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Exposure to violence, chronic stress, nasal DNA methylation, and atopic asthma in children

Qi Yan PhD[1] | Erick Forno MD, MPH[1] | Andres Cardenas PhD[2,3] | Cancan Qi MsC[4,5] | Yueh-Ying Han PhD[1] | Edna Acosta-Pérez PhD, MSc[6] | Soyeon Kim PhD[1] | Rong Zhang PhD[1,7] | Nadia Boutaoui PhD[1] | Glorisa Canino PhD[6] | Judith M. Vonk PhD[5,8] | Cheng-jian Xu PhD[4,5] | Wei Chen PhD[1] | Anna Marsland PhD[9] | Emily Oken MD, MPH[3] | Diane R. Gold MD, MPH[10,11] | Gerard H. Koppelman MD[4,5] | Juan C. Celedón MD, DrPH[1]

[1]Division of Pediatric Pulmonary Medicine, UPMC Children's Hospital of Pittsburgh, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[2]Division of Environmental Health Sciences, University of California, Berkeley, California, USA

[3]Division of Chronic Disease Research Across the Lifecourse, Department of Population Medicine, Harvard Medical School and Harvard Pilgrim Health Care Institute, Boston, Massachusetts, USA

[4]Department of Pediatric Pulmonology and Pediatric Allergy, University Medical Center Groningen, Beatrix Children's Hospital, University of Groningen, Groningen, The Netherlands

[5]University Medical Center Groningen, GRIAC Research Institute, University of Groningen, Groningen, The Netherlands

[6]Behavioral Sciences Research Institute, University of Puerto Rico, San Juan, Puerto Rico

[7]Department of Statistics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[8]Department of Epidemiology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands

[9]Department of Psychology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

[10]Channing Division of Network Medicine, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts, USA

[11]Department of Environmental Health, Harvard School of Public Health, Boston, Massachusetts, USA

Correspondence
Juan C. Celedón, MD, DrPH, Division of Pulmonary Medicine, UPMC Children's Hospital of Pittsburgh, 4401 Penn Ave, Pittsburgh, PA 15224, USA.
Email: juan.celedon@chp.edu

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Abstract

Background: Exposure to violence (ETV) or chronic stress may influence asthma through unclear mechanisms.

Methods: Epigenome-wide association study (EWAS) of ETV or chronic stress measures and DNA methylation in nasal epithelium from 487 Puerto Ricans aged 9–20 years who participated in the Epigenetic Variation and Childhood Asthma in Puerto Ricans study [EVA-PR]). We assessed four measures of ETV and chronic stress in children (ETV scale, gun violence, and perceived stress) and their mothers (perceived stress). Each EWAS was conducted using linear regression, with CpGs as dependent variables and the stress/violence measure as a predictor, adjusting for age, sex, the top five principal components, and SVA latent factors. We then selected the top 100 CpGs (by *p* value) associated with each stress/violence measure

in EVA-PR and conducted a meta-analysis of the selected CpGs and atopic asthma using data from EVA-PR and two additional cohorts (Project Viva and PIAMA).
Results: Three CpGs (in *SNN*, *PTPRN2*, and *LINC01164*) were associated with maternal perceived stress or gun violence ($p = 1.28–3.36 \times 10^{-7}$), but not with atopic asthma, in EVA-PR. In a meta-analysis of three cohorts, which included the top CpGs associated with stress/violence measures in EVA-PR, 12 CpGs (in *STARD3NL*, *SLC35F4*, *TSR3*, *CDC42SE2*, *KLHL25*, *PLCB1*, *BUD13*, *OR2B3*, *GALR1*, *TMEM196*, *TEAD4*, and *ANAPC13*) were associated with atopic asthma at FDR-$p < .05$.
Conclusions: Pending confirmation in longitudinal studies, our findings suggest that nasal epithelial methylation markers associated with measures of ETV and chronic stress may be linked to atopic asthma in children and adolescents.

KEYWORDS
childhood asthma, epigenetics, stress, violence

1 | INTRODUCTION

The United States has the highest rate of firearm-related deaths of all industrialized countries.[1] As recently reviewed, exposure to violence (ETV, including gun violence) affects stress responses, and both ETV and chronic stress may influence asthma pathogenesis.[2,3] Prolonged ETV can lead to chronic and frequent activation of the body's stress responses, disrupting neuroendocrine, autonomic nervous system, and immune responses. Chronic stress, both related and unrelated to ETV, may cause or worsen asthma by altering circulating levels of and response to catecholamines and glucocorticoids, as well as by affecting immune responses, leading to airway inflammation and airflow obstruction.[2–4] ETV and chronic stress may also cause or worsen asthma through indirect effects mediated by other risk factors.[2] For example, community violence may decrease outdoor physical activity, leading to obesity.[5]

Puerto Ricans are often exposed to violence in their communities.[6–8] ETV is associated with asthma in Puerto Rican children,[8,9] in whom ETV is also associated with DNA methylation of the promoter of the gene for the pituitary adenylate cyclase activating polypeptide 1 type 1 receptor (*ADCYAP1R1*) in white blood cells.[8] Moreover, such methylation has been associated with childhood asthma in Puerto Ricans.

