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Prenatal exposure to traffic-related air pollution, the gestational epigenetic clock, and risk of early-life allergic sensitization



To the Editor:

The Developmental Origins of Health and Disease paradigm posits that an adverse early-life environment induces gradients at birth that can alter lifetime health trajectories, including the development of atopic diseases. An important adverse environmental factor that has received considerable scrutiny is air pollution. Meta-analyses of birth cohort studies suggest that early-life exposure to traffic-related air pollution (TRAP) is associated with an increased risk of asthma and allergic sensitization, with a caveat that there is substantial variability across studies.¹ In seeking to understand the underlying biology, the study of epigenetics offers a plausible mechanism by which such environmental exposures in early life can get “under the skin” to regulate the activity of genes relevant to allergy and asthma.²

We used a recently developed biomarker of fetal development—epigenetic gestational age (eGA)—to further characterize the relationship between *in utero* exposure to TRAP and allergic sensitization. As a biomarker, eGA holds great promise because it may identify infants who deviate from the typical developmental profile, allowing targeted early preventative

interventions. In adults, patterns of DNA methylation (DNAm) at specific CpG sites are used to estimate “epigenetic age” and this is highly correlated with chronological age.³ Moreover, deviations between epigenetic and chronological age have been associated with many age-related health outcomes.⁴ Using umbilical cord blood DNAm profiles, similar estimates of eGA have been created.^{5,6} Although deviations in epigenetic age as quantified by DNAm in adults are typically associated with morbidity, the direction of the change in newborns in relation to various health outcomes is only beginning to be defined. In this context, we developed our hypotheses that deviation in eGA from chronological age (a variable referred to as “delta gestational age” or dGA) is associated with both prenatal TRAP exposures and early-life allergic sensitization, and that the relationship between prenatal TRAP exposure and sensitization risk is mediated in part by dGA.

In an atopy-enriched subset of the Canadian Healthy Infant Longitudinal Development (CHILD) cohort ($n = 145$, 29% having a positive skin prick test result to at least 1 of 10 standardized allergens at age 1 year), the mean gestational age (GA) was 276 ± 7.9 days or 39.4 weeks (see this article's Methods section in the Online Repository at www.jacionline.org). The mean eGA was 243 ± 8.2 and 279 ± 5.8 days as calculated by the 2 available gestational epigenetic clock algorithms developed by Knight et al⁵ and Bohlin et al.⁶ Both algorithms resulted in eGAs that were statistically associated with chronological GA (Fig 1, A and B). Given the stronger positive correlation using the method of Bohlin et al ($r = 0.66$; $P < .001$) compared with that of Knight et al ($r = 0.44$; $P = .001$), all further analyses were conducted on the basis of Bohlin et al's eGA.

All subsequent analyses did not control for the following features because no statistical differences were observed: child's sex, maternal factors (age, health status, parity, gestational diabetes, ethnicity, and antibiotic use), and child's birth weight (GA- and sex-standardized z score). Mode of delivery, which was strongly associated with dGA (see Table E1 in this article's Online Repository at www.jacionline.org), was included in our analyses. Children born by elective cesarian section had a statistically larger deviation between their eGA and chronological GA (5.74 days; 95% CI, 0.35-8.23).

Children of mothers exposed to higher levels of TRAP during the first trimester of pregnancy were at increased risk of allergic sensitization at age 1 year ($P = .06$; see Table E1). These children also had a larger deviation, meaning their eGA was larger than their chronological age (2.1 days; 95% CI, 0.11-4.08, for each $10 \mu\text{g}/\text{m}^3$ increase in NO_2 exposure, which approximates an interquartile increase). Proximity to green space (as quantified from satellite imagery using the normalized difference vegetation index) was not associated with a change in dGA (see Table E1).

Building on this observation, we further established that infants with a 1-day deviation in dGA were 8% more likely to be sensitized to allergens at age 1 year (odds ratio, 1.07; 95% CI, 1.00-1.14; $P = .05$) (Fig 1, C). Finally, when estimating the direct effect of air pollution (ie, adjusting for dGA), an *in utero* exposure increase of $10 \mu\text{g}/\text{m}^3$ NO_2 was associated with a 34% increased risk of allergic sensitization (odds ratio, 1.34; 95% CI, 0.97-1.87), compared with the 42% increase (odds ratio, 1.42; 95% CI, 1.02-1.99) without dGA adjustment (see Table E2 in this article's Online Repository at www.jacionline.org).

