TNF-308 Modifies the Effect of Second-Hand Smoke on Respiratory Illness–Related School Absences

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Rationale: Exposure to second-hand smoke (SHS) has been associated with increased risk of respiratory illness in children including respiratory illness–related school absences. The role of genetic susceptibility in risk for adverse effects from SHS has not been extensively investigated in children.

Objective: To determine whether the tumor necrosis factor (TNF) G-308A genotype influences the risk for respiratory illness–related school absences associated with SHS exposure.

Methods: Incident school absences were collected, using an active surveillance system, between January and June 1996, as part of the Air Pollution and Absence Study, a prospective cohort study nested in the Children’s Health Study. Buccal cells and absence reports were collected on 1,351 students from 27 elementary schools in California.

Measurements and Main Results: Illness-related school absences were classified as nonrespiratory and respiratory illness–related, which were further categorized into upper or lower respiratory illness–related absences based on symptoms. The effect of SHS exposure on respiratory illness–related absences differed by TNF genotype (p interaction, 0.02). In children possessing at least one copy of the TNF-308 A variant, exposure to two or more household smokers was associated with a twofold risk of a school absence due to respiratory illness (relative risk, 2.13; 95% confidence interval, 1.34, 3.40) and a fourfold risk of lower respiratory illness–related school absence (relative risk, 4.15; 95% confidence interval, 2.57, 6.71) compared with unexposed children homozygous for the common TNF-308 G allele.

Conclusions: These results indicate that a subgroup of genetically susceptible children are at substantially greater risk of respiratory illness if exposed to SHS.

Keywords: epidemiology; school absence; second-hand smoke; TNF

Exposure to second-hand smoke (SHS) is common among children and causes substantial morbidity. According to the Third National Health and Nutrition Examination Survey, 43% of children between the ages of 4 and 11 yr are exposed to SHS in the home (1). Results from the Behavioral Risk Factor Surveillance System indicate that among smokers in the United States, nearly 88% allow smoking in some or all areas of the home (2). Nearly 10% of current smokers have a child in the home, and state-specific prevalence estimates for SHS exposure in children (< 18 yr) range from 12.3% in California to 34.2% in Kentucky (2).

The contributions of SHS exposure to a wide spectrum of respiratory illnesses in children have been well documented (3–10). Estimates of population-attributable risk for SHS exposure in children range from 9% for asthma prevalence to 25% for hospital admissions due to lower respiratory symptoms (5). We have previously reported that SHS exposure increases respiratory illness–related school absences in children, an important adverse outcome associated with large economic costs (3).

Although SHS is common and is associated with increased respiratory illnesses and respiratory illness–related school absences, variations in susceptibility to SHS have not been extensively studied. We hypothesized that an inflammatory response to SHS underlies the adverse respiratory effects of SHS, and that variation in susceptibility reflects differences in the magnitude of this inflammatory response. Tumor necrosis factor (TNF) α is an important cytokine in the inflammatory response to SHS and its gene is a candidate gene for susceptibility to SHS (11–14). Located on chromosome 6 at location p21, TNF has a common variant in the promoter region, G-308A, which has been associated with TNF expression regulation in some studies (15–17). Although the TNF region is highly polymorphic with a complex pattern of long-range linkage disequilibrium, studies of respiratory conditions have focused on the G-308A single nucleotide polymorphism because of its reported associations with inflammation and inflammatory diseases, including asthma (16–21). To investigate the role of TNF G-308A variant in susceptibility of SHS-exposed children to respiratory illness, we examined data from the Air Pollution and Absence Study (APAS), a prospective cohort study of school absences that was nested within the Children’s Health Study, a multiyear prospective study of the respiratory health development of several thousand children attending public schools (22). We assessed the joint effects of SHS and TNF-308 genotype on the incidence of respiratory illness–related absences in a group of fourth-grade students from 12 southern California communities.

METHODS

APAS was a prospective cohort study conducted as part of the Children’s Health Study (23, 24). This analysis focused on school absence data gathered for 1,935 fourth-grade students from 27 elementary schools across 12 southern Californian communities between January and June 1996. Parents or guardians of study participants completed written informed consent and baseline questionnaires pertaining to their child’s sociodemographics, medical histories, exposure histories, and household characteristics. Of the 1,935 subjects selected for this study, approximately 70% (n = 1,351) provided a buccal cell sample. The University of Southern California’s Institutional Review Board for human studies approved the study protocols.

