ADCYAP1R1 and Asthma in Puerto Rican Children

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Rationale: Epigenetic and/or genetic variation in the gene encoding the receptor for adenylate-cyclase activating polypeptide 1 (*ADCYAP1R1*) has been linked to post-traumatic stress disorder in adults and anxiety in children. Psychosocial stress has been linked to asthma morbidity in Puerto Rican children.

Objectives: To examine whether epigenetic or genetic variation in *ADCYAP1R1* is associated with childhood asthma in Puerto Ricans.

Methods: We conducted a case-control study of 516 children ages 6–14 years living in San Juan, Puerto Rico. We assessed methylation at a CpG site in the promoter of *ADCYAP1R1* (cg11218385) using a pyrosequencing assay in DNA from white blood cells. We tested whether cg11218385 methylation (range, 0.4–6.1%) is associated with asthma using logistic regression. We also examined whether exposure to violence (assessed by the Exposure to Violence [ETV] Scale in children 9 yr and older) is associated with cg11218385 methylation (using linear regression) or asthma (using logistic regression). Logistic regression was used to test for association between a single nucleotide polymorphism in *ADCYAP1R1* (rs2267735) and asthma under an additive model. All multivariate models were adjusted for age, sex, household income, and principal components.

Measurements and Main Results: Each 1% increment in cg11218385 methylation was associated with increased odds of asthma (adjusted odds ratio, 1.3; 95% confidence interval, 1.0–1.6; $P = 0.03$). Among children 9 years and older, exposure to violence was associated with cg11218385 methylation. The C allele of single nucleotide polymorphism rs2267735 was significantly associated with increased odds of asthma (adjusted odds ratio, 1.3; 95% confidence interval, 1.02–1.67; $P = 0.03$).

Conclusions: Epigenetic and genetic variants in *ADCYAP1R1* are associated with asthma in Puerto Rican children.

Keywords: methylation; *ADCYAP1R1*; childhood asthma; Puerto Ricans; violence

Puerto Ricans are disproportionately affected by childhood asthma (1, 2). In the United States, the prevalence of asthma is higher in

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Puerto Rican children share a disproportionate burden of asthma and are often exposed to stressful events. Although psychosocial stress has been linked to childhood asthma in Puerto Ricans and in members of other ethnic groups, little is known about the mechanisms underlying this association. Epigenetic or genetic variation in the promoter of the gene for the receptor for adenylate-cyclase activating polypeptide 1 (*ADCYAP1R1*) has been linked to post-traumatic stress disorder in non-Puerto Rican adults and to anxiety in non-Puerto Rican children.

What This Study Adds to the Field

We show that methylation of a CpG site and a single nucleotide polymorphism in the promoter of *ADCYAP1R1* are each associated with asthma in Puerto Rican children living in San Juan, Puerto Rico. In addition, we report that exposure to violence is associated with *ADCYAP1R1* methylation and asthma in these children.

Puerto Rican children (18.4%) than in non-Hispanic blacks (14.6%) or non-Hispanic whites (8.2%) (3). Puerto Rican children have higher rates of emergency department visits or hospitalizations for asthma than non-Hispanic black or non-Hispanic white children. Exposure to violence and psychosocial stress may contribute to the high burden of asthma in Puerto Rican children (4, 5).

Puerto Ricans living in the island of Puerto Rico and in the US mainland are exposed to significant levels of personal and community violence (6–8). Puerto Ricans may also be highly susceptible to emotional distress after exposure to violence (9, 10). For example, Puerto Ricans were more likely to report symptoms of post-traumatic stress disorder (PTSD) or to have a per-event panic attack after the attacks of September 11, 2001 than members of other ethnic groups (9). PTSD and psychosocial stress have been associated with increased asthma morbidity (11), and we previously showed that physical or sexual abuse (a traumatic event) is associated with increased asthma morbidity in Puerto Rican children of school age living in San Juan, Puerto Rico (4). The biologic mechanisms linking exposure to violence or psychosocial stress and asthma are largely unknown (11).

