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Associations of Exposure to Air Pollution during the Male Programming Window and Mini-Puberty with Anogenital Distance and Penile Width at Birth and at 1 Year of Age in the Multicenter U.S. TIDES Cohort

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Background: Ambient air pollution may be a developmental endocrine disruptor. In animal models, gestational and perinatal exposure to diesel exhaust and concentrated particulate matter alters anogenital distance (AGD), a marker of prenatal androgen activity, in both sexes. Little is known in humans.

Objectives: We examined exposure to fine particulate matter ($PM_{2.5}$) and nitrogen dioxide (NO_2) in relation to human AGD at birth and at 1 year of age, focusing on exposures during critical windows of reproductive development: the male programming window (MPW; gestational weeks 8–14) and mini-puberty (postnatal months 1–3).

Methods: The Infant Development and Environment Study (TIDES) recruited first trimester pregnant women ($n = 687$) at four U.S. sites (Minneapolis, Minnesota; Rochester, New York; San Francisco, California; and Seattle, Washington) from 2010 to 2012. We measured anus to clitoris (AGD-AC) and anus to fourchette (AGD-AF) in female infants at birth; in males, we measured anus to penis (AGD-AP), anus to scrotum (AGD-AS), and penile width at birth and at 1 year of age. Using advanced spatiotemporal models, we estimated maternal exposure to $PM_{2.5}$ and NO_2 in the MPW and mini-puberty. Covariate-adjusted, sex-stratified linear regression models examined associations between $PM_{2.5}$ and NO_2 and AGD.

Results: In males, a 1-$\mu g/m^3$ increase in $PM_{2.5}$ exposure during the MPW was associated with shorter AGD at birth, but a longer AGD at 1 year of age (e.g., birth AGD-AP: $\beta = -0.35$ mm; 95% CI: −0.62, −0.07; AGD-AS: $\beta = 0.37$ mm; 95% CI: 0.02, 0.73). Mini-pubertal $PM_{2.5}$ exposure was also associated with shorter male AGD-AP ($\beta = -0.50$ mm; 95% CI: −0.89, −0.11) at 1 year of age. Although not associated with male AGD measures, 1-ppb increases in NO_2 exposure during the MPW ($\beta = -0.07$ mm; 95% CI: −0.02, −0.12) and mini-puberty ($\beta = -0.04$ mm; 95% CI: −0.08, 0.01) were both associated with smaller penile width at 1 year of age. Results were similar in multipollutant models, where we also observed that in females AGD-AC was inversely associated with $PM_{2.5}$ exposure, but positively associated with NO_2 exposure.

Discussion: $PM_{2.5}$ and NO_2 exposures during critical pre- and postnatal windows may disrupt reproductive development. More work is needed to confirm these novel results and clarify mechanisms. https://doi.org/10.1289/EHP12627

Introduction

Together, indoor and outdoor air pollution are estimated to account for 7 million deaths worldwide per year, mostly due to respiratory and cardiovascular diseases, including lung cancer, heart disease, stroke, and chronic respiratory illness.[1] Often overlooked is the growing body of evidence suggesting that air pollutants adversely impact reproductive health. Numerous epidemiological studies have reported that exposure to air pollution is associated with reduced semen quality,[2–5] ovarian reserve,[6–8] and fecundability.[9–11] Air pollution exposures during pregnancy, furthermore, have been linked to hypertensive disorders of pregnancy,[12,13] shortened gestation and preterm birth,[14] and low birthweight and small for gestational age.[15–18] One possible mechanism by which air pollution may impact reproductive health outcomes is through endocrine disruption. Air pollution typically contains complex mixtures of particulate matter (PM) of varying sizes and compositions, including known endocrine disruptors, such as heavy metals and polycyclic aromatic hydrocarbons.[19]

Although much of the literature on air pollution and reproductive outcomes focuses on adult exposures, the impacts of endocrine disruptors on health may be particularly profound when exposure occurs during key developmental periods, such as gestation and early infancy.[20] The male programming window (MPW) in the first trimester and mini-puberty in infancy are believed to be critical periods for reproductive system development.[21–24] The MPW, which occurs between approximately gestational weeks 8 and 14, is a critical period during which androgen activity programs subsequent development of the male reproductive system.[25] Disruption of testosterone activity (such as through exposure to antiandrogenic compounds) during that period may have permanent adverse impacts on the reproductive organs.[26] Mini-puberty occurs around postnatal months 1–3, and during that time gonadotropin, estrogen, and androgen levels rise transiently as part of typical development in healthy infants, after which they drop to nonmeasurable levels, remaining very low until the onset of puberty.[27,28] A study of male infants observed associations between serum testosterone at 3 months of age and penile growth, suggesting a role for postnatal androgens in male reproductive system development[29]

