

# Association study suggests opposite effects of polymorphisms within *IL8* on bronchial asthma and respiratory syncytial virus bronchiolitis

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**Background:** IL-8 is a strong inducer of inflammation. Accordingly, it plays a pivotal role in acute inflammatory responses during respiratory syncytial virus (RSV) infections and in chronic inflammatory diseases such as bronchial asthma and juvenile idiopathic arthritis. Recently, 2 studies have found association of the polymorphism –251A of *IL8* with RSV bronchiolitis. Furthermore, epidemiologic studies have demonstrated an increased risk for the development of asthma after RSV bronchiolitis, and a common genetic background for the 2 diseases is currently being discussed.

**Objective:** This study investigated whether IL-8 is in association with asthma and/or arthritis and whether the results can confirm a common genetic background of RSV bronchiolitis and asthma.

**Methods:** The polymorphisms –A251T, C781T, C1633T, and A2767T within *IL8* were genotyped in the following 4 populations: children with asthma, atopic children, children with juvenile idiopathic arthritis, and control subjects. Statistical analysis made use of the Armitage trend test and the software program Arlequine.

**Results:** Association of all polymorphisms was found with asthma ( $P = .008$  to  $P = .03$ ). Surprisingly –251T was associated with asthma, which is the opposite allele as described in association with RSV bronchiolitis. Furthermore, all polymorphisms were significantly more common in children with arthritis than in asthmatic children ( $P = .006$  to  $P = .02$ ). No association was seen with the diagnosis of arthritis per se or with atopy.

**Conclusion:** This is the first study to describe association of IL-8 with asthma and a significant inverse distribution of the polymorphisms in juvenile idiopathic arthritis. In addition, the results of this study might suggest that RSV bronchiolitis and bronchial asthma have at least some different genetic factors. (J Allergy Clin Immunol 2004;114:671-6.)

**Key words:** Respiratory syncytial virus, bronchiolitis, IL-8, bronchial asthma

## Abbreviations used

JIA: Juvenile idiopathic arthritis

RSV: Respiratory syncytial virus

SNP: Single nucleotide polymorphism

IL-8 is a member of the family of chemokines. It is mainly involved in the initiation and amplification of acute inflammatory reactions<sup>1</sup> and in chronic inflammatory processes. Therefore, it plays an important role in diseases in which inflammation is a substantial pathophysiological feature—that is, in chronic inflammatory diseases such as bronchial asthma and juvenile idiopathic arthritis (JIA)—and in acute viral and bacterial infections.

Recently, 2 independent studies have reported association of the polymorphism –251T within the promotor of IL-8 with respiratory syncytial virus (RSV) bronchiolitis.<sup>2,3</sup> In addition, higher levels of IL-8 have been found in the serum of children with RSV bronchiolitis.<sup>4</sup> Several epidemiologic studies have demonstrated that severe RSV infections are a risk factor for the development of bronchial asthma and allergic sensitization in children.<sup>5,6</sup> At the same time, acute RSV infections are often accompanied by bronchial hyperreactivity; this resembles the clinical presentation of bronchial asthma. Thus, a common genetic background for these diseases is currently discussed.<sup>6</sup>

The important role of IL-8 in the pathophysiology of bronchial asthma has been confirmed by many studies in humans and animals. Repeated administration of IL-8 into the airways induces bronchial hyperreactivity in guinea pigs,<sup>7,8</sup> and increased levels of IL-8 in sputum precede an exacerbation of asthma in humans.<sup>9</sup> Besides, IL-8 might be especially important in nonatopic asthma, because IL-8-producing cells are more frequently found in this subgroup of patients with asthma.<sup>10</sup> Also, IL-8 selectively inhibits IgE production in atopic patients by inhibiting IL-4 and thus might even protect against the development of atopy.<sup>11,12</sup>

To get an as comprehensive a picture as possible of the role of IL-8 in the pathogenesis of inflammatory conditions, this study also investigated the effect of polymorphisms within IL-8 on another chronic inflammatory disease—JIA. Our interest in the genetics of JIA was also prompted by studies suggesting that chronic inflammatory diseases have some genetic elements in common.<sup>13,14</sup> In fact, we have recently shown that the polymorphism Arg110Gln in IL-13 is significantly more frequent in

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patients with asthma than in children with JIA.<sup>15</sup> This might be the first hint that different inflammatory diseases are indeed influenced by the same gene variant, as has been proposed on the basis of common genetic linkage.

