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Cytokine gene polymorphisms in preterm infants with necrotising enterocolitis: genetic association study

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ABSTRACT

Background: The inflammatory cytokine cascade is implicated in the pathogenesis of necrotising enterocolitis (NEC). Genetic association studies of cytokine polymorphisms may help to detect molecular mechanisms that are causally related to the disease process.

Aim: To examine associations between the common genetic variants in candidate inflammatory cytokine genes and NEC in preterm infants.

Methods: Multi-centre case–control and genetic association study. DNA samples were collected from 50 preterm infants with NEC and 50 controls matched for gestational age and ethnic group recruited to a multi-centre case–control study. Ten candidate single-nucleotide polymorphisms in cytokines previously associated with infectious or inflammatory diseases were genotyped. The findings were included in random-effects meta-analyses with data from previous genetic association studies.

Results: All allele distributions were in Hardy–Weinberg equilibrium. None of the studied cytokine polymorphisms was significantly associated with NEC. Four previous genetic association studies of cytokine polymorphisms and NEC in preterm infants were found. Meta-analyses were possible for several single-nucleotide polymorphisms. These increased the precision of the estimates of effect size but did not reveal any significant associations.

Conclusions: The available data are not consistent with more than modest associations between these candidate cytokine variant alleles and NEC in preterm infants. Data from future association studies of these polymorphisms may be added to the meta-analyses to obtain more precise estimates of effects sizes.

Necrotising enterocolitis (NEC) is a major cause of death and disability in preterm infants but its pathogenesis is incompletely understood. Gut immaturity and sub-optimal gut perfusion, exacerbated by enteral feeding, may be important. Additional factors, including enteric or systemic infection, may then precipitate a cascade of inflammatory events leading to the clinical and pathological end point of NEC.¹

Evidence exists for the involvement of several host inflammatory mediators in the final common pathway leading to NEC. Plasma concentrations and tissue expression of the proinflammatory cytokines, tumour necrosis factor (TNF), interleukin (IL)-1 β , IL6, IL8 and IL18, are increased in infants with NEC.^{2–4} Proinflammatory effects are modulated by receptor antagonists and regulation of receptors as well as by the effects of anti-inflammatory cytokine cascades, principally

What is already known on this topic

- Inflammatory cytokine cascades may play a key role in the pathogenesis of necrotising enterocolitis (NEC) in preterm infants.
- Genetic association studies may help to distinguish causal molecular mechanisms from epiphenomena.

What this study adds

None of the candidate cytokine polymorphisms so far studied is significantly associated with NEC.

IL10.^{5,6} Studies using animal models have suggested that modulating the inflammatory cytokine cascade may be a beneficial adjunctive strategy for treating preterm infants with NEC.^{7–9}

In investigating the contribution of these complex cytokine cascades to the pathogenesis of NEC, it is difficult to distinguish molecular mechanisms that are causal from those that are epiphenomena of the disease process. Raised plasma and tissue concentrations of inflammatory mediators may be a consequence, rather than the cause, of the disease. An alternative strategy that obviates these problems is to examine whether the risk of NEC is associated with genetic factors that regulate cytokine production or function. This approach has been used to study the role of inflammatory mediators in a number of diseases associated with preterm birth, including NEC.^{10–12}

Here we have examined associations between variants in candidate cytokine genes with susceptibility to NEC in preterm infants recruited to a multi-centre case–control study. We focused on common single-nucleotide polymorphisms (SNPs) that are of likely or proved functional significance, or have plausible disease associations described previously. As genetic association studies in this field are often limited by small sample sizes and are underpowered to exclude modest associations, where possible, we have quantitatively synthesised the study findings with data from other studies in order to provide a more precise estimate of the effect size.¹³

METHODS

A case–control study of NEC in preterm infants was conducted in 10 neonatal units in the north of Britain between January 2004 and December 2005.

