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

Ginny Henderson, Stan Craig, John Baier, Nicholas Helps, Peter Brocklehurst and William McGuire


Updated information and services can be found at: http://fn.bmj.com/cgi/content/abstract/adc.2007.119933v2

These include:

Rapid responses You can respond to this article at: http://fn.bmj.com/cgi/eletter-submit/adc.2007.119933v2

Email alerting service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article

Notes

Online First contains unedited articles in manuscript form that have been peer reviewed and accepted for publication but have not yet appeared in the paper journal (edited, typeset versions may be posted when available prior to final publication). Online First articles are citable and establish publication priority; they are indexed by PubMed from initial publication. Citations to Online First articles must include the digital object identifier (DOIs) and date of initial publication.

To order reprints of this article go to: http://journals.bmj.com/cgi/reprintform
To subscribe to Archives of Disease in Childhood - Fetal and Neonatal Edition go to: http://journals.bmj.com/subscriptions/
CYTOKINE GENE POLYMORPHISMS IN PRETERM INFANTS WITH NECROTISING ENTEROCOLITIS: GENETIC ASSOCIATION STUDY

1Henderson G, 2Craig S, 3Baier RJ, 4Helps N, 5Brocklehurst P, 6McGuire W.

1 Department of Health Sciences, Griffith University, Brisbane, Australia
2 Regional Neonatal Unit, Royal Jubilee Maternity Hospital, Belfast, UK
3 Department of Paediatrics, University of Manitoba, Canada.
4 The Sequencing Service, College of Life Sciences, University of Dundee, UK
5 National Perinatal Epidemiology Unit, University of Oxford, UK
6 Centre for Newborn Care, Australian National University, Canberra, Australia

Keywords: necrotising enterocolitis, cytokine, polymorphism
Word count: 2097

Correspondence:
William McGuire
Centre for Newborn Care
The Canberra Hospital
ACT 2606
Australia

Telephone: +61 (0)262443077
Email: william.mcguire@act.gov.au

The Corresponding Author has the right to grant on behalf of all authors and does grant on behalf of all authors, an exclusive licence (or non exclusive for government employees) on a worldwide basis to the BMJ Publishing Group Ltd and its Licensees to permit this article (if accepted) to be published in Archives of Disease in Childhood editions and any other BMJPGL products to exploit all subsidiary rights, as set out in our licence (http://adc.bmjjournals.com/misc/ifora/licenceform.shtml).
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 gestational-age and ethnic group frequency-matched controls recruited to a multi-centre case-control study. 10 candidate single nucleotide polymorphisms (SNPs) 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 SNPs. 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.
BACKGROUND
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 the several host inflammatory mediators in the final common pathway leading to NEC. Plasma levels and tissue expression of pro-inflammatory cytokines, tumour necrosis factor (TNF), interleukin (IL)-1 beta, IL-6, IL-8, and IL-18 are elevated in infants with NEC.2-4 Pro-inflammatory effects are modulated by receptor antagonists and regulation of receptors as well as by the effects of anti-inflammatory cytokine cascades, principally IL-10.5,6 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. Elevated plasma and tissue levels 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 focussed on common single nucleotide polymorphisms (SNP) that are of likely or proved functional significance, or have plausible disease associations described previously. Since 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 ten neonatal units in the north of Britain between January 2004 and December 2005. 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). DNA was extracted using the REDExtract-N-Amp Blood PCR kit (Sigma Chemicals). The relevant sequences were amplified by polymerase chain reaction (PCR) and the products sequenced in an automated sequencer using a standard SNaPshot run setup (see appendix for PCR SNP primer sequences, and PCR run conditions). Alleles were assigned with the ABI GeneMapper software and re-checked manually. Data were
analysed combining individuals homozygous and heterozygous for the variant allele into a single exposure class.