The aim of this study was to investigate the role of polymorphisms within IL-8 in different chronic inflammatory diseases and to test whether there is a common genetic basis for bronchial asthma and RSV bronchiolitis.

## METHODS

### Asthmatic population

Three hundred twenty-one children with suspected asthma (aged 5-18 years) were recruited from the southwestern part of Germany between July 2000 and January 2003. The probands were characterized at the University Children's Hospital, Freiburg, Germany, by using a standardized clinical protocol. Participants were asked in advance to discontinue any asthma or allergy medication before the clinical testing. An extended medical history was recorded, including the occurrence and duration of wheezing symptoms, previous and acute medications, severity of previous asthma attacks, previous allergic rhinitis or conjunctivitis, atopic dermatitis, and any family history of allergic diseases.

### Skin prick test

Skin prick tests to 17 common allergens and positive (histamine) and negative controls were performed. The following allergens were tested: house dust mites; different grass and tree pollens; *Aspergillus fumigatus*; *Alternaria alternata*; *Cladosporium herbarum*; and dog, cat, rabbit, duck, and horse dander. The wheal response diameters were recorded after 15 minutes. The skin prick test was regarded as positive if the wheal of the test allergen was at least half the size of the wheal of the positive control.

### Specific and total IgE

Specific IgE was detected by ELISAs against 2 mixtures of grass pollens and mite allergens (*Dermatophagoides pteronyssinus* and *D. farinae*, respectively), cat and dog dander, and hazel and birch pollens (Magic Lite, Chiron Diagnostics, Fernwald, Germany). The cutoff point for a positive test was 1.43 Magic Lite units.<sup>16</sup> Total serum IgE was measured by an enzyme allergosorbent test (Phadezym, Pharmacia, Uppsala, Sweden).

### Pulmonary function tests

Pulmonary function tests were performed with standard protocols. In addition, exercise-induced asthma was diagnosed by subjecting the probands to physical exercise for 6 minutes under standardized conditions. The first spirometry and peak flow measurements were performed after 2 to 3 minutes and the second after 5 to 6 minutes. After 10 more minutes, the children inhaled salbutamol, and a third set of spirometry and peak flow measurements were taken.

To test for bronchial hyperresponsiveness, inhalations with increasing, doubling concentrations of histamine (from 0.125 to 8 mg/dL) were performed. Testing was stopped after a 15% decrease in FEV<sub>1</sub>.

### Asthma definition

Two hundred thirty-one of the 321 recruited children were diagnosed as positive for bronchial asthma. The diagnosis was based on a clear-cut history of asthmatic symptoms, the use of antiasthmatic medication, and at least some bronchial hyperreactivity. The antiasthmatic drugs included typical betamimetics, such as salbutamol, and standard corticosteroids used in asthma treatment, such as

budenoside. Bronchial hyperreactivity was defined as a decrease in FEV<sub>1</sub> by at least 15% in histamine testing or exercise provocation.

### Atopic population

Specific IgE antibodies to a highly purified crude extract of *Dermatophagoides pteronyssinus* were detected by an ELISA (Magic Lite, ALK-Abelló, Hørsholm, Denmark). The cutoff point for a positive class 1 titer was >1.43 Magic Lite units. The mite-sensitized (atopic) population consisted of 106 unrelated children. The individuals were recruited from a population as described elsewhere.<sup>17</sup> All individuals were recruited from the southwestern part of Germany. None of the atopic patients had asthma according to the asthma definition described previously.

### JIA population

The forms of JIA have been recently classified by the International League of Associations for Rheumatology.<sup>18</sup> This study focused on patients who fulfilled the clinical criteria of chronic arthritis, which refers to arthritis of at least 6 weeks' duration of unknown cause that occurs in children younger than 16 years old. In addition, all patients included in the study tested positive for antinuclear antibodies in serum, ie, titer >1:80. Eighty-six children fulfilling these criteria were recruited. One child in this group had asthma but not increased IgE levels.

### Control population

Two hundred seventy randomly chosen probands were used as controls (aged 19-40 years). They originated from the same area in the southwestern part of Germany. No medical history was taken and no medical testing was performed on controls.

### Single-strand conformation polymorphism analysis

Blood samples to perform screening for polymorphisms were collected from 41 independent adults in a German population recruited without regard to the atopic phenotype. The amplified products were resolved on nondenaturing polyacrylamide gels containing 10% or 0% glycerol at 20°C or 10°C for 2 hours.<sup>19</sup> The gels were silver-stained as described elsewhere.<sup>20</sup>

### Sequencing

Sequencing was performed by the dideoxy chain termination method<sup>21</sup> with the Big Dye Terminator cycle sequencing kit on an ABI 310 sequencer (Applied Biosystems, Foster City, Calif). The same primers were used as for the PCR. All the following studies included reference individuals with known genotypes as confirmed by sequencing.