Table 1 Location of candidate single-nucleotide polymorphism (SNP) relative to transcription start site

Cytokine	SNP position (major/minor allele)	Putative effect of minor allele
TNF	−238 (G/A)	Increased transcription ^{15*}
TNF	−308 (G/A)	Increased transcription ^{15 16*}
IL1β	−31 (T/C)	Decreased transcription ¹⁷
IL1β	−511 (C/T)	Increased transcription ¹⁷
IL4 receptor α	+1902 (G/A)	Enhanced signalling ¹⁸
IL6	−174 (G/C)	Increased production ¹⁹
IL8	−251 (T/A)	Increased production ²⁰
IL10	−1082 (G/A)	Reduced transcription ²¹
IL18	−137 (G/C)	Reduced transcription ²²
IL18	−607 (C/A)	Reduced transcription ²²

*Cell/stimulus specific.

IL, interleukin; TNF, tumour necrosis factor.

Briefly, cases were preterm infants with NEC diagnosed using modified Bell criteria or at laparotomy or autopsy examination. Controls were infants who had not developed NEC by 34 weeks' postmenstrual age. Cases and controls were frequency-matched for gestational age at birth. More details of the methods are published elsewhere.¹⁴ The Northern & Yorkshire multi-centre research ethics committee approved the study.

A dried blood sample from each infant was collected on filter paper (Whatman FTA, Whatman International, Maidstone, Kent, UK). DNA was extracted using the REDExtract-N-Amp Blood PCR kit (Sigma Chemicals, Poole, Dorset, UK). The relevant sequences were amplified by PCR, and the products sequenced in an automated sequencer using a standard SNaPshot run setup (see online supplement for PCR SNP primer sequences and PCR run conditions). Alleles were assigned with the ABI GeneMapper software and rechecked manually. Data were analysed combining individuals homozygous and heterozygous for the variant allele into a single exposure class.

Meta-analysis

Medline (1966–2007) and EMBASE (1980–2007) were searched for genetic association studies using the following text words and MeSH terms: [Enterocolitis, Necrotizing OR necrotising enterocolitis OR NEC] AND [Polymorphism, Genetic OR Cytokines/genetics]. References in previous reviews and studies

Table 2 Variant cytokine genotypes in cases and controls

Allele	Cases	Controls	OR (95% CI)
TNF (−308A)	19 (38%)	17 (34%)	1.19 (0.53 to 2.69)
TNF (−238A)	6 (12%)	9 (18%)	0.62 (0.20 to 1.90)
IL1 (−31G)	28 (56%)	33 (66%)	0.66 (0.29 to 1.47)
IL1 (−511T)	30 (60%)	33 (66%)	0.77 (0.24 to 1.74)
IL4R (+1902G)	15 (30%)	13 (26%)	1.22 (0.51 to 2.93)
IL6 (−174C)	37 (74%)	34 (68%)	1.34 (0.56 to 3.19)
IL8 (−251A)	36 (72%)	40 (80%)	0.64 (0.25 to 1.63)
IL10 (−1082G)	39 (78%)	38 (76%)	1.12 (0.44 to 2.84)
IL18 (−137C)	20 (40%)	25 (50%)	0.67 (0.30 to 1.47)
IL18 (−607A)	31 (62%)	30 (60%)	1.09 (0.49 to 2.43)

IL, interleukin; IL4R, interleukin 4 receptor; TNF, tumour necrosis factor.

were examined. Abstracts presented at the Society for Pediatric Research and European Society for Pediatric Research between 1995 and 2006 were searched.