Respiratory Illness Incidence

Incident absences were documented via an active surveillance system augmented by telephone interviews with parents or guardians to collect
additional information. An incident absence was defined as an absence that followed attendance on the preceding school day and was regarded as an independent event regardless of whether it occurred in the same subject. Participating schools provided daily absence summary information for study subjects within 4 wk of each absence, and each reported absence was categorized as an illness-related or non–illness-related absence. Telephone interviews with parents were conducted within 4 wk of a reported illness-related school absence or absence that could not be categorized based on existing data; non–illness-related absences were not further characterized (25, 26).

Illness-related school absences were categorized into respiratory absences and gastrointestinal absences, based on symptoms information collected by telephone interviews with parents. Respiratory absences were defined as absences with one or more of the following symptoms: runny nose/sneezing, sore throat, cough, earache, wheezing, or asthma attack. These were further classified into mutually exclusive categories of upper and lower respiratory illness–related absences. An upper respiratory illness–related absence was defined as a respiratory illness absence with one or more of the following symptoms: runny nose/sneezing, sore throat, and earache. A lower respiratory illness–related absence was defined as a respiratory illness absence with wet cough, wheezy asthma. Absences in which study participants exhibited both lower and upper respiratory symptoms were placed in the lower respiratory illness category.

Variable Definition

SHS exposure was defined by the child’s exposure to smokers in the home, including regular visitors. The number of reported smokers in the home (none, one, and two or more) was based on parent/guardian written responses on a self-administered questionnaire. The number of cigarettes smoked per day inside the home was categorized as none, 1 to 29, and 30 or greater. Each child’s asthma status was defined by parental response to the question, “Has a doctor ever diagnosed this child as having asthma?” Body mass index (BMI) was calculated from measured height and weight and categorized as underweight, normal, risk of overweight, and overweight by creating age-specific percentiles of BMI (< 5% = underweight, 5–85% = normal, 85–95% = risk of overweight, > 95% = overweight) (27).

Laboratory Methods

Buccal cells were collected as a source of genomic DNA from consenting study subjects. Genomic DNA was isolated from buccal mucosal cells using Purgene DNA isolation kit (Gentra Systems, Minneapolis, MN). The G to A transition polymorphism at position -308 of human TNF was identified by polymerase chain reaction (PCR), and allelic discrimination assays were performed on an ABI Prism 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Standard setup and operation for the allelic discrimination assay were used as detailed by the ABI Prism 7700 Sequence Detection System user’s manual. The QuantiTect Probe PCR kit (Qiagen, Valencia, CA) was used for PCR reactions. Primers and fluorescent probes were designed by using Primer Express applications-based primer design software (Applied Biosystems). The forward primer for human TNF was 5'-GAAATGGAGGCAATAGGTTTGA-3', and the reverse primer was 5'-GTAGGACCCTGGAGCTGGA-3'. The minor groove-binding probe for the G allele was 5’-CCGTCCTCATGCC-3’ and the minor groove-binding probe for the A allele was 5’-CGGTCCTCATGCC-3’ (Applied Biosystems). Ten percent of the samples were selected as quality controls and eight samples of each genotype for TNF were validated by using PCR and restriction fragment length polymorphism methods.

Statistical Analysis

A Poisson regression model was fitted to estimate relative risks (RR) and 95% confidence intervals (95% CI) for illness-related school absences among genotyped subjects (28, 29). An offset term, composed of the log-expected value of the dependent variable, was included in the model to normalize the fitted cell means on a per-subject basis. The deviance divided by its degrees of freedom was used to account for overdispersion in all models (30). Inclusion of potential confounders in the models was based on a review of the literature and changes in effect estimates of at least 10% in multivariate analyses. On the basis of these criteria and the study design, the following variables were selected as potential confounders: community, race, sex, age, asthma status, family income, health insurance status, and BMI.