Based on prior findings,[2,3,8,9] we hypothesized that both ETV and chronic stress would increase asthma risk through DNA methylation of genes regulating autonomic, neuroendocrine, and immune responses in airway epithelium, a tissue relevant to asthma. To examine this hypothesis, we first tested for association between ETV or chronic stress measures and genome-wide DNA methylation in nasal epithelium, as DNA methylation in nasal (airway) epithelium is well correlated with that in bronchial (airway) epithelium,[10–13] and epigenetic regulation of airway epithelial gene expression has been implicated in asthma pathogenesis.[14] We then examined whether the top CpGs associated with ETV or chronic stress were also associated with atopic asthma in Puerto Rican children and in two independent cohorts. We focused on atopic asthma, both because atopic asthma is the most common type of childhood asthma and because we previously showed that DNA methylation profiles can accurately classify children according to the presence of atopic asthma.[14]

2 | METHODS

2.1 | Study population

Subject recruitment and study procedures for the Epigenetic Variation and Childhood Asthma in Puerto Ricans study (EVA-PR) have been previously described.[14] In brief, EVA-PR is a case-control study of asthma in subjects aged 9–20 years. Participants with and without asthma were recruited from households in San Juan (PR) from February 2014 to May 2017, using multistage probability sampling; 638 households had more than or equal to one eligible child, and 543 (85.1%) children (one per household) agreed to participate. The study was approved by the institutional review boards of the University of Puerto Rico (San Juan, PR) and the University of Pittsburgh (Pittsburgh, PA). Written parental consent and assent were obtained from participants less than 18 years old, and consent was obtained from participants more than or equal to ≥18 years old.

The study protocol included administration of questionnaires on ETV and chronic stress in the child and her/his mother, measurement of serum allergen-specific IgEs, and collection of nasal epithelial samples for DNA and RNA extraction. Participating children completed a modified version of the ETV Scale,[15–17] a questionnaire used to assess ETV in children more than or equal to 8 years. The ETV Scale measures both witnessing and direct victimization for five events: shoving, punching, or kicking; knife attacks; shootings; hearing gunshots; and witnessing verbal abuse of the child's primary caregiver. An ETV score (range = 0–15) is obtained by summing the

scores for each section. Internal consistency and test-retest reliability and validity have been established for the English and Spanish versions. The child's exposure to gun violence was derived from the ETV scale.[9] Participating children also completed the Checklist of Children's Distress Symptoms (CCDS), a 28-item scale to assess stress symptoms in the previous 6 months as a result of ETV.[16,18] Answers for each question in the CCDS range from 1 to 5. An overall score is obtained by summing the scores for all 28 questions, and then dividing by the number of questions, so that the score ranges between 1 and 5. Mothers of participating children completed the perceived stress scale (PSS) questionnaire, which includes four items and measures the degree to which mothers believed that their lives were unpredictable, uncontrollable, or overwhelming in the preceding month.[19] Each question in the maternal PSS ranges between 0 and 4, and a total score ranges between 0 and 16.[19]

Serum IgEs to each of five common allergens in Puerto Rico (dust mite [Der p 1], cockroach [Bla g 2], cat dander [Fel d 1], dog dander [Can f 1], and mouse urinary protein [Mus m 1]) were measured using the UniCAP 100 system (Pharmacia & Upjohn, Kalamazoo, Mich). Atopy was defined as an IgE more than or equal to 0.35 IU/ml to more than or equal to one of the allergens tested. Asthma was defined as a physician's diagnosis plus more than or equal to one episode of wheeze in the previous year. Atopic asthma was defined as the presence of both atopy and asthma.

Whole-genome methylation in nasal epithelium was measured using Infinium HumanMethylation450 BeadChip arrays (Illumina). Methylation β-values were calculated as a percentage: $\beta = M/(M + U + \alpha)$, where M and U represent methylated and unmethylated signal intensities, respectively, and α is an arbitrary offset to stabilize β-values where fluorescent intensities are low. The β-values were used in all downstream analyses. We performed the same preprocessing and quality control procedures as in our previous study.[14] In brief, Raw IDAT files were loaded using the R package *minfi*.[20] Samples with low detection values (>10% CpG probes with a detection $p > .01$) were removed. The R package *Enmix*[21] was used to perform background correction and normalization. Cross-reactive ($n = 26{,}098$) and SNP-containing probes[22] ($n = 15{,}834$), sex chromosomal probes ($n = 9{,}490$), and low-quality probes (>10% of samples with detection $p > .01$; $n = 21{,}174$) were also removed. After further filtering, CpG sites with an overall mean β-value of greater than 0.9 or less than 0.1 were removed, leaving 227,901 CpG probes in the analysis.

RNA-Seq was performed using the Illumina NextSeq 500 platform, with paired-end reads at 75 cycles and 80 million reads per sample; reads were aligned to reference human genome (hg19)[23] and TPM (Transcripts Per Kilobase Million) were used as proxy for gene expression level. After QC, 16,737 genes were retained for the analyses of gene expression, which focused on genes in *cis* (within 1 Mb on each side of the transcription start site) with the top CpGs from the methylation analyses. Genome-wide genotyping was conducted using the HumanOmni2.5 BeadChip platform, (Illumina), as previously described.