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and in infants with disorders of sexual development (such as Klinefelter's and Turner syndromes and androgen insensitivity syndrome), hormone concentrations during mini-puberty are disrupted in syndrome-specific ways.[30] Measuring time-sensitive exposures specific to the MPW and mini-puberty is challenging given the limitations of working with human populations, thus most research in this area is based on animal models. In addition, most research has focused on males, with very little known about critical windows for the development or programming of the female reproductive system.

Research on developmental exposure to air pollutants in relation to reproductive health has been limited owing in part to the long lag between gestational exposures and reproductive maturity in humans. However, in recent years, anogenital distance (AGD) has emerged as a marker of prenatal endocrine disruption that is measurable starting *in utero* and may serve as a signal of future reproductive health.[31] AGD has been widely used in toxicological research in rodents, whereby prenatal exposures that interfere with *in utero* hormone signaling (such as anti-androgenic phthalates and flutamide) result in shorter, less androgenized AGD in male offspring at birth.[32,33] This end point has now been translated to humans, with epidemiological studies demonstrating that newborn AGD may be sensitive to a variety of endocrine disruptors, including phthalates, bisphenol A (BPA), per- and polyfluoroalkyl substances, and glyphosate.[34–39] In addition, male infants born with genital anomalies, such as cryptorchidism and hypospadias, typically have shorter AGD, further suggesting a common prenatal link likely related to impaired gestational androgen activity.[40] To the extent that AGD may be stable over the life span (as suggested by animal models and longitudinal studies in young children),[41–44] endocrine disruptor-related alterations in infant AGD may be relevant to adult reproductive function, although longitudinal human data AGD from early life through reproductive maturity is lacking. In cross-sectional studies of adults, shorter AGD in men has been linked to lower semen quality, lower sex steroid hormone concentrations, and reduced fertility in some, but not all, studies.[45–51] In females, longer AGD has been associated with polycystic ovary syndrome,[52] whereas shorter AGD has been associated with endometriosis.[53–55]

To date to our knowledge, a single epidemiological study has examined the relationship between early life exposure to air pollution and AGD in infancy. In the Shanghai-Minhang Birth Cohort Study (S-MBCS), PM $\leq$2.5 μm in aerodynamic diameter [fine particulate matter ($PM_{2.5}$)] exposure was estimated in each trimester (and in pregnancy overall) using a satellite-based modeling approach.[56] In adjusted models, $PM_{2.5}$ in the first and third trimesters was associated with shorter AGD in both male and female infants, suggesting potentially anti-androgenic effects of air pollution in both sexes. By contrast, in early rat studies of gestational exposure to total diesel engine exhaust (5.63 mg/m^3 PM), in both male and female exposed fetuses, AGD was longer, reproductive organ development was delayed, and overall, maternal testosterone and progesterone levels were increased compared with controls.[57] However, in adulthood, the male offspring had lower sperm counts, a reduced spermatid/Sertoli cell ratio, and higher follicle-stimulating hormone concentrations, suggesting reduced fecundity.[58] A mouse study of exposure to 20 mg/m^3 National Institute of Standards and Technology (NIST) diesel exhaust particle Standard Reference Material 2975 on gestational days 7–9 reported no change in sex steroid activity or AGD of the offspring at postnatal day 170; however, sperm production was reduced.[59] More recently, studies of ultrafine PM have shown mixed results. For instance, in mice exposed to ultrafine concentrated PM (at 50 $\mu g/m^3$) during the postnatal period, a trend toward decreased AGD normalized to body weight at postnatal days 26–27 was observed in females, but not in males.[60] In a separate mouse study, exposure to concentrated ambient $PM_{2.5}$ (CAPs) during multiple periods of gestation resulted in shorter AGD in offspring of both sexes at postnatal day 10, with stronger effects observed in females.[61] Importantly by postnatal day 21, differences in AGD in relation to CAPs exposure persisted only in animals that had been exposed during early gestation. These studies, suggesting potentially anti-androgenic effects of prenatal exposure to air pollutants, contrast with results of a recent epidemiological study in which maternal exposure to $PM_{2.5}$ during preconception and in early pregnancy was associated with higher maternal androgenic steroid concentrations in late pregnancy.[62]