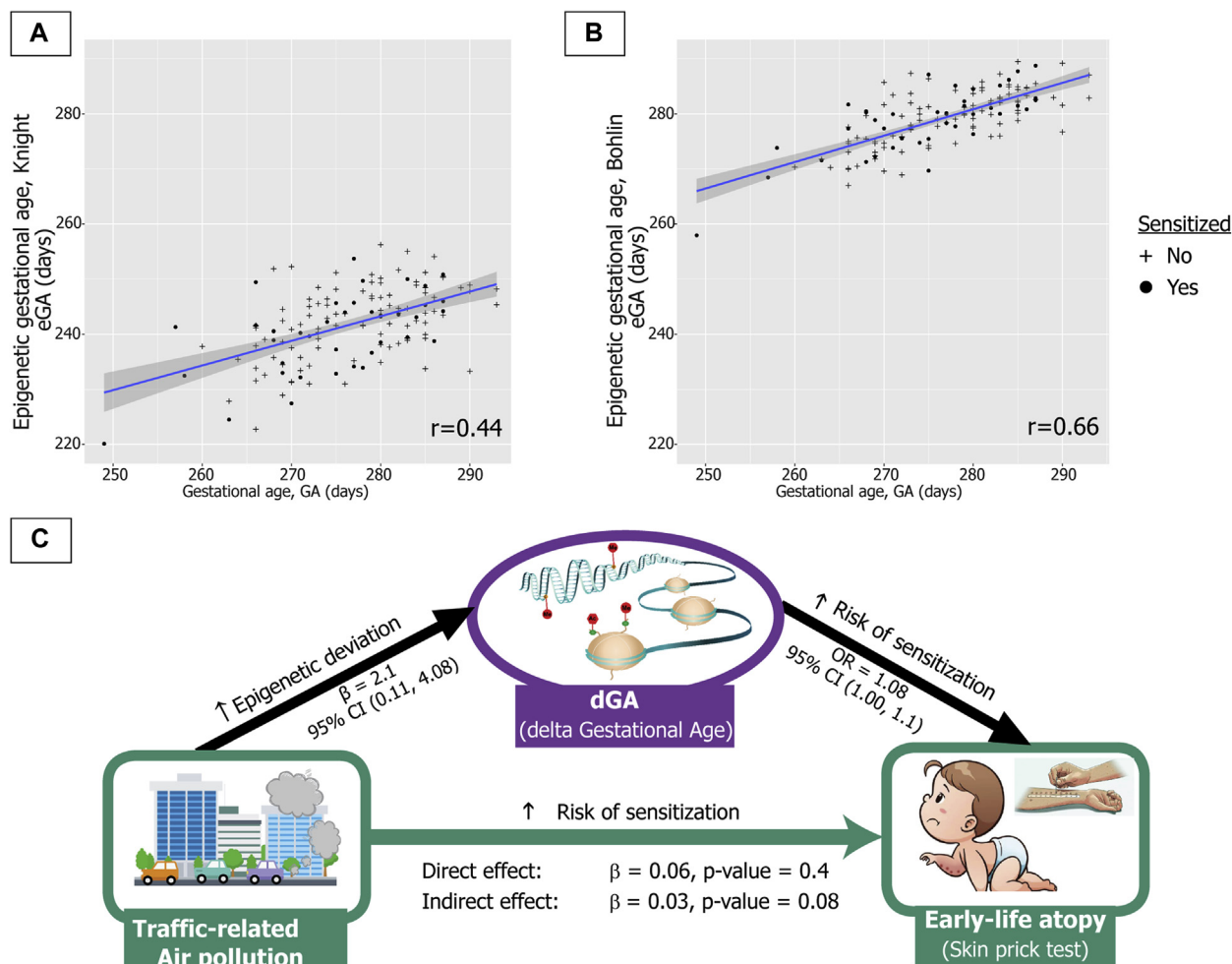


FIG 1. A and B, Association between chronological GA and eGA estimated from DNAm in days, using the methods developed by (Fig 1, A) Knight et al and (Fig 1, B) Bohlin et al. C, Mediation pathway between *in utero* exposure to TRAP (in $\mu\text{g}/\text{m}^3$) and atopy measured using skin prick test at age 1 year via “dGA” (in days) measured in umbilical cord blood.