2.2 | Study cohorts included in the meta-analysis of atopic asthma

2.2.1 | PIAMA

PIAMA is a birth cohort study of children born in the Netherlands in 1996 and 1997. The study protocol has been previously described.[24] The Medical Ethical Committees of the participating institutions approved the study, and the parents and legal guardians of all participants, as well as the participants themselves, gave written informed consent. At age 16 years, nasal epithelial cells were collected at two study centers (Groningen and Utrecht)[25] by brushing the lateral area underneath the right inferior turbinate. Serum IgEs to each of four common allergens (house dust mite, cat, dactylis (grass) and birch) were measured, and atopy was defined as an IgE more than or equal to 0.35 IU/ml to any of the allergens tested. Asthma was defined as physician-diagnosed asthma and either wheeze in the previous year or use of medications for respiratory or lung problems. Atopic asthma was defined as the presence of both asthma and atopy.

In total, 479 nasal epithelial samples were hybridized to the Infinium HumanMethylation450 BeadChip arrays. DNA methylation data were pre-processed with Bioconductor package *minfi*.[20] Samples with call rate less than 99% were removed. Sixty-five SNP probes were used to check for concordance between paired DNA samples (nasal and blood DNA samples from the same subjects were hybridized in the same experiments); paired samples with Pearson correlation coefficient less than 0.9 were excluded, as were probes on sex chromosomes, probes that mapped to multiple loci, 65 SNP-probes, and probes containing SNPs at the target CpG sites with a MAF more than 5%.[22] "DASEN"[26] was used to perform signal correction and normalization. After QC, 455 samples and 436,824 probes remained in the analysis, as previously described.[27] Of these 455 samples, 246 samples corresponded to subjects with atopic asthma and nonatopic control subjects, and were thus included in the meta-analysis.

2.2.2 | Project Viva

Project Viva is a prospective pre-birth cohort study of mother–child pairs recruited between 1999 and 2002 during the mothers' first prenatal visits at Atrius Harvard Vanguard Medical Associates, a medical practice in eastern Massachusetts (USA).[28] The Institutional Review Board of Harvard Pilgrim Health Care reviewed and approved all study protocols. Asthma and atopy were assessed at the same time-point as collection of nasal samples. Atopy was defined as ≥1 IgE ≥ 0.35 IU/mL to the allergens tested (Dermatophagoides farinae, cat dander, dog dander, *Alternaria* or *Aspergillus* species, rye grass, ragweed, and oak and silver birch).

Nasal swabs were collected from the anterior nares, previously demonstrated to yield respiratory epithelial cells.[29] Epigenome-wide methylation was measured on DNA extracted from nasal samples,

using Infinium MethylationEPIC BeadChip arrays (Illumina). DNA methylation data was imported into the *R* statistical software for preprocessing using *minfi*.[20] QC was first performed at the sample level, excluding samples that: had overall low intensity (< 10.5; $n = 3$), were mismatched on recorded sex ($n = 4$), or had mixed genotype distributions on the measured SNP probes ($n = 59$) indicating possible sample contamination ($n = 8$). In addition, 35 technical duplicates were excluded. Of 547 high-quality samples, 156 corresponded to subjects with atopic asthma and non-atopic controls and were thus retained for the meta-analysis. QC was then performed at the probe level by computing a detection *p* value relative to control probes. In total, 40,377 cross-reactive probes previously identified in the MethylationEPIC BeadChip[30] were excluded, leaving 719,075 high-quality probes included in the analysis. DNA methylation heterogeneity was estimated by including potential cell-type variation, using 10 principal components derived via ReFACTor.[31]

2.3 | Statistical analysis

We analyzed four measures of ETV and chronic stress. As in prior work, the child's ETV scale was analyzed as continuous, the child's exposure to gun violence was analyzed as binary (having heard gunshots at least twice vs. no more than once),[9] the child's perceived stress scale was analyzed as binary (upper quartile [≥ 2.6 points] vs. lowest three quartiles [< 2.6 points]),[32] and maternal perceived stress (assessed by the PSS) was analyzed as binary (upper quartile [> 7 points] vs. lowest three quartiles [≤ 7 points]).[32] Of the 487 participants in EVA-PR who had nasal genome-wide methylation (GWM) data and complete data on relevant covariates, 470 had ETV data, 475 had data on gun violence, 471 had CCDS data, and 476 had maternal PSS data.

We conducted epigenome-wide association studies (EWAS) of ETV or chronic stress measures among subjects in EVA-PR using linear regression models, as follows:

$$\begin{aligned} CpG \sim\ & ETV/\text{stress} + \text{age} + \text{sex} + \text{body mass index } (BMI)\,z\text{-score} \\ & + \text{residential distance to a major road} \\ & + \text{annual household income} + 5\ PCs + SVA \text{ latent factors} \end{aligned} \quad (1)$$

where CpG is the β-value for a CpG methylation, with known batches adjusted by R function *Combat*,[33] BMI z-score is calculated using CDC growth charts, residential distance to a road (a proxy for traffic-related air pollution) is a binary covariate (as in prior work, < 444.7 vs. ≥ 444.7 meters or quartiles 1–3 vs. quartile 4), annual household income is a binary covariate (≥ $15,000 vs. < $15,000 per year, near the median household income in Puerto Rico in 2008-2009), five PCs are the top five principal components from genome-wide genotypic data, and latent factors (LFs) are estimated by *sva*[34] (which capture unknown data heterogeneity).