Taken as a whole, this literature, although limited, suggests that early exposure to air pollution may alter fetal and early infant androgen activity and by extension, androgen-sensitive development; however, directionality is unclear and human evidence is scarce. Here, using data from a large, multicenter U.S. pregnancy cohort study and advanced spatiotemporal models of air pollution exposure, we build upon the single human study on this topic. To advance beyond the somewhat arbitrary exposure windows (e.g., trimesters) considered in the prior human study, we leverage temporal resolution in air pollution models to examine $PM_{2.5}$ and NO_2 exposures during known critical periods of early hormone activity (the MPW and mini-puberty) in relation to AGD and penile width (PW) at birth and at 1 year of age. In males, we hypothesize that higher exposure to air pollutants during these critical periods will be associated with shorter AGD and smaller PW. Given the paucity of research on female reproductive development, no *a priori* hypotheses were developed regarding females.

Methods

Study Overview and Population

The Infant Development and the Environment Study (TIDES) recruited pregnant individuals from prenatal clinics in four clinical sites based at academic medical centers (University of California-San Francisco, California; University of Minnesota, Minnesota; University of Rochester, New York; and University of Washington, Washington) from 2010 to 2012.[63] Eligibility criteria included being >18 years of age, able to read and write in English, and at <13 wk gestation, as well as planning to deliver in a participating study hospital. Study visits occurred in each trimester during which questionnaire data and biospecimens were collected. Children born into the study were followed postnatally through periodic in-person visits. In the present analysis, we report on data collected during visits at birth and at 1 year of age; only male offspring participated in the 1-year-of-age visits. TIDES was approved by all participating institutions, including the clinical sites and the coordinating center at the Icahn School of Medicine at Mount Sinai. All participants provided written informed consent before engaging in any study activities.

Air Pollution Assessment

At enrollment, each TIDES participant reported their residential address. Using the address data, we estimated average $PM_{2.5}$ and NO_2 exposures specific to each time window of interest based on spatiotemporal models with point-based spatial resolution and a 2-wk temporal-resolution scale. Details of these models, summarized here, have been explained further elsewhere.[64–66] Models used data from stationary air monitoring stations, as well as a substantial volume of measurements from research cohort-specific monitors at various locations nationwide. The temporal variations in these data were leveraged, along with a large suite of other variables, to predict

changes in pollutant levels over time at each given point in space. Specifically, a geographic information system was used to identify covariates representing land use characteristics that could reflect spatial variability in air pollution distributions; next, the dimension-reduced regression covariates were obtained using partial least squares from >400 of these geographic variables. The spatiotemporal features of pollution concentrations were decomposed into spatially varying long-term averages, spatially varying seasonal and long-term trends, and spatially correlated but temporally independent residuals, and these components were fitted jointly in a likelihood-based spatiotemporal extension of universal kriging. Using these models, biweekly $PM_{2.5}$ and NO_2 exposures were estimated at all TIDES participants' addresses. Finally, these biweekly estimates were averaged to correspond to the time windows of interest. Given the interest in disruption of androgen activity during critical windows for the present analysis, our primary models estimated average pollutant concentrations during the MPW (gestational weeks 8–14) and, in males only, mini-puberty (postnatal months 1–3, when testosterone peaks in male infants[28]). Secondarily, we examined estimated pollutant levels averaged over each trimester of pregnancy.

AGD Assessment

At birth, study coordinators conducted TIDES birth exams as described elsewhere.[67] In most cases, these exams occurred in the birth hospital before discharge. Following standard protocols, a trained examiner used dial calipers to assess two AGD measures on each child. In females, we measured *a*) ano-fourchette distance (AGD-AF), the distance from the center of the anus to the posterior fourchette; and *b*) ano-clitoral distance (AGD-AC), the distance from the center of the anus to the anterior clitoral hood. In males, we measured *a*) ano-penile distance (AGD-AP), the distance from the center of the anus to the anterior base of the penis; and *b*) ano-scrotal distance (AGD-AS), the distance from the center of the anus to the base of the scrotum. We also measured PW in males. At 1 year of age, male children participated in a follow-up assessment including AGD measurements performed as described above. All measurements were made in triplicate, and the mean was used in analyses.