For each potentially eligible study, information on setting, design, inclusion/exclusion criteria and genotyping method was extracted. Study investigators were contacted to obtain additional information if necessary. Case–control and cohort studies were eligible for inclusion provided that (a) NEC was defined using standard criteria (Bell staging or modifications), (b) the enrolment of participants was not made on the basis of prior knowledge of genotype, (c) genotyping had been blinded to clinical status, (d) the study reported the ethnic ancestry of participants, (e) the reported genotype distributions were in Hardy–Weinberg equilibrium, and (f) the report provided data sufficient to calculate an odds ratio (OR). Included data were synthesised in random-effects meta-analyses—that is, with no a priori assumption of effect homogeneity—using RevMan software (version 4.2). Heterogeneity was assessed using the χ^2 test ($p < 0.1$ considered significant).

RESULTS

Genetic association study

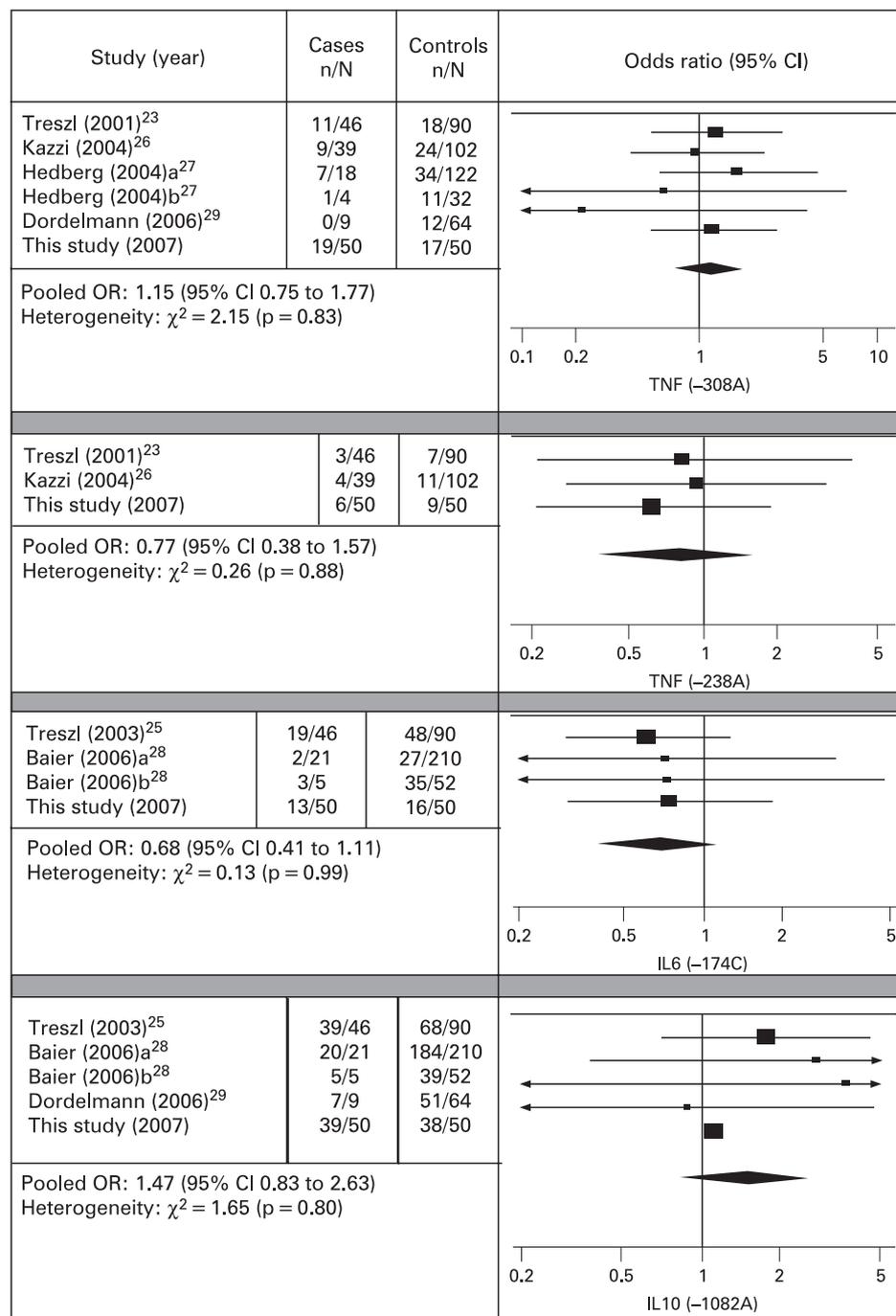
Ten polymorphisms were genotyped in 50 cases and 50 matched controls (table 1). Allele distribution was in Hardy–Weinberg equilibrium for all polymorphisms. The proportion of infants with the variant allele did not differ significantly between cases and controls for any of the comparisons (table 2). The findings did not change when analysis was restricted to infants with stage 2/3 NEC ($n = 38$).