Because of limited statistical power in EVA-PR and lack of a replication cohort with data on stress/violence and nasal DNA methylation, we selected the top 100 CpG sites from each of the four EWAS (one for each measure of ETV or chronic stress) in EVA-PR for the analysis of atopic asthma, to reduce multiple testing. For this analysis in EVA-PR, we included 272 participants (167 subjects with atopic asthma and 105 nonatopic controls). Next, we used linear regression modeling to test whether the top methylation signals for ETV or chronic stress were also associated with atopic asthma in each of the three study cohorts (EVA-PR, Viva, and PIAMA), as follows

$$CpG \sim \text{atopic asthma} + \text{age} + \text{sex} + 5\ PCs + SVA \text{ latent factors} \quad (2)$$

We then conducted a meta-analysis of the three EWAS of atopic asthma. In this meta-analysis, *p* values from each of the three independent studies were taken as input, with effect direction considered. First, a Z-score was calculated based on the *p* value and direction of effect in each study, as follows

$$Z_i = \Phi^{-1}(1 - P_i/2) \times \text{sign}(\Delta_i),$$

where Z_i is the Z-score for study *i*, P_i is the *p* value for study *i*, Δ_i is the direction of effect for the study *i*, and Φ^{-1} gives the percentile of a standard normal distribution. Combined coefficients were calculated by averaging the study-specific coefficients, with weights reflecting the standard errors (*SE*) from each study, as follows

$$\beta = \frac{\sum_i \beta_i w_i}{\sum_i w_i}$$

where β is the combined coefficient, β_i is the study-specific coefficient, and $w_i = 1/(SE_i)^2$ is the weight for study *i*. An overall *p* value was then calculated as:

$$P = 2\Phi\left(\left| - \frac{\beta}{1/\sqrt{\sum_i w_i}} \right| \right)$$

where *P* is the overall *p* value. The false discovery rate (FDR) approach was used to adjust for multiple testing in the meta-analysis of the selected CpG sites and atopic asthma.

To assess the biological relevance of the top CpG sites for atopic asthma, we conducted an expression quantitative trait methylation (eQTM) analysis, to test whether those CpGs affected the expression of *cis*-genes (i.e., those with transcription start sites located within 1 Mb of the CpG, on each side) in EVA-PR. This analysis was adjusted for age, sex, asthma and atopy status, the top five PCs, RNA sample sorting protocol (i.e., whole-cells or CD326-positive nasal epithelial cells),[35] methylation and RNA-Seq batch, and LFs estimated from *sva*.[34]

3 | RESULTS

The main characteristics of the EVA-PR participants included in the four EWAS of ETV and stress measures (ETV, gun violence, CCDS and PSS) are shown in Table S1. In reviewing the results for each of these EWAS, the Q-Q plots showed no evidence of inflation (Figure 1). In these EWAS, methylation of cg18961589 in *LINC01164* (on chromosome 10) was associated with gun violence exposure