Covariates

Covariate data were obtained from maternal questionnaires and clinical records as follows. At enrollment in early pregnancy, mothers reported their age (continuous), parity (nulliparous/parous), and highest level of educational attainment, which was categorized here as some college or less, graduated college, and some graduate education. Mothers self-reported smoking habits during pregnancy in each trimester, which was subsequently categorized as any/no smoking during pregnancy. At birth, the infant's biological sex was recorded and mothers reported their child's race (as American Indian or Alaska Native, Asian, Black or African-American, Native Hawaiian or Pacific Islander, White, or other) and ethnicity (as Hispanic or Latino vs. Not Hispanic or Latino). We recoded race and ethnicity here as non-Hispanic black, non-Hispanic white, and other. Race and ethnicity are used as proxies for systemic racism and discrimination that may underlie higher exposure to pollutants and contribute to alterations in prenatal hormone pathways. Gestational age at birth (based on clinical records) and age at exam were combined to create a single variable for the child's postconception age at AGD exam. In light of the strong association between child's body size and AGD, we calculated weight-for-length/height *z*-scores at birth and at 1 year of age based on World Health Organization sex-specific growth curves.[68]

Statistical Analysis

We conducted descriptive analyses (mean, median, standard deviation, range, missingness) for all exposures, outcomes, and covariates. Subsequent analyses were based on data from complete cases. In preliminary analyses, we examined correlations between key variables, including $PM_{2.5}$ and NO_2 within time points, and between exposures at adjacent time points. To understand the nature of the association of each outcome with $PM_{2.5}$, NO_2, and covariates, partial dependence plots from a random forest model were examined for evidence of possible nonlinearity.[69]

We first examined the five continuous outcome measures (2 AGD measures each in males and females, as well as PW in male) at birth in relation to $PM_{2.5}$ and NO_2 concentrations averaged over the MPW and secondarily, each trimester. To do so, we fitted separate unadjusted, sex-stratified linear regression models for each exposure–outcome relationship and then fitted additional models that adjusted for the covariates. Because the random forest plots showed that some associations were nonlinear, we used generalized additive models (GAMs) using the gam() function in the mgcv package in R to allow smooth nonlinear relationships between all continuous covariates and each outcome. To report slopes for $PM_{2.5}$ and NO_2, additional GAM models were fitted in which $PM_{2.5}$ and NO_2 were assumed to have linear associations with outcomes. In our final models, covariates that demonstrated nonlinear associations with AGD were fitted with smooth terms, whereas the others were included as linear terms.

We then examined three reproductive outcomes (2 AGD measures and PW) measured at 1 year of age in males only. Our primary exposures were estimated $PM_{2.5}$ and NO_2 concentrations averaged over the MPW and postnatal mini-puberty. We again fitted unadjusted models, followed by GAM models adjusting for the covariates listed above, as well as the corresponding AGD or PW measurement at birth (as a precision variable).

We conducted a number of sensitivity analyses based on our primary models. First, we fitted models additionally including average temperature and humidity during the relevant exposure windows as determined based on the National Oceanic and Atmospheric Administration's Local Climatological Data.[70] Although there is little evidence that these factors are associated with AGD and they are therefore unlikely to confound associations, their importance to air pollution modeling warranted further consideration. Second, we adapted our primary models as multipollutant models that simultaneously adjusted for both pollutants as measured during the same windows of interest. Third, we fitted multitemporal models that included estimated air pollutant exposure at both time points (MPW and mini-puberty) in relation to male genital measures at 1 year of age. For these analyses, we assumed a linear relation between the genital measures at birth and 1 year of age. Fourth, we refitted models excluding the 49 (7.1%) participants who reported any smoking during pregnancy, given some prior evidence that maternal smoking may be associated with longer AGD in both sexes.[71] In a final sensitivity analysis focused on AGD at 1 year of age only, we excluded the corresponding AGD or PW measurement at birth.