The dominant coding of the -308 A allele was used in the analysis of TNF and the interaction between TNF and SHS exposure. The dominant model was chosen based on functional assays that supported a dominant effect for the inflammatory effects of the variant alleles. Three SHS variables were considered, including a simple “yes/no” variable for exposure at home, a second variable coding the number of smokers in the home (0, 1, 2+, ) and a third variable coding the number of cigarettes smoked at home (0, 1, 29, 30+). Interactions between TNF and each smoking variable were evaluated by adding the corresponding product term to the model and using a likelihood ratio test to test its significance. Within genotype category, a one degree-of-freedom trend test was used to evaluate possible exposure–response relationships across categories of the SHS variables. All tests assumed a two-sided alternative hypothesis and a 0.05 significance level. All analyses were conducted using SAS/STAT software, version 9.1 (SAS Institute, Cary, NC).

RESULTS

The distribution of selected characteristics among APAS participants and relative risks for illness-related absences are shown in Table 1. At study enrollment, 75% of study subjects were 10 yr
of age. Approximately 15% of the study participants reported physician-diagnosed asthma, which was associated with a 50% increase in risk for illness-related school absences. More than half of the study participants were non-Hispanic whites and most had health insurance (51 and 82%, respectively). Genotyping for TNF was completed for 1,351 subjects, and 24% possessed one or two copies of the variant A allele.

Children exposed to SHS had increased risk for lower respiratory illness–related school absences (Table 2). Among this group of fourth-grade children, 20% were exposed to SHS and nearly 6% of subjects had two or more smokers in the home. There was a 51% greater risk of lower respiratory illness–related school absences among children with SHS exposure compared with those unexposed to SHS. With increasing number of household smokers and number of cigarettes smoked, lower respiratory illness–related absence rates also increased (p trend < 0.01). TNF-308 genotype was not strongly associated with respiratory illness–related school absences.

The association of SHS exposure with respiratory illness–related school absences was most apparent in subjects who inherited at least one copy of the variant TNF-308 A allele. Examining the crude respiratory absence incidence rates among children grouped by TNF and SHS exposure status (Figure 1) showed children with the GG genotype exhibited similar incidence rates whether exposed or unexposed to SHS. Unexposed children with at least one copy of the variant A allele had similar incidence rates of respiratory illness–related school absences compared with children with the GG genotype; however, absence rates among those possessing the variant A allele showed a marked increase with SHS exposure. This pattern is similar for illness-related absences, and upper respiratory and lower respiratory illness–related absences.

To further examine the role of the TNF genotype in the association of absence rates with SHS, we estimated the relative risk, adjusted for community, age, race, sex, income, insurance, asthma status, and BMI (Table 3). We found that exposed children with the variant A allele had a 75% increase in risk for illness-related absences compared with unexposed children with the GG genotype. The increase in illness-related absence risk associated with SHS exposure was larger in the TNF-308 A allele population than in the G allele group (p interaction, 0.01). Among subjects carrying the variant A allele, illness-related absence risk increased as the number of smokers increased (p trend < 0.01). We observed no increase in risk among children possessing the GG genotype.

The relationship of SHS exposure and TNF-308 genotype with respiratory illness–related absences and lower respiratory illness–related absences (but not upper respiratory illness–related absences) was comparable to that found for illness absences. A strong dose–response relationship was seen for respiratory (p trend < 0.01) and lower respiratory illness (p trend < 0.01) with increasing number of household smokers. The effect of SHS exposure and number of smokers on lower respiratory illness differed significantly by TNF genotype (p interaction < 0.01). Children with at least one variant A allele who were exposed to 30 or more cigarettes per day had a relative risk of 2.75 (95% CI, 1.26, 5.98) for respiratory illness–related school absences compared with nonexposed children with the GG genotype. A dose–response relationship was observed among children with the variant allele who were exposed to increasing numbers of cigarettes per day (p trend, 0.03). Restricting the analyses to children without asthma did not substantially alter the above findings (see Table E1 in the online supplement). No significant relationships were found between SHS exposure and absences due to upper respiratory symptoms.