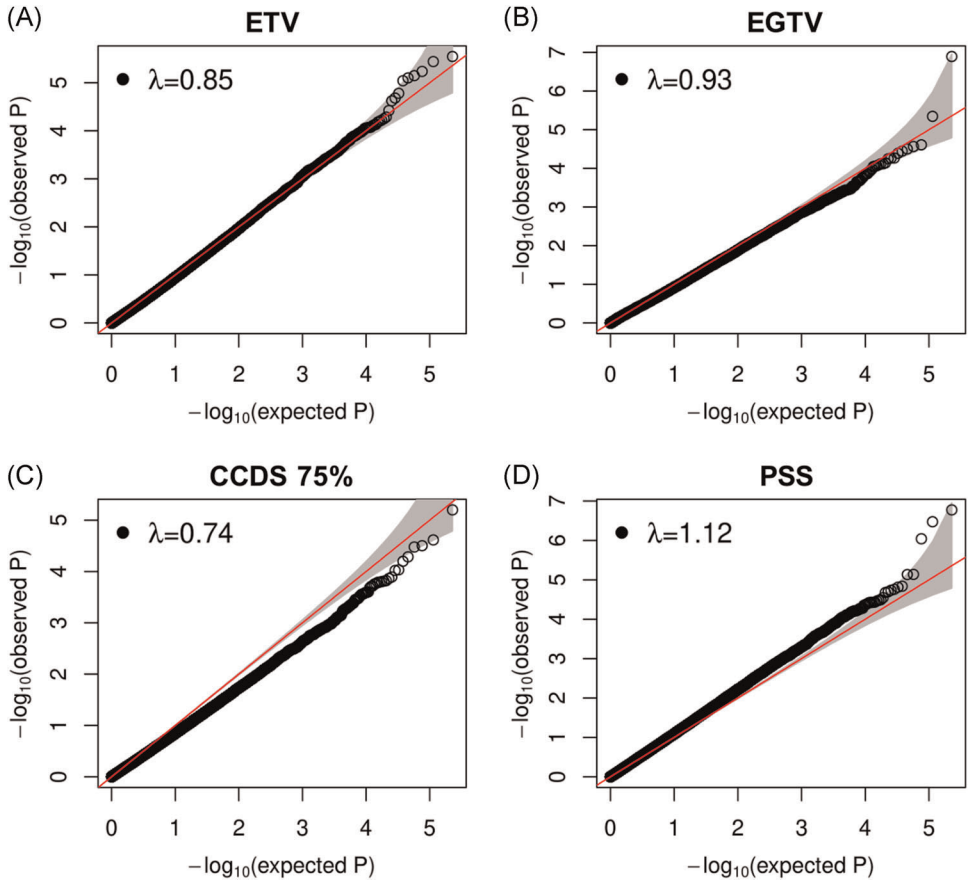

FIGURE 1 Q-Q plots of four epigenome-wide analyses of stress/violence measures in nasal epithelium from participants in the Epigenetic Variation of Childhood Asthma in Puerto Ricans study (EVA-PR). CCDS, checklist of children's distress symptoms; ETGV, exposure to gun violence; ETV, exposure to violence; PSS, Perceived Stress Scale

($\beta = 0.03$, $p = 1.28 \times 10^{-7}$ at FDR-$p < .05$), and methylation of cg03402351 in *SNN* (on chromosome 16, $\beta = 0.04$, $p = 1.69 \times 10^{-7}$) and cg19064846 in *PTPRN2* (on chromosome 7, $\beta = 0.03$, $p = 3.36 \times 10^{-7}$) were associated with maternal perceived stress at FDR-$p < .05$ (Figure 2).

We then selected the top 100 CpG sites (by P-value) from each of the four EWAS in EVA-PR for testing for association with atopic asthma in a meta-analysis including data from EVA-PR, PIAMA, and Project Viva. A total of 336 CpG sites were included in this meta-analysis, as 4 CpG sites overlapped across EWAS and 60 CpG sites were missing in Project Viva; see Tables S2 and S3). The main characteristics of participants in the three cohorts included in the meta-analysis of atopic asthma and nasal epithelial DNA methylation are shown in Table 1. As expected, EVA-PR (a case-control study of asthma) had a larger proportion of participants with atopic asthma than PIAMA or Viva (both unselected for asthma). The studies were conducted in different geographic locations, ranging from Puerto Rico to Boston to the Netherlands. While all participants in EVA-PR were Puerto Rican and most participants in PIAMA were European, Viva was ethnically diverse. (Table 2).

We first performed separate analyses of the 336 CpG sites and atopic asthma in each of the three study cohorts (EVA-PR, PIAMA, and Viva), and then combined the results from the three cohorts in a meta-analysis. In this meta-analysis, we identified seven CpGs (cg02695349 in *STARD3NL*, cg18146152 in *TSR3*, cg03541903 in *CDC42SE2*, cg21376795 in *KLHL25*, cg26772788 in *GALR1*, cg03729152 in *TMEM196*, and cg16848072 in *ANAPC13*) associated with higher odds of atopic asthma, and five CpGs (cg04990977 in *SLC35F4*, cg27178677 in *PLCB1*, cg17076485 in *BUD13*, cg17335499 in *OR2B3*, and cg01039401 in *TEAD4*) associated with lower odds of atopic asthma, all at FDR-$p < .05$.

We then conducted a *cis*-expression quantitative trait methylation (*cis*-eQTM) analysis of the 12 CpGs associated with atopic asthma in nasal epithelial cells from participants in EVA-PR. In this analysis, we identified 16 nominally significant *cis*-acting eQTM probes in nasal epithelium (Table S4).

Our EWAS of ETV or stress measures in EVA-PR included children with and without asthma. We did not adjust for asthma status in these analyses, as this could have attenuated our effect estimates for the analysis of atopic asthma. In a sensitivity analysis, we repeated the four EWAS of ETV or stress measures after additional adjustment for asthma status, obtaining similar results (Figures S1 and S2). Moreover, when the top 100 CpGs from each of these four EWAS