Pituitary adenylate cyclase-activating polypeptide (PACAP) broadly regulates cellular stress responses. A recent study examined the role of the PACAP-PAC1 receptor pathway in the pathogenesis of PTSD in adults with significant exposure to lifetime traumatic events (12). Using DNA from white blood cells (WBCs), Ressler and coworkers (12) showed that methylation of a site in a CpG island in the 5' untranslated region of the gene for the PAC1 receptor (*ADCYAP1R1*) was associated with PTSD. In the same

(Received in original form October 4, 2012; accepted in final form December 26, 2012)

Supported by Grant R01HL079966 from the NIH and the Department of Pediatrics, Children's Hospital of Pittsburgh of University of Pittsburgh Medical Center.

Author Contributions: W.C., G.C., and J.C.C. participated in study design and implementation. W.C., J.M.B., Y.-Y.H., A.A.B., D.E.W., J.K.K., and J.C.C. participated in the data analysis. E.A.-P., N.B., C.S., A.C., M.A., and A.C.-S. participated in sample processing and data generation. W.C. and J.C.C. wrote the initial draft of the manuscript. All coauthors participated in the review of the manuscript and approved its final version.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 187, Iss. 6, pp 584–588, Mar 15, 2013

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Originally Published in Press as DOI: 10.1164/rccm.201210-1789OC on January 17, 2013

Internet address: www.atsjournals.org

study, results from a concurrent genetic association study and experiments in murine models further implicated alterations in the PACAP-PAC1 receptor pathway in the causality of PTSD. Given those novel findings, a reported link between PTSD and asthma morbidity (13), and our previous findings in Puerto Rican children, we hypothesized that DNA methylation or genetic variation in *ADCYAP1R1* would be associated with childhood asthma in Puerto Ricans. We examined this hypothesis in a case-control study of asthma in children living in San Juan, Puerto Rico.

METHODS

Subject Recruitment

Subject recruitment has been previously described in detail elsewhere (14). From March 2009 to June 2010, children were chosen from randomly selected households, using a scheme similar to that of a prior study (15). In brief, households in the Standard Metropolitan Area of San Juan were selected by a multistage probability sample design (15). Primary sampling units were randomly selected neighborhood clusters based on the 2000 US census, and secondary sampling units were randomly selected households within each individual primary sampling units. A household was eligible if at least one resident was a child 6–14 years old. Within each housing unit selected, one child was selected for screening. In households with multiple eligible children, one child was randomly selected by using Kish tables. On the basis of the sampling design, a total of 7,073 households were selected, and 6,401 (90.5%) were contacted. Of these 6,401 households, 1,111 had at least one child within the age range of the study who met other inclusion criteria (see below). To reach our target sample size (~700 children), we attempted to enroll a random sample ($n = 783$) of these 1,111 children. Parents of 105 of these 783 eligible households refused to participate or could not be reached. There were no significant differences in age, sex, or area of residence between eligible children who did ($n = 678$ [86.6%]) and did not ($n = 105$ [13.4%]) participate. Of the 678 participants, 583 had blood samples collected for DNA extraction and were included in the initial analysis.

The main recruitment tool was a screening questionnaire given to parents of children ages 6–14 years to obtain information about the child's respiratory health and Puerto Rican ancestry. We selected as cases children who had physician-diagnosed asthma, wheeze in the prior year, and four Puerto Rican grandparents. We selected as control subjects children who had no physician-diagnosed asthma, no wheeze in the prior year, and four Puerto Rican grandparents.

Study Procedures

Participants completed a protocol that included questionnaires and collection of blood for DNA extraction. The parents of all participants completed a slightly modified version of a questionnaire used in the Genetics of Asthma in Costa Rica Study (16). Children ages 9 years and older completed the Spanish version of the validated Exposure to Violence (ETV) Scale questionnaire (see Table E1 in the online supplement) (17–19).

A pyrosequencing assay was conducted to assess methylation at a CpG site (cg11218385) located within 1,136 base pairs and in the same CpG island (in the promoter of *ADCYAP1R1*, Chr7: 31,091,717–31,093,077) as the CpG site (cg27076139) previously associated with PTSD in adults (12). The assay was performed in DNA extracted from WBCs using PyroMark Q24 (Qiagen, Valencia, CA). In brief, diluted bisulfite-converted DNA was subjected to polymerase chain reaction amplification and samples were randomly allocated. Polymerase chain reaction products were checked on agarose gel, and then immobilized to streptavidin sepharose HP beads and denatured. The released pyrophosphate (PPi), in a quantity equimolar to the amount of incorporated nucleotide, was converted to light by an enzyme cascade. The light was detected by charge-coupled device sensors and seen as a peak in the raw data output (Pyrogram). CpG analysis was conducted using PyroMark Q24 software. The percentage of methylation was reported from the built-in software. Replicated samples and artificial control subjects (at 0%, 25%, 50%, 75%, and 100% methylation) were added for quality control and experimental validation. The subject pass rate was greater than 99.5% and the reproducibility among technical replicates was 99.3%.