Residual diagnostics for all models were conducted using standard methods, including graphical checks for linearity, constant variance, and normality of the residuals.[72] Cook's distance and other plots were used to check for potentially influential observations. All models were fit using R (version 4.1.1; R Development Core Team). Values of $p < 0.05$ were considered statistically significant.

Results

Descriptive Statistics and Correlations

There were 794 babies born to mothers who consented to participate in TIDES. Of those, 55 had no AGD or no air pollution data

available and 52 were missing covariate data. Three dyads provided AGD data at 1 year of age, but not at birth. Ultimately, 687 dyads were included in models considering AGD at birth, and 268 dyads (male offspring only) were included in models considering 1-year-of-age AGD. Participants with missing data did not differ from those included in models in terms of maternal age, infant age at exam, infant weight-for-length z-scores, parity, race/ethnicity, education, and smoking (Table S1). However, we observed differences by center whereby Seattle participants comprised 18% of participants included in models, but 41% of participants missing covariate data.

Among the 687 dyads in the analytic data set, on average, mothers were 31.1 ± 5.5 y old and 45.9% had a prior live birth. The cohort was predominantly non-Hispanic white (59.7%) and 43.5% had some graduate education, whereas 24.9% had less than a college education (Table 1). A small fraction (7.1%) of mothers reported smoking during pregnancy. Half (49.3%) of the infants born were female and on average, the birth exam occurred at 40.2 ± 2.2 wk postconception. At 1 year of age, 268 male offspring participated in exams and provided data for this analysis; these exams occurred at 95.3 ± 6.4 wk postconception on average. Compared with the full cohort who participated at birth, mothers who participated in the 1-year-of-age visit more often had less than a college degree (32.5% vs. 24.9% among birth-visit participants) and were less likely to be of "other" races/ethnicities (23.1% vs. 27.5% among birth-visit participants; Table 1).

Summary statistics for estimated $PM_{2.5}$ and NO_2 concentrations averaged across the main temporal windows of interest are presented in Table 2. Among all participants who completed birth visits, mean $PM_{2.5}$ was $7.86 \pm 2.21\ \mu g/m^3$ and mean NO_2 was 9.56 ± 4.43 ppb during the MPW. Estimated exposures for the same time intervals were similar among the subset of participants who completed birth visits. Average estimated exposures during mini-puberty tended to be higher ($PM_{2.5}$: $9.39 \pm 4.24\ \mu g/m^3$; NO_2: 10.32 ± 4.90 ppb) than those observed for the MPW. We observed low-to-moderate positive correlations between estimated exposures in the MPW and mini-puberty ($PM_{2.5}$: $r = 0.25$; NO_2: $r = 0.44$; Figure S1). Correlations between the two pollutants ($PM_{2.5}$ and NO_2) measured at the same time points were similarly moderate (MPW: $r = 0.26$; mini-puberty: $r = 0.47$). Estimated $PM_{2.5}$ exposures tended to be highest in San Francisco and Minneapolis and lowest in Seattle, whereas NO_2 exposures tended to be highest in Seattle and lowest in Rochester (Table S2; Figure S2). Estimated exposures by trimester are presented in Table S3 and estimated exposures by participant characteristics are presented in Table S4.

At birth, AGD measures were longer in males than females (Table 1). On average, in males, AGD-AP was 49.8 ± 5.9 mm and AGD-AS was 24.8 ± 4.5 mm, whereas in females, AGD-AC was 36.9 ± 3.7 mm and AGD-AF was 16.0 ± 3.1 mm. The correlation between the two AGD measures in was 0.65 in males and 0.48 in females. At 1 year of age, in males, average AGD-AP was 74.3 ± 7.1 mm and AGD-AS was 37.7 ± 6.4 mm. Correlations between AGD measures at birth and at 1 year of age in males was weak to moderate (AGD-AP: $r = 0.32$; AGD-AS: $r = 0.51$). PW similarly increased from birth to 1 year of age (birth: 10.8 ± 1.3 mm; 1 year of age: 13.5 ± 1.7 mm), and measurements taken at the two time points were weakly correlated ($r = 0.23$).

Unadjusted models are summarized in Table S5 and our primary adjusted models are presented in Figures 1 and 2 and Table S6. In general, in male infants, associations were similar, but stronger, in adjusted models compared with unadjusted. In female infants, associations tended to be attenuated after adjustment for covariates. All estimates presented are per 1-$\mu g/m^3$ increase in $PM_{2.5}$ or 1-ppb increase in NO_2.

