.5%)	23 (8.7%)	265	
	Kenyan	Control	468 (89.3%)	55 (10.5%)	1 (0.2%)	524	0.908[c]
		Bacteraemia	587 (88.7%)	73 (11.0%)	2 (0.3%)	662	
		Gram-positive bacteraemia	287 (89.7%)	32 (10.0%)	1 (0.3%)	320	0.956[c]
		Pneumococcal bacteraemia (IPD)	159 (94.1%)	10 (5.9%)	0 (0%)	169	0.159[c]
rs600718 Intron 2 -565 [T/A]	UK	Control	430 (59.6%)	262 (36.3%)	30 (4.2%)	722	9.138 (**0.010**)
		IPD	164 (63.1%)	75 (28.8%)	21 (8.1%)	260	
	Kenyan	Control	487 (89.2%)	59 (10.8%)	0 (0%)	546	0.147 [c]
		Bacteraemia	603 (88.3%)	75 (11.0%)	5 (0.7%)	683	
		Gram-positive bacteraemia	297 (88.9%)	33 (9.9%)	4 (1.2%)	334	**0.040** [c]
		Pneumococcal bacteraemia (IPD)	162 (93.6%)	10 (5.8%)	1 (0.6%)	173	**0.022** [c]

[a] Number of individuals (%); AA, wild-type homozygote; Aa, heterozygote; aa, mutant homozygote.

[b] Genotypic 3×2 Chi-squared comparison, 2 degrees of freedom.

[c] Genotypic two-sided Fisher's exact test P value.