**DISCUSSION**

Previous studies have reported that exposure to SHS contributes to the occurrence of respiratory symptoms and diseases among infants and younger children (3–10). Adverse respiratory health outcomes caused by SHS exposure include increased occurrence and severity of respiratory symptoms, respiratory infections, physician visits, emergency room visits, hospital admissions, and

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**TABLE 2. THE RELATIVE RISK OF SECOND-HAND SMOKE, NUMBER OF SMOKERS, NUMBER OF CIGARETTES SMOKED, AND TNF ON SCHOOL ABSENCES: AIR POLLUTION AND ABSENCE STUDY, 1996**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Illness RR (95% CI)</th>
<th>Any Respiratory RR (95% CI)</th>
<th>Lower Respiratory² RR (95% CI)</th>
<th>Upper Respiratory³ RR (95% CI)</th>
<th>Nonillness RR (95% CI)</th>
</tr>
</thead>
<tbody>
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<td>Second-hand smoke*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<tr>
<td>Yes</td>
<td>1.16 (0.94, 1.43)</td>
<td>1.05 (0.82, 1.34)</td>
<td>1.51 (1.12, 2.04)</td>
<td>1.02 (0.76, 1.36)</td>
<td>1.05 (0.83, 1.33)</td>
</tr>
<tr>
<td>No. smokers*</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
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<td>1.00</td>
</tr>
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<td>1</td>
<td>1.01 (0.78, 1.33)</td>
<td>0.90 (0.65, 1.24)</td>
<td>1.23 (0.84, 1.81)</td>
<td>0.91 (0.63, 1.32)</td>
<td>1.14 (0.87, 1.50)</td>
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<tr>
<td>&gt; 2</td>
<td>1.44 (1.07, 1.93)</td>
<td>1.38 (0.98, 1.94)</td>
<td>2.05 (1.37, 3.06)</td>
<td>1.29 (0.86, 1.93)</td>
<td>0.98 (0.68, 1.42)</td>
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<td>0.22</td>
<td>0.0013</td>
<td>0.43</td>
<td>0.73</td>
</tr>
<tr>
<td>No. cigarettes*</td>
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<td>1–29</td>
<td>1.14 (0.92, 1.42)</td>
<td>1.03 (0.79, 1.34)</td>
<td>1.16 (0.82, 1.64)</td>
<td>1.13 (0.84, 1.51)</td>
<td>1.11 (0.87, 1.41)</td>
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<td>&gt; 30</td>
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<td>1.36 (0.71, 2.60)</td>
<td>3.83 (2.07, 7.11)</td>
<td>0.91 (0.37, 2.21)</td>
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</tr>
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<td>0.0032</td>
<td>0.59</td>
<td>0.30</td>
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<td>TNF¹</td>
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<td></td>
<td></td>
<td></td>
</tr>
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<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>G/A or A/A</td>
<td>1.16 (0.97, 1.39)</td>
<td>1.15 (0.93, 1.41)</td>
<td>1.22 (0.93, 1.60)</td>
<td>1.10 (0.87, 1.41)</td>
<td>0.83 (0.67, 1.03)</td>
</tr>
</tbody>
</table>

*Adjusted for community, age, race, sex, income, insurance, asthma, and body mass index (BMI).
† Adjusted for community, age, race, sex, income, insurance, asthma, BMI, and second-hand smoke.
‡ Subjects that exhibited only upper respiratory symptoms are included in this category.
§ Subjects that exhibited only upper respiratory symptoms are included in this category.

**Definition of abbreviations:** CI = confidence interval; RR = relative risk.
transient changes in lung function. In this prospective study of school absences, we found that SHS-exposed children who carried a TNF-308 variant allele were at highest risk for respiratory illness–related school absences, especially lower respiratory illness–related school absences. Among this group of genetically susceptible children, a strong dose–response relationship was apparent for respiratory illness–related absence risk, and the risk increased as the number of household smokers and the number of cigarettes smoked increased. Because exposure to SHS is widespread and illness-related absences from school are common events that represent a broad spectrum of morbidity from mild transient illnesses to the most severe illnesses requiring emergency room visits or hospital admissions, the identification of a group of children who are genetically susceptible to SHS exposure has clinical and public health implications for prevention of adverse respiratory health effects (10, 31, 32).