Our hypothesis that dGA might indicate a role for epigenetics on the causal pathway between prenatal exposures and later development of allergic sensitization was then formally tested using the counterfactual framework-based mediation analysis (details are provided in this article's Online Repository at www.jacionline.org). Based on 1000 bootstrapped samples and after controlling for mode of delivery, the average causal mediation effect was 0.03 ($P = .08$), whereas the average direct effect was 0.06 ($P = .4$)—results compatible with mediation for the prespecified $\alpha = 0.10$ given the limited sample size (Fig 1; see also Fig E1 in this article's Online Repository at www.jacionline.org).

Our results suggest that, in our modest sample of 145 children, exposure to TRAP during the first trimester of pregnancy increases the risk of allergic sensitization at age 1 year, and that the impact of exposure to air pollution can be detected through epigenetic patterns present at birth. Specifically, children whose mothers were exposed to higher TRAP during the first trimester had larger dGA. Importantly, a formal statistical test of mediation showed that 31% of the association between TRAP and the risk of

allergic sensitization at age 1 year is mediated by modification of DNAm-based eGA (Fig 1, C).

Studies have only recently begun to examine the relationship between eGA deviations and exposures or health outcomes.⁷⁻⁹ Although more studies are needed, currently available data indicate that various adverse prenatal environmental exposures (including TRAP, maternal depression, extreme prematurity, and gestational diabetes mellitus) are associated with alterations in eGA.

The results of this investigation are based on several strengths. We used novel and comprehensive approaches to determine epigenetic age. In addition, the availability of extensive data from the CHILD cohort enabled the assessment of potential confounders such as maternal age, GA, ethnicity, and child's sex. Finally, the environmental exposures were rigorously assessed at the individual's reported home addresses throughout pregnancy up to the time of birth. Although greenness exposure was averaged across the whole pregnancy, TRAP prediction models at a fine spatiotemporal scale allowed us to report that the first trimester of pregnancy is likely a critical time window when the methylome is

particularly vulnerable to environmental insults such as traffic air pollution.

In conclusion, we report data that indicate an important trend where prenatal exposure to TRAP is linked to an increased risk of allergic sensitization, which is potentially mediated by altering the gestational epigenetic clock. Our “proof-of-concept” investigation strongly suggests that this relationship deserves reexamination in larger populations to confirm the plausible mechanism of the impact of prenatal TRAP exposure on allergic sensitization via epigenetic modifications.

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IL-12/IL-23 neutralization is ineffective for alopecia areata in mice and humans



To the Editor:

Alopecia areata (AA) is a common autoimmune disease resulting in nonscarring hair loss. Recent work involving genome-wide association studies,¹ whole-genome gene expression assessments,² and mechanistic studies in animal models³ has revealed the role of IFN- γ and common γ -chain cytokines, including IL-15 and IL-2, in the pathogenesis of AA, as well as the central role of natural killer group 2D-expressing CD8 T cells as pathogenic disease mediators. These data have spurred investigations into novel treatments and have uncovered the potential for repurposing agents already approved for human use to treat AA, including Janus kinase inhibitors.^{4,5}

It was recently reported that expression of the genes encoding the shared p40 subunit of IL-12 and IL-23 and the IL-23-specific p19 subunit was increased in skin biopsy samples from AA lesions when compared with that from nonlesional scalp⁶ and that effective treatment of AA lesions with intralesional administration of triamcinolone led to a reduction in the expression of p40 but not p19.⁷ In addition, patients with AA treated with ustekinumab, a human mAb that binds the shared p40 subunit of IL-12 and IL-23 and antagonizes their activities, demonstrated hair regrowth.⁸ These data and subsequent case series^{9,10} suggest that ustekinumab may be a potential therapy for AA. Interestingly, despite data indicating that IFN- γ , a T_H1 cytokine, is critical for AA pathogenesis,³ it was reported that the p35 subunit specific for the T_H1-inducing IL-12 cytokine was not elevated in lesional AA samples compared with nonlesional control samples. These data hint that the IL-23-altering effects of ustekinumab may be responsible for its efficacy in AA.