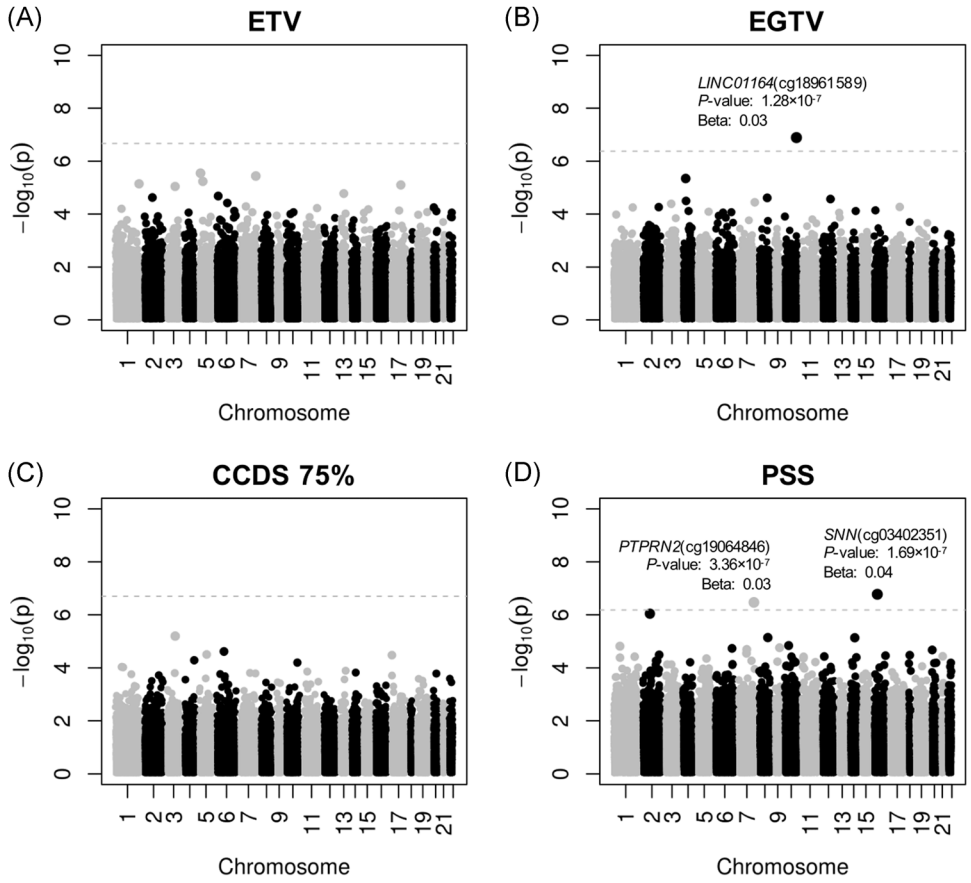

FIGURE 2 Manhattan plots for the epigenome-wide association studies (EWAS) of four ETV/stress measures in nasal epithelial samples from participants in the Epigenetic Variation of Childhood Asthma in Puerto Ricans study (EVA-PR). (A) Exposure to Violence scale (ETV), (B) Exposure to gun violence (ETGV), (C) Checklist of Child Distress Symptoms (CCDS), and (D) maternal perceived stress symptoms (PSS). The chromosomal position of each CpG site is displayed along the *x*-axis and the negative logarithm of the association *p* value is displayed on the *y*-axis. The dotted line represents the threshold for genome-wide significance line (false-discovery rate-adjusted $p < .05$)

TABLE 1 Summary of characteristics of participants in the studies included in the meta-analysis of CpG sites (associated with stress or violence measures in EVA-PR) and atopic asthma

	EVA-PR (*n* = 272)	PIAMA (*n* = 246)	Project Viva (*n* = 156)
Age in years (mean ± *SD*)	15.5 ± 3.1	16.3 ± 0.2	12.9 ± 0.6
Male gender (*n*, %)	144 (52.9)	111 (47.0)	79 (50.6)
Race/ethnicity			
White		239 (97.2)	110 (70.5)
Black			21 (13.5)
Hispanic or Latino	272 (100)		8 (5.1)
Asian			5 (3.2)
More than one race/unknown		7 (2.8)	12 (7.7)
Atopic asthma (*n*, %)	167 (61.4)	27 (11.0)	36 (23.1)
Study sites	San Juan (Puerto Rico)	The Netherlands	Boston (MA)
Methylation platform	Infinium HumanMethylation450 BeadChips	Infinium HumanMethylation450 BeadChips	Infinium MethylationEPIC BeadChip

Abbreviations: EVA-PR, Epigenetic Variation of Childhood Asthma in Puerto Ricans study; *SD*, standard deviation.

TABLE 2 CpG sites associated with atopic asthma (at *FDR-p* < .05) in a meta-analysis of data from three cohorts (EVA-PR, Project Viva, and PIAMA)