**Table 1. Location of candidate SNP (relative to transcription start site)**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>SNP position (major/minor allele)</th>
<th>Putative effect of minor allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF</td>
<td>-238 (G/A)</td>
<td>*Increased transcription</td>
</tr>
<tr>
<td>TNF</td>
<td>-308 (G/A)</td>
<td>*Increased transcription</td>
</tr>
<tr>
<td>IL-1 beta</td>
<td>-31 (T/C)</td>
<td>Decreased transcription</td>
</tr>
<tr>
<td>IL-1 beta</td>
<td>-511 (C/T)</td>
<td>Increased transcription</td>
</tr>
<tr>
<td>IL-4 receptor alpha</td>
<td>+1902 (G/A)</td>
<td>Enhanced signalling</td>
</tr>
<tr>
<td>IL-6</td>
<td>-174 (G/C)</td>
<td>Increased production</td>
</tr>
<tr>
<td>IL-8</td>
<td>-251 (T/A)</td>
<td>Increased production</td>
</tr>
<tr>
<td>IL-10</td>
<td>-1082 (G/A)</td>
<td>Reduced transcription</td>
</tr>
<tr>
<td>IL-18</td>
<td>-137 (G/C)</td>
<td>Reduced transcription</td>
</tr>
<tr>
<td>IL-18</td>
<td>-607 (C/A)</td>
<td>Reduced transcription</td>
</tr>
</tbody>
</table>

*cell/stimulus specific

**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 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 (i) NEC was defined using standard criteria (Bell staging or modifications), (ii) the enrolment of participants was not made based on prior knowledge of genotype, (iii) genotyping had been blinded to clinical status, (iv) the study reported the ethnic ancestry of participants, (vi) the reported genotype distributions were in Hardy–Weinberg equilibrium, and (vi) 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 Chi squared test (p < 0.1 considered significant).

**RESULTS**

**Genetic association study**

Ten polymorphisms were genotyped in 50 cases and 50 matched controls. 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).

**Systematic literature search**

Four genetic association studies of cytokine polymorphisms and NEC in preterm infants were found (table 3).
Table 2. Variant cytokine genotypes in cases and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF –308A</td>
<td>19 (38%)</td>
<td>17 (34%)</td>
<td>1.19 (0.53 to 2.69)</td>
</tr>
<tr>
<td>TNF –238A</td>
<td>6 (12%)</td>
<td>9 (18%)</td>
<td>0.62 (0.20 to 1.90)</td>
</tr>
<tr>
<td>IL-1 –31G</td>
<td>28 (56%)</td>
<td>33 (66%)</td>
<td>0.66 (0.29 to 1.47)</td>
</tr>
<tr>
<td>IL-1 –511T</td>
<td>30 (60%)</td>
<td>33 (66%)</td>
<td>0.77 (0.24 to 1.74)</td>
</tr>
<tr>
<td>IL-4R +1902G</td>
<td>15 (30%)</td>
<td>13 (26%)</td>
<td>1.22 (0.51 to 2.93)</td>
</tr>
<tr>
<td>IL-6 –174C</td>
<td>37 (74%)</td>
<td>34 (68%)</td>
<td>1.34 (0.56 to 3.19)</td>
</tr>
<tr>
<td>IL-8 –251A</td>
<td>36 (72%)</td>
<td>40 (80%)</td>
<td>0.64 (0.25 to 1.63)</td>
</tr>
<tr>
<td>IL-10 –1082G</td>
<td>39 (78%)</td>
<td>38 (76%)</td>
<td>1.12 (0.44 to 2.84)</td>
</tr>
<tr>
<td>IL-18 –137C</td>
<td>20 (40%)</td>
<td>25 (50%)</td>
<td>0.67 (0.30 to 1.47)</td>
</tr>
<tr>
<td>IL-18 –607A</td>
<td>31 (62%)</td>
<td>30 (60%)</td>
<td>1.09 (0.49 to 2.43)</td>
</tr>
</tbody>
</table>