Table 1. Characteristics [mean ± SD or N (%)] of the TIDES study population (2010–2012).

Characteristics	Provided data for models examining AGD at birth ($n = 687$)	Provided data for models examining AGD at 1 year of age (boys only) ($n = 268$)
Maternal characteristics		
Maternal age (y)	31.1 ± 5.5	31.1 ± 5.3
Parity		126 (47.0)
Parous	315 (45.9)	—
Nulliparous	372 (54.1)	—
Education		
Some college or less	171 (24.9)	87 (32.5)
Graduated college	217 (31.6)	63 (23.5)
Some graduate education	299 (43.5)	118 (44.0)
Smoking during pregnancy		
Any	49 (7.1)	17 (6.3)
None	638 (92.9)	251 (93.7)
Study center		
Minneapolis, Minnesota	192 (27.9)	87 (32.5)
Rochester, New York	199 (29.0)	76 (28.4)
San Francisco, California	173 (25.2)	63 (23.5)
Seattle, Washington	123 (17.9)	42 (15.7)
Infant characteristics		
Infant sex		
Female	339 (49.3)	—
Male	348 (50.7)	268 (100)
Infant race/ethnicity		
Non-Hispanic white	410 (59.7)	165 (61.6)
Non-Hispanic black	88 (12.8)	41 (15.3)
Other	189 (27.5)	62 (23.1)
Preterm birth (<37 wk)		
Yes	57 (8.3)	29 (10.8)
No	630 (91.7)	239 (89.2)
Birth exam characteristics		
Postconception age at AGD exam (wk)	40.2 ± 2.2	40.3 ± 2.4
Weight-for-length/height z-score	−0.4 ± 1.2	−0.4 ± 1.1
AGD-AP [male (mm)]	49.8 ± 5.9	49.7 ± 6.0
AGD-AS [male (mm)]	24.8 ± 4.5	24.7 ± 4.5
PW [male (mm)]	10.8 ± 1.3	10.7 ± 1.3
AGD-AC [female (mm)]	36.9 ± 3.7	—
AGD-AF [female (mm)]	16.0 ± 3.1	—
One-year-of-age exam characteristics (males only)		
Postconception age at AGD exam (wk)	—	95.3 ± 6.4
Weight-for-length/height z-scores	—	0.31 ± 1.0
AGD-AP (mm)	—	74.3 ± 7.1
AGD-AS (mm)		37.7 ± 6.4
PW (mm)	—	13.5 ± 1.7

Note: Complete case data are presented in Table 1. There were 106 dyads missing data on one or more variables who were therefore excluded from the present analyses. Missingness is as follows: gestational air pollution estimates ($n = 32$), AGD-AP at birth ($n = 20$), AGD-AS at birth ($n = 19$), PW at birth ($n = 19$), AGD-AC at birth ($n = 20$), AGD-AF at birth ($n = 18$), AGD-AP at 1 year of age ($n = 102$), AGD-AS at 1 year of age ($n = 101$), PW at 1 year of age ($n = 104$), race/ethnicity ($n = 37$), maternal education ($n = 9$), parity ($n = 26$), weight-for-length/height z-score at birth ($n = 59$), age at birth exam ($n = 38$), maternal age ($n = 2$), weight-for-length/height z-score at 1 year of age ($n = 102$), age at 1-year-of-age exam ($n = 102$). —, Not applicable; AGD, anogenital distance; AGD-AC, anogenital distance, anus to clitoris; AGD-AF, anogenital distance, anus to fourchette; AGD-AP, anogenital distance, anus to penis; AGD-AS, anogenital distance, anus to scrotum; mm, millimeters; PW, penile width; SD, standard deviation; TIDES, The Infant Development and Environment Study; wk, weeks; y, years.

$PM_{2.5}$ and Genital Measurements

In adjusted models, estimated $PM_{2.5}$ during the MPW was inversely associated with AGD-AP [$\beta = -0.35$ mm; 95% confidence interval (CI): −0.62, −0.07] and, less strongly, with AGD-AS ($\beta = -0.14$ mm; 95% CI: −0.37, 0.09) at birth in male infants (Figure 1). In models examining AGD in boys at 1 year of age,

Table 2. Distributions of maternal exposures to NO_2 and $PM_{2.5}$ during the male programming window and mini-puberty in the TIDES cohort (2010–2012).