### Genotyping

Genotyping of the 4 polymorphisms -A251T, C781T, C1633T, and A2767T was performed by RFLP. Polymorphisms were numbered according to Hull et al.<sup>3</sup>

The polymorphism -A251T was typed by using the primer pair 5'-CCA TCA TGA TAG CAT CTG TA-3' and 5'-CCA CAA TTT GGT GAA TTA T\*TA A-3' (annealing temperature 55°C; \*change to the original sequence to create a restriction site for an endonuclease). After PCR, the product was digested with 1 U of *AseI* (New England Biolabs, Beverly, Mass) overnight, and the fragments were resolved on a 2% agarose gel.

The polymorphism C781T was typed by using the primer pair 5'-CTC TAA CTC TTT ATA TAG GAA T\*T-3' and 5'-GAT TGA TTT TAT CAA CAG GCA-3' (annealing temperature 52°C). After PCR, the product was digested with 1 U of *EcoRI* (New

**TABLE I.** Results of genotyping on the 4 different populations\*

| Polymorphism | Population | Homozygote WT (n) | Heterozygote (n) | Homozygote MU (n) | Frequency WT |
|--------------|------------|-------------------|------------------|-------------------|--------------|
| -251T        | Asthma     | 76                | 109              | 41                | 0.577        |
|              | Atopy      | 25                | 56               | 18                | 0.535        |
|              | Controls   | 74                | 124              | 70                | 0.507        |
|              | JIA        | 23                | 34               | 28                | 0.471        |
| 781C         | Asthma     | 90                | 110              | 31                | 0.628        |
|              | Atopy      | 32                | 56               | 17                | 0.571        |
|              | Controls   | 84                | 124              | 61                | 0.543        |
|              | JIA        | 27                | 32               | 26                | 0.506        |
| 1633T        | Asthma     | 86                | 106              | 33                | 0.618        |
|              | Atopy      | 31                | 57               | 17                | 0.567        |
|              | Controls   | 83                | 121              | 62                | 0.539        |
|              | JIA        | 26                | 27               | 28                | 0.488        |
| 2767T        | Asthma     | 90                | 109              | 31                | 0.628        |
|              | Atopy      | 32                | 57               | 16                | 0.576        |
|              | Controls   | 87                | 123              | 59                | 0.552        |
|              | JIA        | 27                | 32               | 26                | 0.506        |

\*Given are the numbers of individuals who are homozygous for the wild-type allele (WT) and heterozygous or homozygous for the mutant allele (MU). The last column lists the allelic frequency of the wild-type allele.

England Biolabs) overnight, and the fragments were resolved on a 2% agarose gel.

The polymorphism C1633T was typed by using the primer pair 5'-CTG ATG GAA GAG AGC TCT GT-3' and 5'-TGT TAG AAA TGC TCT ATA TTC TC-3' (annealing temperature 56°C). After PCR, the product was digested with 2 U of *Nla*III (New England Biolabs) overnight, and the fragments were resolved on a 2% agarose gel.

The polymorphism A2767T was typed by using the primer pair 5'-CCA GTT AAA TTT TCA TTT CAG G\*TA-3' and 5'-CAA CCA GCA AGA AAT TAC TAA-3' (annealing temperature 53°C). After PCR, the product was digested with 1 U of *Bst*Z17 I (New England Biolabs) overnight, and the fragments were resolved on a 2% agarose gel. The genotyping was performed blind by 2 investigators who were unaware of the phenotype.

## Statistical analysis

An association analysis based on the case-control design was performed for each single nucleotide polymorphism (SNP) by using the Armitage trend test, taking into account the individuals' genotypes rather than just the alleles and following the guidelines given by Sasieni<sup>22</sup> as implemented in the program FINETTI (Thomas F. Wienker, unpublished data; <http://ihg.gsf.de/cgi-bin/hw/hwa1.pl> and <http://ihg.gsf.de/linkage/download/finetti.zip>). Separate analyses were performed for the asthmatic versus the control and the JIA populations, for the JIA versus the control populations, and for the atopic versus the control and asthmatic populations. The Armitage trend test does not rely on the assumption of Hardy-Weinberg equilibrium (HWE) when testing for associations between a marker and the disease of interest. Although deviation from HWE has been shown to inflate the chance of false-positive results when performing the allele-based test,<sup>23</sup> the Armitage trend test based on the genotypes remains valid even if HWE does not hold. Still, for each of the 4 SNPs, we tested whether the genotype distribution for each of the 4 populations was in HWE by using the program FINETTI.