Although we did not directly study the mechanisms for the effects of SHS exposure on absenteeism in children with the TNF-308 variant, it is likely that SHS exposure increases the risk of an absence by increasing the risk and severity of respiratory infections, as well as increasing asthma-related airflow obstruction, inflammation, and symptoms. Increased susceptibility for SHS associated with the TNF-308 variant was apparent in children without asthma, suggesting that increased risk may arise from common acute illnesses, such as from viral infections that affect the lower respiratory tract. We were unable to assess whether children with asthma had the same increased susceptibility to SHS associated with the TNF-308 variant as children

### Table 3. The Relative Risk of Second-Hand Smoke and TNF, and Number of Smokers and TNF, on School Absences: Air Pollution and Absence Study, 1996

<table>
<thead>
<tr>
<th>Variables</th>
<th>Type of School Absence</th>
<th>n</th>
<th>Any Respiratory Illness RR (95% CI)</th>
<th>Lower Respiratory Illness RR (95% CI)</th>
<th>Upper Respiratory Illness RR (95% CI)</th>
<th>Nonillness RR (95% CI)</th>
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</thead>
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</tr>
<tr>
<td>G/G</td>
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<td>824</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>173</td>
<td>0.97 (0.75, 1.26)</td>
<td>0.86 (0.64, 1.17)</td>
<td>1.02 (0.70, 1.50)</td>
<td>0.96 (0.68, 1.35)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>p interaction 0.0131</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>G/A or A/A</td>
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<td>266</td>
<td>1.04 (0.85, 1.27)</td>
<td>1.02 (0.81, 1.29)</td>
<td>0.90 (0.66, 1.25)</td>
<td>1.07 (0.82, 1.40)</td>
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<td>50</td>
<td>1.75 (1.26, 2.41)</td>
<td>1.64 (1.12, 2.39)</td>
<td>3.00 (1.97, 4.56)</td>
<td>1.24 (0.76, 2.02)</td>
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<td></td>
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<td>p trend 0.0001</td>
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<td></td>
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<tr>
<td>G/G</td>
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<td>1.00</td>
<td>1.00</td>
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<td>1.00</td>
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<td>119</td>
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<td>1.10 (0.71, 1.69)</td>
<td>0.88 (0.58, 1.35)</td>
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<td>≥ 2</td>
<td>55</td>
<td>1.04 (0.69, 1.57)</td>
<td>1.01 (0.63, 1.63)</td>
<td>0.84 (0.41, 1.72)</td>
<td>1.24 (0.75, 2.04)</td>
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<tr>
<td>G/A or A/A</td>
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<td>27</td>
<td>1.03 (0.81, 1.30)</td>
<td>0.91 (0.66, 1.25)</td>
<td>1.08 (0.82, 1.41)</td>
<td>0.86 (0.68, 1.08)</td>
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<tr>
<td></td>
<td>≥ 2</td>
<td>22</td>
<td>2.27 (1.52, 3.38)</td>
<td>2.13 (1.34, 3.40)</td>
<td>4.15 (2.57, 6.71)</td>
<td>1.45 (0.77, 2.73)</td>
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<td>p interaction 0.0366</td>
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For definition of abbreviations, see Table 2.
Values are adjusted for community, age, race, sex, income, insurance, asthma, and body mass index.
* Subjects who exhibited lower respiratory symptoms only or both lower and upper respiratory symptoms are included in this category.
† Subjects that exhibited only upper respiratory symptoms are included in this category.
without asthma due to insufficient numbers of exposed subjects with asthma possessing the variant genotype.

The role of the TNF-308 variant in respiratory susceptibility to tobacco smoke has not been extensively studied. The genetic susceptibility associated with the TNF-308 variant is likely mediated by variation in inflammatory responses to SHS (11–14). Smoke from cigarettes contains a wide variety of chemicals, including nitric oxide (NO), nitrogen dioxide (NO2), carbon monoxide (CO), transition metals, and reactive organic compounds, such as polycyclic aromatic hydrocarbons (PAHs), that promote oxidative stress (33–37). Oxidative stress is intimately involved in multiple inflammation processes, which are regulated, in part, by cytokines such as TNF (11, 13). Second-hand smoke exposure is associated with the activation of redox-sensitive transcription factors, nuclear factor-kappa B, and activator protein-1, which activates the genes of proinflammatory mediators such as TNF (19, 38). The TNF-308 variant allele may alter gene expression and amplify the intensity of the inflammatory response to SHS, leading to increased risk and severity of respiratory illness in susceptible groups (19, 20, 39).