Although we have genome-wide genotypic data in study participants (20), the single nucleotide polymorphism (SNP) in *ADCYAP1R1* that has been implicated in PTSD and childhood anxiety (rs2267735) is neither included in our genotyping platform (HumanOmni2.5 Bead-Chip; Illumina, Inc, San Diego, CA) nor in moderate-to-high linkage disequilibrium ($r^2 \geq 0.6$) with any SNP in that platform. We thus genotyped SNP rs2267735 using the TaqMan 5' exonuclease assay (Life Technologies, Carlsbad, CA) using primers provided by ABI (ABI, Foster City, CA). The subject pass rate was greater than 97% and the reproducibility among replicates was 100%.

Written parental consent was obtained for participating children, from whom written assent was also obtained. The study was approved by the Institutional Review Boards of the University of Puerto Rico (San Juan, PR); Brigham and Women's Hospital (Boston, MA); and the University of Pittsburgh (Pittsburgh, PA).

Statistical Analysis

Bivariate statistical analyses were conducted using Fisher exact tests for categorical variables, and two-tailed t tests for pairs of binary and continuous variables. Logistic regression was used for the multivariate analysis of methylation at the *ADCYAP1R1* CpG site (expressed as a percentage) and asthma (physician-diagnosed and wheeze in the prior year). Because of their potential association with DNA methylation or asthma in Puerto Rican children, the following covariates were included in all multivariate models: age, sex, household income ($<$ vs. $\geq$ \$15,000 [near the median income for households in Puerto Rico in 2008–2009 (21)]), and principal components (22) (calculated from available genome-wide genotypic data (20) [using EIGENSTRAT (22)] to adjust for potential population stratification caused by differences in DNA methylation and asthma prevalence among ancestral populations for Puerto Ricans [20]).

We examined whether ETV is associated with methylation at the CpG site of *ADCYAP1R1* using multivariate linear regression. For this analysis, we used a summary score (ranging from 0 to 15) obtained from the ETV Scale questionnaire (17–19) (administered to children ≥ 9 yr old). Next, we examined *ADCYAP1R1* methylation, ETV, and asthma jointly within a causal inference framework using structural equation models [SEMs] (23) (see Figure E1). We aimed to compare two models relating ETV to asthma, one with the mediator (methylation) in the model, and the other one without. Both SEMs were adjusted for the same variables as those included in the final multivariate analysis of ETV or *ADCYAP1R1* methylation and asthma. We estimated the proportion of the ETV effect on asthma that is mediated by *ADCYAP1R1* methylation, calculated as indirect effect of ETV (see Figure E1, path b) divided by total effect of ETV (see Figure E1, paths b and c). All of the estimated coefficients were rescaled before computing indirect effects (24). Finally, we tested for association between SNP rs2267735 and asthma under an additive model using logistic regression. The multivariate logistic regression model was adjusted for age, sex, household income, and principal components. All analyses were conducted in R (www.r-project.org, version 2.14.0) and STATA (StataCorp LP, College Station, TX; version 12.0, SEM module).

RESULTS

After excluding 66 subjects without sufficient DNA for the pyrosequencing assay ($n = 44$) or who failed quality control for our genome-wide association study (20) (e.g., sex mismatch, duplicate samples [$n = 22$]), 517 (89%) of the 583 participants remained in this analysis, comprising 272 children with asthma (cases) and 245 children without asthma. One case in San Juan had extremely high methylation at the CpG site of interest (~20%, confirmed in additional replication experiments; all other samples had methylation levels between 0.4% and 6.1% [see Figure E2]) and was excluded from all primary analyses to avoid losing generalizability of the models (despite obtaining similar results when including this subject [see Table E2]). A comparison of children who were and were not included in this analysis is shown in Table E3. There were no significant differences in age, sex, or household income between children (cases or control subjects) who were and were not included in this analysis.