Exposure	Window	Mean ± SD	Min	Percentile 25th	50th	75th	Max
Participants who provided data for models examining AGD at birth ($n = 687$)—mothers of males and females							
$PM_{2.5}$ ($\mu g/m^3$)	MPW (gestational weeks 8–14)	7.86 ± 2.21	2.25	6.39	7.63	9.29	16.69
NO_2 (ppb)	MPW (gestational weeks 8–14)	9.56 ± 4.43	1.80	6.06	8.98	12.36	31.37
Participants who provided data for models examining AGD at 1 year of age ($n = 268$)—mothers of males only[a]							
$PM_{2.5}$ ($\mu g/m^3$)	MPW (gestational weeks 8–14)	7.89 ± 2.13	2.25	6.64	7.57	9.39	13.93
	Mini-puberty (postnatal months 1–3)	8.16 ± 2.39	2.38	6.79	7.93	9.17	17.6
NO_2 (ppb)	MPW (gestational weeks 8–14)	9.39 ± 4.24	2.41	5.94	8.71	12.16	24.37
	Mini-puberty (postnatal months 1–3)	10.32 ± 4.90	2.19	6.82	9.67	13.02	30.42

Note: AGD, anogenital distance; max, maximum; min, minimum; MPW, male programming window; m^3, meters cubed; NO_2, nitrogen dioxide; ppb, parts per billion; $PM_{2.5}$, fine particulate matter; SD, standard deviation; TIDES, The Infant Development and Environment Study.
[a]Postnatal exposures are limited to mother–son dyads with AGD measures at 1 year of age only, whereas prenatal exposures include all infants, male and female.

associations with $PM_{2.5}$ during the MPW were positive (AGD-AP: $\beta = 0.28$ mm; 95% CI: −0.13, 0.68; AGD-AS: $\beta = 0.37$ mm; 95% CI: 0.02, 0.73), whereas associations with $PM_{2.5}$ estimated during mini-puberty were negative [AGD-AP: $\beta = -0.50$ mm; 95% CI: −0.89, −0.11; AGD-AS: $\beta = -0.21$ mm; 95% CI: −0.55, 0.14 (Figure 2)]. All CIs for the association between $PM_{2.5}$ and PW included the null. In secondary analyses examining estimated exposures by trimesters, associations in the first trimester were similar to those observed during the MPW, and we additionally observed an inverse association between $PM_{2.5}$ in the third trimester and PW at birth (Table S6). In addition, in models examining AGD in boys at 1 year of age, positive associations were observed between $PM_{2.5}$ in the third trimester and AGD-AP ($\beta = 0.46$ mm; 95% CI: 0.02, 0.89), as well as AGD-AS ($\beta = 0.35$ mm; 95% CI: −0.03, 0.72; Table S7).

Associations between $PM_{2.5}$ during the MPW and both female AGD measures showed evidence of nonlinearity and were nonsignificant in both linear and nonlinear models (Figure 1; Table S6, Figure S3, and Excel Table S1). In secondary analyses examining estimated exposures by trimesters in females, the only notable association observed was a positive association between $PM_{2.5}$ in the third trimester and AGD-AF ($\beta = 0.26$ mm; 95% CI: 0.08, 0.45; Table S7).

NO_2 and Genital Measurements

In males, no associations between prenatal exposures to estimated NO_2 and genital measurements at birth were observed (Figure 1). Some evidence of nonlinearity in models examining exposures during the MPW was again observed; however, associations were not significant in linear or nonlinear models (Figure S3). Similarly, few associations were observed between prenatal exposures to NO_2 and AGD in males at 1 year of age (Figure 2; Table S6). However, higher NO_2 exposures in both the MPW ($\beta = -0.07$ mm; 95% CI: −0.12, −0.02) and mini-puberty ($\beta = -0.04$ mm; 95% CI: −0.08, 0.01) were associated with smaller 1-year-of-age PW (Figure 2; Table S6). In secondary analyses examining estimated exposures by trimester, no associations were observed, with the exception of an inverse association between first trimester NO_2 and PW at 1 year of age ($\beta = -0.07$ mm; 95% CI: −0.12, −0.02; Tables S7 and S8).