Studies of the TNF-308 variant have shown a direct association of this proinflammatory variant with wheezing and asthma outcomes, conditions in which airway inflammation plays a central role, and which increase the rates of absences (16, 17, 40–44). Furthermore, SHS exposure is associated with childhood asthma risk and exacerbations. On the basis of the pathophysiology of asthma and the pathways that mediate SHS effects, we speculate that the risk associated with the TNF-308 variant and SHS may be larger in children with asthma than those without asthma. Because asthma and SHS exposure are common in childhood, studies examining the role of the TNF-308 variant in susceptibility for asthma exacerbations as well as new-onset asthma among children exposed to household SHS warrant a high priority.

Our results should be interpreted in light of some study limitations. SHS exposure assessment was based on self-report of parental smoking. Although estimates based on self-reported smoking behavior have associated measurement error, studies have shown that questionnaires provide reasonably valid long-term estimates of exposure (45–47). Because there is little evidence that short-term measurement or “snapshots” of exposure improve estimates of long-term SHS exposure from questionnaires, we did not measure household levels or biomarkers of SHS exposure such as cotinine. We limited the possibility of differential misclassification of SHS exposure by collecting exposure information prospectively and assessed parental reporting by comparing analyses using parental responses to those based on independent reports made by the children at school. To explain our findings, exposure misclassification would need to vary by both children’s risk and genotype. On the basis of these considerations and sensitivity analyses, it is unlikely that bias from differential exposure misclassification explains our findings. Of the total number of eligible subjects, approximately 30% did not provide buccal cell samples for this study, which may give rise to selection bias. However, comparing those subjects who were and were not genotyped did not reveal any marked differences in demographic factors or absence rates, making “healthy student bias” or selection bias an unlikely explanation for our findings.

Furthermore, other cofactors of inflammation may potentially confound the relationship between SHS and respiratory illness. Some that have been well described in the literature include air pollution, personal smoking, indoor allergens, allergic disease, and family history of asthma (25, 48–54). Air pollution was indirectly taken into account by adjusting for community of residence; and adjusting for cats, dogs, allergy, and history of asthma individually and in combination resulted in a negligible change in the effect of SHS, TNF, and respiratory illness–related school absences.

Conducting a candidate-gene association study to assess the role of the TNF-308 variant and susceptibility to SHS requires careful interpretation in light of the potential for population stratification and linkage disequilibrium. In our analyses of TNF genotype, SHS, and school absences, we considered the effects of ethnicity (non-Hispanic white and Hispanic white) and found the risk pattern of school absences showed neither important confounding by ethnicity nor substantial differences in effects by ethnic status. In addition, the minor allele frequency showed no marked difference by ethnicity (non-Hispanic white and Hispanic white). Taken together, population stratification is unlikely to explain our findings. We note that the region spanning the TNF loci has long-range linkage disequilibrium (over 45 kb) and the associations with the -308 variant may be due to other linked variants in this region (15). The TNF region comprises fewer than 20 common haplotypes. The -308 variant occurs on two long-range haplotypes in populations with European ancestry, one of which is the most common haplotype in the linked region (15). On the basis of the available information on locus-region linkage structure, the associations with the -308 A variant reflect a comparison of these two haplotypes containing the -308 variant to the remaining haplotypes across the 45-kb region and indicate that the -308 A variant or another variant on the two haplotypes underlies the increased susceptibility to SHS.

In summary, the detrimental effects of SHS on respiratory illness absences in children having at least one copy of the variant TNF A allele suggest that a relatively common genetic variant, or haplotype marked by this variant, places healthy children at increased risk for the adverse effects of SHS. Because of the high prevalence of SHS exposure and the adverse effects of SHS on respiratory illness, further research is warranted to identify genetically susceptible groups and to develop targeted interventions that reduce exposure among those at greatest risk for adverse events.

Conflict of Interest Statement: None of the authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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