To further investigate the role of IL-23 and IL-12 in the pathogenesis of AA and the potential for targeting the shared p40 subunit for treatment purposes, we first assessed the levels of the p40 subunit in the C3H/HeJ mouse model of AA. Levels of p40 in the skin of AA mice were found to be elevated when compared with those in unaffected controls (Fig 1, A). Similarly, we found that levels of the IL-23-specific p19 subunit and the intact IL-12 heterodimer were elevated in affected skin of AA mice compared with unaffected controls (Fig 1, A). On the basis of these data, we next determined whether neutralization of the shared p40 subunit or the IL-23-specific p19 subunit was effective at suppressing the development of AA. Using a

METHODS

Ethics approval

The human ethics board at all universities directly involved with the Canadian Healthy Infant Longitudinal Development (CHILD) cohort (McMaster University, University of British Columbia, University of Toronto, University of Manitoba, and University of Alberta) approved this study.

Study population

The 145 study participants ($n = 145$) constitute an atopy-enriched subset of the CHILD cohort.^{E1-E3}

Assessing allergic sensitization

Allergic sensitization was assessed by standardized epicutaneous skin tests administered to each infant at age 1 year using a panel of 6 inhalant (*Alternaria alternata*, cat hair, dog epithelium, house-dust mites [*Dermatophagoides pteronyssinus* and *Dermatophagoides farinae*], and German cockroach) and 4 food (whole cow's milk, egg white, soybean, and peanut) allergens. Histamine and glycerin were used as positive and negative controls, respectively. The wheal responses were measured at 10 (histamine) and 15 (allergen) minutes, and the maximum diameter and its orthogonal were averaged. A child was diagnosed with allergic sensitization (or atopy) if they developed a wheal greater or equal to 2 mm in diameter for any of the 10 allergens tested. Subjects testing negative to histamine were not included in this cohort unless they tested positive (with a wheal diameter of ≥ 2 mm) for 1 of the 10 allergens. If a subject tested positive to glycerin, the wheal size for glycerin was subtracted from the wheal size of any positive allergen response.

Quantifying eGA

Umbilical cord blood samples were obtained in the delivery room, processed to isolate cord blood mononuclear cells (CBMCs), and frozen as previously described.^{E3} DNAm data were generated on frozen CBMCs using the Illumina HumanMethylation450 array, as previously described.^{E4} Briefly, DNA was extracted from CBMC pellet using the Qiagen DNAeasy kit, per manufacturer's instructions. The Zymo EZ DNAm gold kit was used to bisulfite convert 750 ng of this DNA, and 160 ng of bisulfite-converted genomic DNA was applied to the 450k arrays as recommended by the manufacturer. Next, we used the Illumina GenomeStudio software to perform background subtraction and color correction.

Two published methods were used for deriving eGA. Bohlin eGA calculation required unfiltered but normalized data, and so we BMIQ normalized the unfiltered data,^{E5} then applied the eGA predictor using the 96 sites as previously reported.^{E6} Knight eGA was calculated according to previous reports, using filtered but unnormalized data.^{E7} To filter, we removed X and Y chromosome probes, probes where the CpG is a single nucleotide polymorphism or the probe binds *in silico* to the X or Y chromosome.^{E8} Next, values where fewer than 3 beads contributed to signal or with a detection P value of less than .01 were set to not available (NA), and any probe with more than 3 NA values was removed. Eight of the required 149 CpGs had been filtered out, which were added back to the data with all values set as NA.

Generation of individualized environmental exposure data

Individual exposure estimates to greenness and TRAP were assessed at the home address(es) throughout pregnancy. Green space exposure was quantified from satellite imagery using the normalized difference vegetation index (NDVI), which is a metric of overall greenness, as previously described.^{E9} NDVI was based on satellite imagery (Landsat 5) of land surface reflectance of visible and near-infrared parts of spectrum, with values ranging from -1 to 1 , with the higher numbers indicating more greenness. These values were computed at mothers' residential addresses during the 9 months of pregnancy. A time-weighted average from all addresses during pregnancy (11.5% of participants moved) was calculated for different buffer distances (ie, 100, 250,

and 500 m) around addresses to capture various representations of green space exposures.

Air pollution levels were estimated using highly spatially resolved surfaces of nitrogen dioxide (NO_2). NO_2 is of primary interest because this pollutant demonstrates fine-scale spatial variability. City-specific land use regression models to estimate NO_2 concentrations were used to assign exposures at the residential locations of all participants. The methodology to adjust both temporally and spatially all geo-referenced residences reported by participants is described elsewhere.^{E10} Briefly, exposure to land use regression-derived NO_2 was estimated using a time-weighted average exposure based on the time each participant resided in each reported address and temporally adjusted on the local fixed-site ambient monitoring data on a biweekly basis. Our exposure assessment approach enabled the computation of trimester-specific individual estimates of TRAP, whereas the greenness measure was derived for the entire pregnancy, which could potentially dilute the signal for an association (compared with that for TRAP), should one exist because NDVI varies by season.