The main characteristics of study participants are shown in Table 1. Compared with control subjects, cases were significantly more likely to be boys and to have higher methylation of the CpG site at *ADCYAP1R1* than control subjects. There were no significant differences in age, household income, or ETV Scale score between cases and control subjects.

The results of the bivariate analysis of *ADCYAP1R1* methylation and asthma are shown in Figure E3. In this analysis, methylation of the CpG site in *ADCYAP1R1* was significantly associated with asthma. In a multivariate analysis (Table 2), each 1% increment in methylation was associated with approximately 30% higher odds of asthma.

We then examined the relationship between ETV and *ADCYAP1R1* methylation among children ages 9 years and older (324 of the 516 children in the methylation analysis). Control subjects who were 9 years and older were more likely to have a household income greater than or equal to \$15,000 per year than control subjects younger than 9 years (38% vs. 19%; $P < 0.05$); there were no other significant differences in sex or household income between cases or control subjects who were 9 years and older and cases or control subjects younger than 9 years. In the multivariate analysis of ETV and *ADCYAP1R1* methylation (Table 3), each one-point increment in the ETV Scale score was associated with significantly higher percentage of methylation of the *ADCYAP1R1* CpG site in control subjects and in all participants (cases and control subjects). For example, a difference of plus-10 points in the ETV Scale score is associated with an increment of 0.5% in methylation at the CpG site of interest. Although there was no significant linear association in cases, an ETV Scale score at or above the median (≥ 3 points) was associated with an increment of 0.3% in *ADCYAP1R1* methylation in cases (95% confidence interval [CI], 0.01–0.57; $P = 0.04$). Given the results noted above and our prior findings, we examined the relationship between ETV and asthma in the 324 participants aged 9 years and older (Table 4). After adjustment for age, sex, and other covariates, each one-point increment in the ETV Scale was significantly associated with approximately 10% excess odds of asthma.

On the basis of our findings for ETV, *ADCYAP1R1* methylation, and asthma, we used a more flexible nonparametric SEM to quantify the mediation effect of the *ADCYAP1R1* methylation within a causal inference framework. After adjustment for age, sex, and household income, we estimated that approximately 4% of the effect of ETV on asthma is mediated by the *ADCYAP1R1* methylation. However, this was not statistically significant ($P = 0.08$; see Table E4).

We tested for association between SNP rs2267735 and asthma in the 516 children included in the analysis of *ADCYAP1R1* methylation. SNP rs2267735 was in Hardy-Weinberg equilibrium in control subjects ($P = 0.90$). The minor allele (G) frequency for this SNP was 0.43 in cases and 0.49 in control subjects. The

TABLE 1. CHARACTERISTICS OF PARTICIPATING CHILDREN*

Variable	Control Subjects (n = 245)	Cases (n = 271)
Sex, male	121 (49%)	162 (60%) [†]
Age, yrs	11 (2.7)	10 (2.6)
Household income, $\geq$ \$15,000/yr	77 (33%)	84 (32%)
Exposure to Violence Scale score [‡]	3.0 (2.2)	3.4 (2.0)
Methylation (%) of CpG site in <i>ADCYAP1R1</i>	2.3 (0.78)	2.5 (0.88) [†]

* Mean (standard deviation) and number (percentage) are shown for continuous and binary traits, respectively.

[†] $P < 0.05$ for the comparison of cases and control subjects.

[‡] Available for 324 children ages 9 years and older (165 cases and 159 control subjects).

TABLE 2. *ADCYAP1R1* METHYLATION AND ASTHMA (N = 516)*

	Odds Ratio (95% Confidence Interval)	P Value
Each 1% increment in methylation at <i>ADCYAP1R1</i> CpG site	1.3 (1.0–1.6)	0.03
Age, yrs	0.9 (0.9–1.0)	0.07
Sex, male	1.5 (1.0–2.1)	0.04
Household income, $\geq$ \$15,000/yr	1.0 (0.7–1.5)	0.97

* Multivariate analysis conducted using logistic regression. The model was additionally adjusted for principal components.