In addition to the analyses based on a single SNP, we performed haplotype frequency estimation, taking into account haplotypes of all 4 markers. This was done by using the program Arlequine (Genetics and Biology Lab, Geneva, Switzerland).

**TABLE II.** Hardy-Weinberg equilibrium as calculated by the Finetti program\*

| Polymorphism | HWE asthma | HWE atopy | HWE controls | HWE JIA |
|--------------|------------|-----------|--------------|---------|
| -251T        | .86        | .17       | .22          | .07     |
| 781C         | .77        | .36       | .24          | .02     |
| 1633T        | .97        | .28       | .17          | .003    |
| 2767T        | .82        | .25       | .22          | .02     |

\*P values.

## Approval

The collection of serum and the subsequent DNA material and the experimental procedures were approved by the Ethical Commission of the University of Freiburg. A statement of informed consent was signed by all participants or, in the case of children, by their parents.

## RESULTS

### Detection of polymorphisms

Screening the coding region and the exon-intron boundaries of the gene coding for IL-8, we did not identify any new polymorphisms.

### Genotyping

The 4 polymorphisms -A251T, C781T, C1633T, and A2767T were genotyped in 4 different populations: children with asthma, atopic children, children with JIA, and a control population. The allelic frequencies of the 4 polymorphisms and the genotype distribution are given in Table I. All polymorphisms were in HWE in the asthmatic, atopic, and control populations. However, the polymorphisms were out of HWE in the populations of children with JIA (see Table II).

**TABLE III.** Results of association studies by the Armitage trend test\*

| Polymorphism | Asthma (controls) | Asthma (JIA) | JIA (controls) |
|--------------|-------------------|--------------|----------------|
| –A251T       | .032              | .0212        | .425           |
| T781C        | .008              | .007         | .426           |
| C1633T       | .016              | .006         | .280           |
| A2767T       | .017              | .007         | .319           |

\**P* values.

## Association studies

A significant association with asthma was found for all 4 polymorphisms (Table III). The strongest association was seen with C781T ( $P = .008$ ); the C allele was more frequent in patients with asthma. The weakest association was shown with –A251T ( $P = .03$ ); thereby, the T allele was more common in patients with asthma.

There was no association of any polymorphism with JIA when compared with controls (Table III). However, the pattern of the frequencies of the polymorphisms was the same for all 4 polymorphisms, with the highest frequency in patients with JIA, the lowest in the patients with asthma, and the control population in between. The difference in frequency between JIA and asthma was significant for all polymorphisms ( $P = .02$  to  $.006$ ; Table III).

Regarding the population of children sensitized to house dust mites, no association was found with IL-8 (data not shown). However, as seen in Table I, the allelic frequency of all 4 polymorphisms was between the frequency of the polymorphisms in the asthmatic and the control populations.

All 4 polymorphisms were in very tight linkage disequilibrium (data not shown). Therefore, a correction of the *P* values by the factor 4 because of multiple testing of 4 polymorphisms would be far too conservative. However, even applying this correction and assuming a *P* value of .0125 as statistically significant, we still find association of C781T with bronchial asthma and an inverse association with JIA for 3 polymorphisms.

## Haplotype analysis

The haplotypes as calculated for all 4 populations are given in Table IV. There was no significant difference in haplotype distribution among the 4 populations (data not shown).

## DISCUSSION

The role of IL-8 in chronic inflammatory diseases in general and in bronchial asthma in particular has been shown in many studies (see review<sup>24</sup>). This study found for the first time an association of polymorphisms of IL-8 with bronchial asthma. The –251T and 781C alleles were significantly more common in patients with asthma than in controls and might be a risk factor for the development of

**TABLE IV.** Haplotypes as calculated by the Arlequine program\*

| Haplotype | Asthma | Atopy | Controls | JIA   |
|-----------|--------|-------|----------|-------|
| ACCA      | 0.045  | 0.031 | 0.032    | 0.031 |
| ACTT      | 0.007  | 0.005 | 0.006    | 0.006 |
| ATTA      | 0.005  | 0.020 | 0.011    | 0.006 |
| ATTT      | 0.369  | 0.418 | 0.445    | 0.493 |
| TCCA      | 0.572  | 0.514 | 0.504    | 0.457 |
| TCTA      | 0.002  | 0.021 | 0.002    | 0.006 |

\*Frequency of the haplotypes in the 4 populations.

asthma. It is interesting to note that 2 recent association studies of severe RSV infections in infancy found opposite results<sup>2,3</sup>; here the –A251 allele conferred risk for severe RSV infection and RSV bronchiolitis. Thus, the results of this study are in contrast to the currently discussed hypothesis that both diseases have a common genetic background. Furthermore, because asthma and RSV bronchiolitis share some clinical features, such as inflammation and wheezing, one would have expected that the same gene variants would give susceptibility to both diseases.