Statistical analysis

For both Bohlin and Knight methods, deviations in eGA from chronological age (referred to as dGA) were calculated by taking the difference between eGA in days and chronological GA in days. To estimate the total, direct, and indirect effects of environmental exposures on sensitization through dGA, we first examined the risk of having allergic sensitization associated with prenatal exposures to TRAP and greenness. Next, we tested whether these environmental factors affected dGA. Finally, dGA was tested against allergic sensitization. Effect estimates are presented for a $10 \mu\text{g}/\text{m}^3$ increase in NO_2 and for 0.1 increase in NDVI, which approximate the interquartile range for each of these exposures. With 145 participants, the statistical testing of differences in sensitization status had 80% power to detect differences with 90% confidence.^{E11} Before investigating the association between TRAP, dGA, and atopy, we assessed bivariate associations between potential confounders and each of dGA and atopy. In light of the sequential pathway between exposures, cord blood DNAm, and 1-year outcome, the selection of covariates pertained to the prenatal period up to the time of birth and included maternal age and gestational diabetes as well as the mode of delivery, which was defined as vaginal birth or cesarian section with and without labor, respectively. In addition, maternal health, in particular atopic status, has been shown to be predictive of offspring atopy; thus, maternal self-reported asthma and atopy (via skin prick test) as well as antibiotic use during pregnancy were also examined. In line with putative risk factors for allergic disorders shown in the literature, we also considered parity and education, the latter serving as a surrogate for socioeconomic status. DNAm is highly associated with genotype, so in the absence of genotyping data, we used self-reported ethnicity.

We tested the associations of dGA against TRAP and greenness independently on the risk of sensitization to allergens in this population by examining the effect of (1) each exposure on atopic outcomes with and without adjustment for dGA, (2) the dGA on the risk of atopy, and (3) each exposure on the dGA (Table E2).

Counterfactual-based causal mediation analysis^{E12} was used to dissect the total effect of a treatment (here TRAP exposure, a continuous variable) into direct and indirect effects, where the latter is transmitted via a mediator (here dGA, a continuous variable) to the outcome (atopy, a binary variable). To complete this analysis, the mediation package,^{E13} designed to perform causal mediation analysis under the assumption of sequential ignorability, was used. Nonparametric bootstrapping was used for variance estimation with 1000 resamples.

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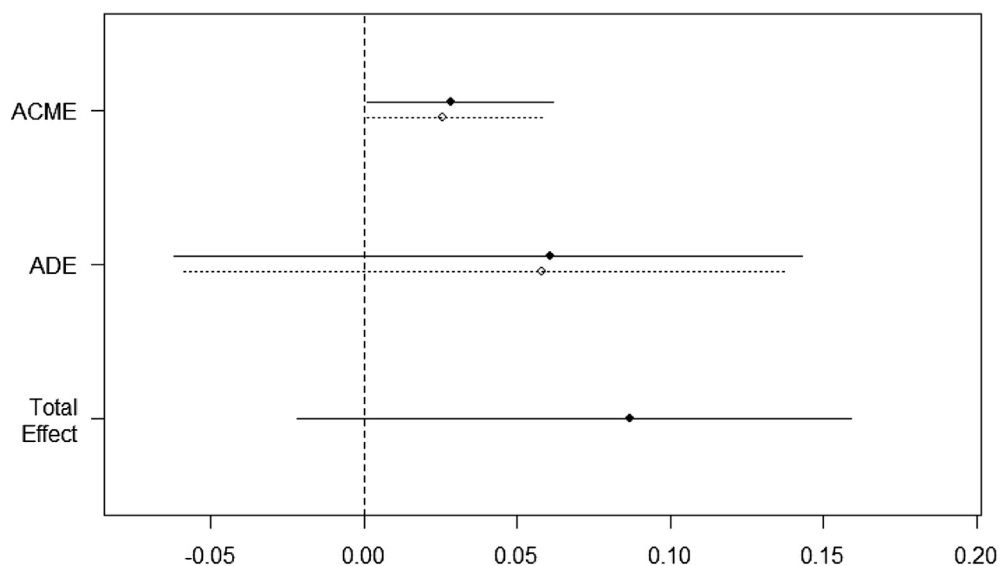


FIG E1. Visualization of CMA results, with prespecified $\alpha = 0.1$, shows direct (average direct effect, ADE) and indirect (average causal-mediated effect, ACME) effects of TRAP exposure (solid line, highest quartile; dashed line, lowest) through dGA. The average of indirect effect (ACME P value = .08) explains 31% of the total effect. CMA, Causal mediation analysis.