C allele of SNP rs2267735 was significantly associated with increased odds of asthma in unadjusted (odds ratio [OR], 1.28; 95% CI, 1.01–1.61; $P = 0.04$) and adjusted (OR, 1.30; 95% CI, 1.02–1.67; $P = 0.03$) analyses. There was no significant modification of the effect of the SNP on asthma by sex or other covariates. SNP rs2267735 was not significantly associated with methylation of cg11218385. Consistent with prior results for PTSD in non-Puerto Rican adults, there was no significant association between any other SNP in *ADCYAP1R1* (available from our genome-wide genotypic data) and asthma.

DISCUSSION

To our knowledge, this is the first report implicating epigenetic or genetic variation in *ADCYAP1R1* and asthma. This is also the first study of DNA methylation and asthma in Puerto Ricans, an ethnic group heavily affected by this disease. We found a significant association between increased methylation of a CpG site (cg11218385) in the promoter of *ADCYAP1R1* and asthma among Puerto Rican children in San Juan, a city on the island of Puerto Rico. Among children in San Juan, increased exposure to violence (a chronic psychological stressor) was significantly associated with asthma. In these children, exposure to violence was also associated with increased methylation of cg11218385 in *ADCYAP1R1*. Among study participants, the C allele of SNP rs2267735 in *ADCYAP1R1* was associated with asthma but not with cg11218385 methylation.

Psychosocial stress has been associated with asthma or asthma morbidity in children in general and in Puerto Rican children in particular. Among 160 predominantly white and African American children (ages 4–6 yr) in Michigan, symptoms of traumatic stress were associated with fourfold increased odds of asthma (25). In a previous study of 678 young twins living in Puerto Rico, we showed that paternal PTSD is associated with increased asthma symptoms at age 1 year (5). In another study of school-aged children in San Juan, we reported that exposure to physical or sexual abuse in the previous year (a highly traumatic event) was significantly associated with approximately twofold increased odds of asthma and asthma morbidity (4). Our current results provide a plausible mechanism for the observed link between psychosocial stress and asthma.

In humans, signaling of PACAP regulates physiologic stress reactions and, together with corticotrophin-releasing hormone, modulates anxiety-related behavior (12, 26). A recent study implicated genetic-epigenetic variation in the gene for the PAC1 receptor (*ADCYAP1R1*) and PTSD and stress responses in heavily traumatized adults of predominantly African American descent (12). In that study, a SNP in an estrogen response element of *ADCYAP1R1* (rs2267735) was significantly associated with PTSD in women but not in men (12). In contrast to this female-specific genetic association, methylation of a CpG site in the promoter of *ADCYAP1R1* (assessed using DNA from WBCs) was significantly associated with PTSD in adults of both sexes. Whereas there has been no replication study of *ADCYAP1R1* methylation and PTSD,

TABLE 3. EXPOSURE TO VIOLENCE AND *ADCYAP1R1* METHYLATION IN CHILDREN OLDER THAN 9 YEARS (N = 324)*

	Beta (95% Confidence Interval)	P
All children		
Each one-point increment in ETV Scale score	0.05 (0.01 to 0.09)	0.02
Age, yrs	−0.008 (−0.06 to 0.04)	0.74
Sex, male	−0.003 (−0.18 to 0.17)	0.98
Household income, ≥\$15,000 per yr	−0.014 (−0.20 to 0.18)	0.89
Cases		
Each one-point increment in ETV Scale score	0.032 (−0.03 to 0.10)	0.35
Age, yrs	0.001 (−0.07 to 0.07)	0.97
Sex, male	0.068 (−0.19 to 0.33)	0.61
Household income, ≥\$15,000 per yr	−0.12 (−0.4 to 0.16)	0.41
Control subjects		
Each one-point increment in ETV Scale score	0.07 (0.012 to 0.13)	0.02
Age, yrs	−0.015 (−0.081 to 0.051)	0.66
Sex, male	−0.065 (−0.32 to 0.19)	0.62
Household income, ≥\$15,000 per yr	0.07 (−0.19 to 0.33)	0.60

Definition of abbreviation: ETV = Exposure to Violence.