The opposite results could be simply due to genotyping errors or a mix-up of genotypes in this study. However, we found the same haplotype structure as described by Hull et al,<sup>3</sup> ie, the –251A allele transmitted in 90% together with 781T and in 10% transmitted with 781C. Thus, a simple mix-up of genotypes can be excluded. Furthermore, all variants were in HWE in both populations. Therefore, the results seem to be valid and suggest that the genetic basis of asthma and RSV bronchiolitis might be different; for some gene variants, even opposite effects on the development of diseases can be found.

The strongest association with asthma was seen with the polymorphism C781T. However, all 4 polymorphisms were in tight linkage disequilibrium with each other. As a consequence, only 6 distinct haplotypes of *IL8* were found. This is in accordance with the study conducted by Hull et al<sup>3</sup> that showed the same haplotype structure in a European sample. Because of the tight linkage disequilibrium of all 4 polymorphisms, it is not yet clear which polymorphism accounts for the observed association. In fact, it is possible that a so-far unknown polymorphism in *IL8* or even in an adjacent gene is the true disease-causing allele. We screened the coding region of the gene and did not identify any coding polymorphisms. Thus, it seems unlikely that a coding polymorphism exists in *IL8* in our populations. Further studies—especially functional analyses—are needed to identify the polymorphism that is responsible for the association.

In contrast, we did not find an association of *IL8* with sensitization to the house dust mite. The gene coding for IL-8 is located on chromosome 4q12, where linkage to house dust mite sensitization has been reported in the English population but was not seen in our German population.<sup>25</sup> This might suggest that IL-8 is predominantly responsible for the inflammatory component that leads to asthma but not for the atopic background of

asthma. This is also in accordance with the above-mentioned findings that IL-8 inhibits the production of IgE and thus might even protect before the development of increased IgE levels.<sup>11,12</sup> However, one should bear in mind that our atopic population was smaller than the asthmatic sample, so the lack of association could be simply due to the smaller size of the population.

The role of IL-8 in the pathogenesis of JIA is less clear, although several studies have investigated its role in arthritis. Some reported higher expression of IL-8 in peripheral blood cells and synovial fibroblasts of patients with rheumatoid arthritis<sup>26,27</sup> and JIA.<sup>28</sup> However, others were unable to replicate these findings in JIA.<sup>29-31</sup> The association results of our study imply an opposite effect of polymorphisms within IL-8 in JIA and asthma. That is, all polymorphisms were significantly more common in children with JIA compared with patients with asthma. Studies of IL-8 in different subtypes of arthritis have shown different levels of IL-8 depending on the disease subtype. Our study population consisted of children with antinuclear antibody-positive JIA. This represents a rather clearly defined subtype of arthritis in which the role of IL-8 is not yet clarified. Another possible explanation for the significant difference of alleles between asthma and JIA might be the different predominant immune response of both diseases. Asthma is a  $T_H2$  disease with high production of IL-4 and IL-13, which stimulate human bronchial epithelial cells to release IL-8.<sup>32</sup> In contrast, JIA is a typical  $T_H1$  disease with production of IFN- $\gamma$ . IFN- $\gamma$  inhibits the production of IL-8 in arthritic osteoblasts.<sup>33</sup> Thus, the surrounding cytokine milieu might essentially influence the production and function of IL-8 independently of the polymorphisms.

In this study, the polymorphisms were not in HWE in the JIA population. Because the population was recruited from the same area as the 3 other populations and has been shown to be in HWE in other genes, this is most likely due to the small sample size.

In conclusion, we demonstrated for the first time an association of polymorphisms in *IL8* with bronchial asthma. In addition, the results suggest that RSV bronchiolitis and bronchial asthma have at least some different genetic factors: the same promotor polymorphism in *IL8* that causes susceptibility to RSV bronchiolitis might protect against asthma. Finally, the results suggest a distinct and perhaps even opposite role of IL-8 in asthma and JIA.

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