Table 3 Cytokine genetic association studies in NEC

Reference	Country and predominant ethnic groups	Participants	Design (cases/controls)	Alleles studied
23–25	Hungary/Caucasian	BW < 1500 g NEC = Bell stage 1–3	Case–control (46/90)	TNF (−308A) TNF (−238A) IL4R (+1902G) IL6 (−174C) IL10 (−1082G) IL18 (−607A)
26	USA/Caucasian and African–American	BW < 1250 g NEC = Bell stage 1–3	Prospective cohort (39/102)	TNF (−308A) TNF (−238A)
27,28	USA/Caucasian and African–American	BW < 1500 g Mechanically ventilated NEC = Bell stage 1–3	Retrospective cohort (26/262)	TNF (−308A) IL6 (−174C) IL10 (−1082G)
29	Germany/Caucasian	GA < 32 weeks NEC = Bell stage 1–3	Retrospective cohort (9/64)	TNF (−308A) IL10 (−1082G)

BW, birth weight; GA, gestational age; IL, interleukin; IL4R, interleukin 4 receptor; NEC, necrotising enterocolitis; TNF, tumour necrosis factor.

Figure 1 Meta-analyses (random effects) of genetic association studies. IL, interleukin; TNF, tumour necrosis factor. a, African-American; b, Caucasian.



Systematic literature search

Four genetic association studies of cytokine polymorphisms and NEC in preterm infants were found (table 3).^{25–29}

Meta-analyses

TNF (–308A), TNF (–238A), IL6 (–174C) and IL10 (–1082A)

Random-effects meta-analyses of data from this study with those from previous studies did not reveal any significant differences (fig 1). None of the meta-analyses revealed significant heterogeneity.

IL4 receptor (+1902G)

The only previous study to have examined the association of the IL4 receptor (+1902G) with NEC reported that the variant allele

was significantly less common in cases than controls (cases 10/46; controls 37/90; OR 0.40 (95% CI 0.18 to 0.90)).²⁵ We did not find any significant difference in the present study (cases 15/50; controls 13/50; OR 1.22 (95% CI 0.51 to 2.93)). Meta-analysis of combined data did not find a significant association (pooled OR 0.66 (95% CI 0.37 to 1.18)).

IL18 (–607A)

One study (in a post hoc analysis) reported that the proportion of infants homozygous for the IL18 (–607A) allele (AA genotype) was significantly higher in infants with stage 3 NEC (cases 4/8; controls 7/90; OR 11.9 (95% CI 2.4 to 57.9)).²⁴ We did not find a significant difference in the present study (cases 1/7; controls 7/50; OR 0.77 (95% CI 0.08 to 7.12)). When

these data were meta-analysed, the association was not statistically significant (pooled OR 3.36 (95% CI 0.21 to 53.6)).