				EVA-PR		Project Viva		PIAMA				
CpG	Gene	Chr	Position	*β*	*p* value	*β*	*p* value	*β*	*p* value	*β* meta-analysis	*p* value meta-analysis	*FDR_p* meta-analysis
cg02695349	*STARD3NL*	chr7	38269241	0.0205	1.04E-02	0.0488	1.10E-05	0.0138	7.44E-02	0.0237	1.40E-06	2.80E-04
cg04990977	*SLC35F4*	chr14	58221774	−0.0478	4.67E-06	−0.0134	1.87E-02	−0.0170	2.62E-02	−0.0199	1.66E-06	2.80E-04
cg18146152	*TSR3*	chr16	1400772	−0.0042	5.24E-01	0.0073	8.90E-03	0.0131	5.79E-05	0.0084	2.62E-05	2.93E-03
cg03541903	*CDC42SE2*	chr5	130651434	0.0071	5.39E-01	0.0095	8.64E-03	0.0209	1.90E-03	0.0117	1.24E-04	1.05E-02
cg21376795	*KLHL25*	chr15	86302121	0.0050	3.25E-01	0.0027	4.01E-01	0.0154	7.41E-06	0.0080	1.74E-04	1.17E-02
cg27178677	*PLCB1*	chr20	8834803	−0.0328	3.60E-05	0.0028	7.65E-01	−0.0119	2.03E-02	−0.0144	2.30E-04	1.29E-02
cg17076485	*BUD13*	chr11	116580712	−0.0112	1.18E-01	−0.0014	8.88E-01	−0.0225	2.28E-04	−0.0150	4.09E-04	1.96E-02
cg17335499	*OR2B3*	chr6	29054325	−0.0179	9.49E-03	−0.0085	1.07E-02	−0.0040	2.90E-01	−0.0078	7.54E-04	3.13E-02
cg26772788	*GALR1*	chr18	75609054	0.0042	4.81E-01	0.0045	3.84E-01	0.0224	3.35E-05	0.0106	8.39E-04	3.13E-02
cg03729152	*TMEM196*	chr7	19961027	0.0247	3.27E-03	0.0054	2.28E-01	0.0148	2.00E-02	0.0110	9.56E-04	3.15E-02
cg01039401	*TEAD4*	chr12	3070406	−0.0612	1.80E-05	0.0036	8.14E-01	−0.0192	1.15E-01	−0.0260	1.03E-03	3.15E-02
cg16848072	*ANAPC13*	chr3	134182665	0.0074	1.91E-01	0.0044	1.58E-01	0.0100	6.30E-03	0.0068	1.72E-03	4.80E-02

Abbreviations: EVA-PR, Epigenetic Variation of Childhood Asthma in Puerto Ricans study; FDR, false discovery rate.

(adjusted for asthma status) were tested for association with atopic asthma in a meta-analysis of the three study cohorts, 7 of the 12 CpGs from the primary analysis were also significant at FDR-*p* < .05 (in *STARD3NL*, *TSR3*, *CDC42SE2*, *PLCB1*, *OR2B3*, *GALR1*, and *TMEM196*).

4 | DISCUSSION

ETV and chronic stress have been linked to childhood asthma.[2] We previously showed that ETV and gun violence are associated with childhood asthma in Puerto Ricans, a high-risk group.[8,9,36] To our knowledge, this is the first report of an association between chronic stress or ETV and DNA methylation in nasal epithelium, as well as the first association study of stress- or violence-related methylation markers in nasal epithelium and atopic asthma.

Among Puerto Rican children, exposure to gun violence was significantly associated with increased methylation of a CpG site (cg18961589) in the gene for long intergenic non-protein coding 1164 (*LINC01164*). *LINC01164* is most highly expressed in brain tissue from GTEx[37] (Figure S3), and SNPs in this gene have been associated with cognitive function and educational attainment.[35] Although cg18961589 was not significantly associated with atopic asthma in the current analysis, methylation of another CpG site in *LINC01164* (cg15491439) was significantly associated with atopic asthma in our recent EWAS in EVA-PR (FDR-$p = 4.20 \times 10^{-4}$).[14] Moreover, this long noncoding RNA is adjacent (~128Kbp) to *PPP2R2D*, a gene that codes for a regulatory subunit of the PPP2A phosphatase family that has been linked to T-cell proliferation and apoptosis in mice.[38]

Increased methylation of cg03402351 in *SNN* (stannin) and increased methylation of cg19064846 in *PTPRN2* (receptor-type tyrosine-protein phosphatase N2) in nasal epithelium were associated with maternal perceived stress but not with atopic asthma. Both *SNN* and *PTPRN2* are highly expressed in brain tissue from GTEx.[37] *PTPRN2* methylation and expression have been associated with Parkinson's Disease,[39–41] and a meta-analysis of epigenome-wide data showed that a differentially methylated region in *PTPRN2* was associated with childhood asthma.[42]

In the meta-analysis of selected stress- or violence-associated CpG sites in the study cohorts, we identified 12 CpG sites associated with atopic asthma (in genes *STARD3NL*, *TSR3*, *CDC42SE2*, *KLHL25*, *GALR1*, *TMEM196*, *ANAPC13*, *SLC35F4*, *PLCB1*, *BUD13*, *OR2B3*, and *TEAD4*). Of note, expression levels of *STARD3NL* (log2FC [fold-change] = −0.16; FDR-$p = 6.24 \times 10^{-6}$), *TSR3* (log2FC = 0.07; FDR-*p* = .04), *CDC42SE2* (log2FC = −0.18; FDR-$p = 2.62 \times 10^{-4}$), *KLHL25* (log2FC = 0.21; FDR-$p = 3.11 \times 10^{-6}$), *PLCB1* (log2FC = −0.31; FDR-*p* = .01), and *ANAPC13* (log2FC = −0.13; FDR-$p = 1.27 \times 10^{-3}$) were associated with atopic asthma in our recent transcriptome-wide association study using nasal epithelial samples from Puerto Ricans.[43]