*Multivariate analysis conducted using linear regression. All models are additionally adjusted for principal components.

there was no significant association between SNP rs2267735 and PTSD in a study of US adults (27). Lack of replication of the findings for PTSD in adults may be explained by differences in age, ethnicity, and socioeconomic status between subjects in the original study and those in the replication study. Compared with subjects in the replication cohorts, those in the study by Ressler and coworkers were more likely to be younger, to be African American, and to be heavily traumatized and economically disadvantaged inner-city residents (12, 27).

Compared with women carrying at least one G allele of SNP rs2267735 or with men, women with the CC genotype have reduced expression of *ADCYAP1R1* in brain tissue (12). More recently, the CC genotype of SNP rs2267735 was shown to be significantly associated with dark-enhanced startle (a marker of anxiety) (28) in boys and girls (ages 6–13 yr) of traumatized mothers (29). This genetic association is consistent with our sex-independent findings for the C allele of SNP rs2267735 in *ADCYAP1R1* and asthma in the current study. Sex-independent findings among participants in the prior study (29) and ours may be caused by lack of peripuberal or post-puberal hormonal effects among most children in both studies.

Our study has major strengths, including its multistage probability sampling design; genotypic and methylation data on a susceptibility gene for anxiety and PTSD; and assessment of ETV in children at risk for asthma morbidity (Puerto Ricans). We also recognize several limitations to our findings. First, we had limited statistical power to examine the relation among exposure to violence, methylation, and asthma because the ETV Scale questionnaire was only administered to participants aged 9 years or older. Similarly, we had limited power to test for an interaction between SNP rs2267735 and exposure to violence. Second, this is a cross-sectional study and we cannot determine whether *ADCYAP1R1* methylation precedes or follows the development of asthma. However, the observed association between exposure to violence and *ADCYAP1R1* methylation or asthma, together with our results for SNP rs2267735 and prior findings (for *ADCYAP1R1*, exposure to violence, PTSD, and asthma) suggest a causal link and merit follow-up. Third, methylation of DNA from WBCs comprises methylation of various WBC subtypes (e.g., lymphocytes and eosinophils) that were not separately assessed in our study. Fourth, we have no data on adherence to medications that may potentially

TABLE 4. EXPOSURE TO VIOLENCE AND ASTHMA IN CHILDREN AGED 9 YEARS AND OLDER (N = 324)*

Variable	Odds Ratio (95% Confidence Interval)	P Value
Each one-point increment in the Exposure to Violence Scale	1.1 (1.0–1.2)	0.04
Age, yrs	0.85 (0.8–1.0)	0.007
Sex, male	1.30 (0.9–2.0)	0.23
Household income, ≥\$15,000/yr	0.91 (0.6–1.4)	0.68

*Multivariate analysis conducted using logistic regression. The model was additionally adjusted for principal components.

affect DNA methylation (e.g., inhaled corticosteroids). However, only approximately 30% of our study participants had used inhaled corticosteroids in the previous 6 months. Current use of systemic corticosteroids has been associated with methylation of certain genes in adults with chronic obstructive pulmonary disease (30) but none of our study participants was on chronic systemic corticosteroids. Moreover, we obtained similar results for the analysis of *ADCYAP1R1* methylation and asthma in children who had (OR, 1.25; 95% CI, 1.0–1.64) and had not (OR, 1.29; 95% CI, −1.0 to 1.67) received at least one course of systemic corticosteroids in the previous year. Fifth, we only assessed one CpG site and thus methylation of other sites in *ADCYAP1R1* may be influenced by SNP rs2267735 or be associated with asthma. Finally, our findings must be interpreted with caution pending replication in other cohorts. Our results may not be generalizable to children of other ethnicities or to Puerto Rican children living in the US mainland. However, they may be relevant to children in economically disadvantaged communities who are highly exposed to violence.

In summary, our findings suggest that epigenetic or genetic variation in the gene for the PAC1 receptor (*ADCYAP1R1*) influences asthma in Puerto Rican children. Our results further suggest that exposure to violence is associated with childhood asthma in Puerto Ricans. Our findings warrant examination in longitudinal studies of epigenetic-genetic variants in the PACAP-PAC1 receptor pathway and asthma in Puerto Rican children, and in children in other ethnic groups that are often exposed to stress and violence.

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank participating children and their families. All analyses were conducted at the Children's Hospital of Pittsburgh of the University of Pittsburgh Medical Center.

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