DISCUSSION

The available data suggest that these common cytokine genetic polymorphisms are not strongly associated with the risk of NEC in preterm infants. In most cases, the estimates of effect size suggest that modest effects have not been missed. For some associations, use of meta-analytical techniques commonly used for synthesising the findings of controlled trials allowed us to increase the precision of the estimates. Meta-analyses were possible because the studies recruited broadly similar populations of infants using standard case definitions of NEC as inclusion criteria. Controls were selected from ethnically similar populations, or data provided to allow stratified analysis by ethnic background to account for the potential confounding effect of population admixture.¹³ Although data were pooled from different study designs, we did not find statistical evidence of heterogeneity in the meta-analyses, suggesting that these estimates are reliable.

The TNF promoter polymorphisms (−308A and −238A) have been the most commonly studied cytokine gene variants. Our findings are consistent with those of three previous studies, which did not detect any significant associations with NEC.^{23 24 29} Although the estimates of effect size from each individual study were wide, meta-analysis of all of these data suggests that, if any associations do exist, they are likely to be very modest. This lack of association does not rule out the possibility that TNF and other proinflammatory cytokines are important in the pathogenesis of NEC in preterm infants. True associations may exist but with very modest effect sizes too small to be excluded by the available data. Alternatively, it may be that the candidate polymorphisms that we have studied are not directly involved in gene regulation, or that any functional effect is dependent on the haplotypic background for which neither we, nor others, have data.

Meta-analysis of association studies also suggests that the common IL6 (−174C) SNP is very unlikely to increase susceptibility to NEC. However, the lower bound of the 95% CI for the OR was consistent with a modest protective effect (halving of odds). Evidence exists that this polymorphism increases IL6 production in neonatal lymphocytes stimulated with lipopolysaccharide.¹⁷ Carriage of the variant is associated with protection against invasive infection in preterm infants, presumably by enhancing the immune response.³⁰ Although it is biologically plausible that a polymorphism that enhances the inflammatory response may increase the risk (or severity) of NEC, another possibility is that such a polymorphism may reduce the risk of NEC by preventing enteric or systemic infections that trigger the inflammatory cascade. Similarly, the IL10 (−1082A) variant has been associated with an increase in susceptibility to late-onset invasive infection in preterm infants.²⁸ Meta-analysis of data from five studies did not reveal a significant association with NEC, but the upper bound of the 95% CI of the OR (2.6) does not exclude a modest effect size. Data from future association studies may be added to this meta-analysis to increase the precision of these estimates of effect size.

Only two studies have previously reported significant associations between cytokine polymorphisms and NEC. Treszl and colleagues found that genetic variation in the IL4 receptor (+1902G) was associated with a lower risk of NEC.²⁵ The investigators suggested that the enhanced production of IL4 protected the immature gastrointestinal tract from inflammation and that screening for this allele would allow targeted

surveillance and intervention to prevent NEC in at-risk infants. In the present study, and on meta-analysis of data from both studies, we did not find a significant association, suggesting that such an approach is not justified at present.

The same investigators reported an association between homozygosity of the IL18 (−607A) allele with stage 3 NEC.²⁴ However, this association appears to have been the result of a post hoc subgroup analysis, and therefore may have been spurious. We did not detect a similar effect, and meta-analysing the data suggests there to be no significant association with NEC. These findings support the need to confirm reports of genetic associations in independent populations, particularly if the first reported effect size is modest, derived from a post hoc or subgroup analysis, and was detected as part of a larger association study where multiple comparisons were made.¹³

Genetic association studies may also be useful in examining the role of other inflammatory mediators in the pathogenesis of NEC. Candidates for further investigation include platelet-activating factor (a phospholipid with complex biological functions that may be a key mediator in the process leading to NEC), cyclo-oxygenase-2, and various forms of nitric oxide synthase that mediate the downstream vascular effects of the inflammatory cascade.¹ Such studies should aim to study similar populations of infants (using standard case definitions) and use measures to avoid confounding, particularly ethnic heterogeneity. The use of family-based studies, which allow multiple genetic markers to be examined without the possibility of confounding due to population admixture, may be particularly suitable for investigating complex diseases of preterm infants.

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Competing interests: None.

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