PLCB1 has been associated with bronchodilator response in children with asthma,[44] and is differentially expressed in children with treatment-resistant asthma compared with children with controlled persistent asthma or healthy controls.[45] Silencing *PLCB1* can attenuate lipopolysaccharide-induced endothelial cell inflammation by inhibiting expression of pro-inflammatory cytokines.[46] In the brain, *PLCB1* is expressed mainly in the cortex, hippocampus, and amygdala; the protein it codes for, PLCB1, is a mediator of synaptic plasticity that plays an important role in cognitive behavior and emotions[47] and may influence the pathogenesis of stress-related

disorders. Indeed, expression of *PLCB1* is decreased in the frontal cortex of rats subjected to chronic stress, and this downregulation is reversed by quetiapine, a drug used to treat major depression.[48] *PLCB1* has also been implicated in depression, bipolar disorder, epilepsy, and schizophrenia.[49]

Four SNPs in *TMEM196* have been shown to be associated with decreased odds of nonsteroidal anti-inflammatory drug (NSAID)-exacerbated respiratory disease (NERD) in asthma.[50] Moreover, *TMEM196* has been shown to regulate autophagy and apoptosis of cancer and inflammatory cells.[51] Of note, disruption of the balance between autophagy and apoptosis induces hyperplasia of the airways, a typical feature of nasal polyps and airway eosinophilia in NERD.[52–54]

We recognize several study limitations. First, we had limited statistical power to examine ETV or chronic stress and DNA methylation in EVA-PR and lacked a cohort with data on stress/ETV for replication analyses. Although top methylation markers in the EWAS of stress/violence measures were associated with atopic asthma in the meta-analysis of three study cohorts, no such marker achieved genome-wide significance for ETV or stress measures in EVA-PR. Thus, false positive results cannot be excluded pending replication studies. Second, we cannot assess temporal relationships or causality in this cross-sectional study. Third, covariates correlated with stress or violence may partly explain our results. However, our EWAS of stress or violence measures in EVA-PR was adjusted for indicators of socioeconomic status (annual income), adiposity (BMI), and traffic-related air pollution. Moreover, some of the top genes associated with atopic asthma have been implicated in neuropsychiatric function or disorders (i.e., *PLCB1)*. Fourth, our statistical power may have been reduced by variability in race/ethnicity, geographic location, and exposure to psychosocial stressors across the cohorts included in the meta-analysis.

In summary, we show that measures of chronic stress or violence are associated with nasal DNA methylation markers in Puerto Rican youth. Moreover, we show that some stress- or violence-related methylation markers are also associated with atopic asthma in a meta-analysis of data from three cohorts of youth in diverse racial/ethnic groups. Although our findings must await confirmation in other studies, they offer "proof of concept" and support longitudinal studies of chronic stress or violence, DNA methylation in nasal epithelium, and stress- or violence-linked methylation markers and asthma in children.

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CONFLICT OF INTERESTS

Dr. Celedón has received research materials from GSK and Merck (inhaled steroids) and Pharmavite (vitamin D and placebo capsules), to provide medications free of cost to participants in NIH-funded studies, unrelated to the current work. The other authors declare no conflicts of interests.

AUTHOR CONTRIBUTIONS

Qi Yan: formal analysis (lead); writing original draft (lead); writing review & editing (equal). **Erick Forno**: project administration (equal); supervision (supporting); writing review & editing (supporting). **Andres Cardenas**: data curation (supporting); formal analysis (lead); writing review & editing (supporting). **Cancan Qi**: data curation (equal); formal analysis (equal); writing review & editing (equal). **Yueh-Ying Han**: data curation (lead); formal analysis (supporting); writing review & editing (equal). **Edna Acosta-Perez**: data curation (supporting); project administration (equal); writing review & editing (supporting). **Soyeon Kim**: data curation (equal); formal analysis (supporting); writing review & editing (supporting). **Rong Zhang**: data curation (supporting); formal analysis (supporting); software (equal); writing review & editing (supporting). **Nadia Boutaoui**: methodology (lead); project administration (supporting); writing review & editing (supporting). **Glorisa Canino**: conceptualization (supporting); funding acquisition (equal); project administration (lead); writing review & editing (supporting). **Judith M. Vonk**: conceptualization (equal); funding acquisition (supporting); supervision (supporting); writing review & editing (supporting). **Chengjian Xu**: data curation (supporting); formal analysis (equal); supervision (equal); writing review & editing (supporting). **Wei Chen**: formal analysis (equal); methodology (supporting); supervision (supporting); writing review & editing (supporting). **Anna Marsland**: conceptualization (equal); formal analysis (supporting); methodology (supporting); writing review & editing (supporting). **Emily Oken**: conceptualization (supporting); funding acquisition (supporting); writing review & editing (equal). **Diane Gold**: conceptualization (supporting); funding acquisition (supporting); project administration (supporting); supervision (supporting); writing review & editing (supporting). **Gerard H Koppelman**: conceptualization (supporting); funding acquisition (supporting); resources (supporting); supervision (lead); writing review & editing (supporting). **Juan Carlos Celedon**: conceptualization (lead); formal analysis (supporting); funding acquisition (lead); investigation (lead); resources (lead); supervision (lead); writing review & editing (lead).

ORCID

Qi Yan https://orcid.org/0000-0002-5236-9673

Erick Forno https://orcid.org/0000-0001-6497-9885

Andres Cardenas https://orcid.org/0000-0003-2284-3298

Juan C. Celedón https://orcid.org/0000-0002-6139-5320

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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