Table 3. Cytokine genetic association studies in NEC.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country and predominant ethnic groups</th>
<th>Participants</th>
<th>Design (cases/controls)</th>
<th>Alleles studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>23-25</td>
<td>Hungary-Caucasian</td>
<td>BW&lt;1500g NEC= Bell stage I to III</td>
<td>Case-control (46/90)</td>
<td>TNF (-308A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TNF (-238A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-4 R (+1902G)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-6 (-174C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-10 (-1082G)</td>
</tr>
<tr>
<td>26</td>
<td>USA-Caucasian and African-american</td>
<td>BW&lt;1250g NEC= Bell stage I to III</td>
<td>Prospective cohort (39/102)</td>
<td>TNF (-308A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>TNF (-238A)</td>
</tr>
<tr>
<td>27,28</td>
<td>USA-Caucasian and African-american</td>
<td>BW&lt;1500g Mechanically ventilated. NEC= Bell stage I to III</td>
<td>Retrospective cohort (26/262)</td>
<td>TNF (-308A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-6 (-174C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-10 (-1082G)</td>
</tr>
<tr>
<td>29</td>
<td>Germany-Caucasian</td>
<td>GA&lt;32 weeks NEC= Bell stage I to III</td>
<td>Retrospective cohort (9/64)</td>
<td>TNF (-308A)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IL-10 (-1082G)</td>
</tr>
</tbody>
</table>

Meta-analyses

*TNF*(-308A), TNF*(-238A), IL-6 (-174C), and IL-10 (-1082A)*

Random effects meta-analyses of data from the present study with those from previous studies did not reveal any significant differences (figure). None of the meta-analyses demonstrated significant heterogeneity.

*IL-4R (+1902G)*

The only previous study to have examined the association of IL-4R (+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 (95% CI): 0.66 (95% CI 0.37 to 1.18)].

**IL-18** (-607A)

One study (in a post-hoc analysis) reported that the proportion of infants homozygous for the IL-18 (-607A) allele (AA genotype) was significantly higher in infants with stage III NEC [cases: 4/8; controls: 7/90; OR 11.9 (95% CI 2.4 to 57.9)]. 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 (doubling of odds) have not been missed. For some associations, use of meta-analytic 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. 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. Although the estimates of effect size from each individual study were wide, meta-analysis of all of these data suggest 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 pro-inflammatory 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 IL-6 (-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 IL-6 production in neonatal lymphocytes stimulated with lipopolysaccharide. Carriage of the variant is associated with protection against invasive infection in preterm infants, presumably by enhancing the immune response. While 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 IL-10 (-1082A) variant has been associated with an increase in susceptibility to late-onset invasive infection in preterm infants. 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 IL-4 receptor (+1902G) was associated with a lower risk of NEC. The investigators suggested that the enhanced production of IL-4 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 current 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 IL-18 (-607A) allele with stage III NEC. 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.

Genetic association studies may be also be useful in examining the role of other inflammatory mediators in the pathogenesis of NEC. Candidates for further investigation include platelet-activating factor (PAF), a phospholipid with complex biological functions that may be a key mediator in the process leading to NEC, cyclooxygenase-2 and various forms of nitric oxide synthase that mediate the downstream vascular effects of the inflammatory cascade. Such studies should aim to study similar populations of infants (using standard case definitions), and employ measures to avoid confounding, particularly ethnic heterogeneity. The use of family-based studies that 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.

**Competing interests**
We declare that we have no competing interests.

**Funding**
The study was funded by Tenovus (Scotland). The funder had no role in the collection, analysis, and interpretation of data, or in the writing of the report and the decision to submit the paper for publication. The National Perinatal Epidemiology Unit receives funding from the Department of Health. The views expressed in this publication are those of the authors and not necessarily those of the Department of Health.

**Acknowledgements**
We thank the principal investigators of the cited genetic association studies for providing further data for inclusion in the meta-analyses.
"What is already known on this topic"

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

"What this study adds"

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