TABLE E1. Selected characteristics of the study population by atopic status* and by dGA†

Characteristic	Nonatopic (n = 102)	Atopic (n = 42)		dGA
Bohlin dGA (d)	2.26 ± 5.33	4.58 ± 7.16	P = .06	
Study center (n, %)			P = .33	P = .09
Vancouver (21, 14%)	67%	33%		5.31 ± 8.3
Edmonton (38, 26%)	66%	34%		3.23 ± 5.2
Winnipeg (43, 30%)	81%	19%		1.31 ± 5.6
Toronto (43, 30%)	67%	33%		3.04 ± 5.3
Sex (n, %)			P = .19	P = .27
Male (84, 58%)	67%	33%		3.4 ± 5.3
Female (60, 42%)	77%	23%		2.2 ± 6.8
Mother ethnicity (n, %)			P = .11	P = .83
White (n = 81)	75%	25%		2.35 ± 5.3
Asian (n = 11)	45%	54%		3.09 ± 4.1
Other (n = 18)	56%	44%		2.54 ± 6.35
Mother education			P = .61	P = .9
No university degree (63, 44%)	73%	27%		3.0 ± 6.5
University degree (80, 56%)	68%	32%		2.9 ± 5.5
Mother atopy			P = .77	P = .17 ^{E12}
Nonsensitized (51, 35%)	73%	27%		2.0 ± 5.1
Sensitized (94, 65%)	70%	30%		3.4 ± 6.4
Mother asthma			P = .22	P = .44
No (99, 69%)	74%	26%		2.72 ± 6.2
Yes (44, 31%)	64%	36%		3.53 ± 5.6
Mother antibiotic use			P = .7	P = .56
No (75, 82%)	71%	29%		3.22 ± 6.6
Yes (16, 18%)	75%	25%		4.25 ± 6.2
Birth weight (Z score)	0.18 ± 1.00	0.27 ± 0.95	P = .30	P = .6 ↓
Maternal age (y)	31.97 ± 4.3	32.55 ± 4.5	P = .24	P = .7 ↑
Mode of delivery (n, %)		30.8%	P = .31	P = .02
Vaginal (n=107)	69.2%	10%		2.31 ± 6.1
C-section with labor (n = 10)	90%	28.6%		1.79 ± 6.3
C-section without labor (n = 28)	71.4%			5.74 ± 5.0
Greenness	0.36 ± 0.16	0.32 (0.19)	P = .25	P = .15 ↑
NO ₂ first trimester (μg/m ³)	20.3 ± 12.9	25.4 ± 12.6	P = .06	P = .04 ↑
NO ₂ second trimester (μg/m ³)	23.3 ± 13.7	24.6 ± 13.6	P = .56	P = .20 ↑
NO ₂ third trimester (μg/m ³)	22.6 ± 14.2	23.7 ± 13.0	P = .68	P = .30 ↑

*Categorical variables: n and % with χ^2 P value; continuous variables: mean and SD with t test P value.

†Categorical variable: mean and SD, P value of t test and ANOVA for dichotomous and multiple categories, respectively; continuous variable: P value of simple linear regression; arrow indicates direction of effect. Statistically significant associations are shown in boldface.

TABLE E2. Effect estimates of TRAP and greenness on both allergic sensitization at age 1 year (atopy) (adjusted and nonadjusted for dGA) and deviation of cord blood DNAm from GA or dGA

	Observed outcomes*			
	Atopy, OR (95% CI)	P value	DGA, b (95% CI)	P value
NO ₂ (first trimester of pregnancy, 10 µg/m ³ increase)				
Unadjusted	1.42 (1.02 to 1.99)	.037	2.1 (0.11 to 4.08)	.038
Adjusted	1.34 (0.97 to 1.87)	.08		
Greenness in pregnancy				
Unadjusted	2.42 (0.05 to 110.5)	.65	1.22 (−0.32 to 2.76)	.12
Adjusted	1.47 (0.05 to 59.4)	.92		

OR, Odds ratio.

*All models are adjusted for mode of delivery.