In females, we observed nonlinear positive associations between prenatal exposures to NO_2 and AGD measures at birth, specifically at exposures >25 ppb (Figure S3). Estimated NO_2 concentrations during the MPW were associated with longer AGD-AC ($\beta = 0.08$ mm; 95% CI: −0.01, 0.16; Figure 1; Table S6), a relationship that appeared to be influenced by a single high (but plausible) NO_2 value (Figure S3). In secondary analyses by trimester, we additionally observed positive associations between NO_2 in the first trimester and AGD-AC ($\beta = 0.10$ mm; 95% CI: 0.01, 0.19), second trimester NO_2 and AGD-AF ($\beta = 0.07$ mm; 95% CI: −0.01, 0.15), and third trimester NO_2 and AGD-AF ($\beta = 0.09$ mm; 95% CI: 0.01, 0.17) at birth (Table S7).

Sensitivity Analyses

Inclusion of meteorological factors. We fitted a set of models additionally adjusted for temperature and humidity averaged over the relevant exposure windows (Table S9). The association between $PM_{2.5}$ in the MPW and AGD-AP was strengthened with the inclusion of temperature and humidity ($\beta = -0.46$ mm; 95% CI: −0.78, −0.15), whereas all other estimates for the prenatal air pollutants and AGD measures in males and females at birth were similar to the main models and CIs included the null. In models examining AGD in male offspring at 1 year of age in relation to $PM_{2.5}$, results were similar to, but generally stronger than, the main models that did not include meteorological covariates. Inclusion of meteorological factors resulted in a slight attenuation of the inverse associations between NO_2 and PW at 1 year of age, whereas associations with NO_2 during mini-puberty were stronger (Table S9).

Multipollutant models mutually adjusted for $PM_{2.5}$ and NO_2. In models mutually adjusted for both pollutants (as estimated during the same time period), the inverse associations between $PM_{2.5}$ and AGD measures in male infants at birth persisted (AGD-AP: $\beta = -0.38$ mm; 95% CI: −0.66, −0.09; AGD-AS: $\beta = -0.18$ mm; 95% CI: −0.42, 0.06; Table S10). Associations with AGD measures in female infants were strengthened in mutually adjusted models, with both pollutants showing associations with AGD-AC, but in opposite directions ($PM_{2.5}$: $\beta = -0.23$ mm; 95% CI: −0.44, −0.02; NO_2: $\beta = 0.12$ mm; 95% CI: 0.03, 0.22). Associations between $PM_{2.5}$ and 1-year-of-age AGD measures in boys were also slightly strengthened in mutually adjusted models. For example, similar to single-pollutant models, $PM_{2.5}$ during the MPW was positively associated with AGD-AP in boys at 1 year of age ($\beta = 0.41$ mm; 95% CI: −0.02, 0.84), whereas associations with $PM_{2.5}$ during mini-puberty were inverse ($\beta = -0.59$ mm; 95% CI: −1.05, −0.13). As in single-pollutant models, associations between NO_2 and AGD measures at 1 year of age were null, whereas NO_2 during both the MPW and mini-puberty were inversely associated with PW (MPW: $\beta = -0.07$ mm; 95% CI: −0.12, −0.02; mini-puberty: $\beta = -0.05$ mm; 95% CI: −0.10, 0.00).

Multitemporal models mutually adjusted for exposures at both the MPW and mini-puberty. In models concurrently examining exposures during both periods of interest (MPW and mini-puberty) in relation to AGD at 1 year of age in boys, results were similar or slightly attenuated compared with single time point models (Table S11). For example, $PM_{2.5}$ during the MPW was positively associated with AGD-AP in boys at 1 year of age ($\beta = 0.26$ mm; 95% CI: −0.14, 0.67), whereas $PM_{2.5}$ exposure during mini-puberty showed inverse associations ($\beta = -0.50$ mm; 95% CI: −0.89, −0.10). Once again, no associations between NO_2 and AGD were observed; however, associations between NO_2 and smaller PW persisted and were stronger during the MPW ($\beta = -0.06$ mm; 95% CI: −0.11, −0.01) compared with during mini-puberty ($\beta = -0.02$ mm; 95% CI: −0.07, 0.03).

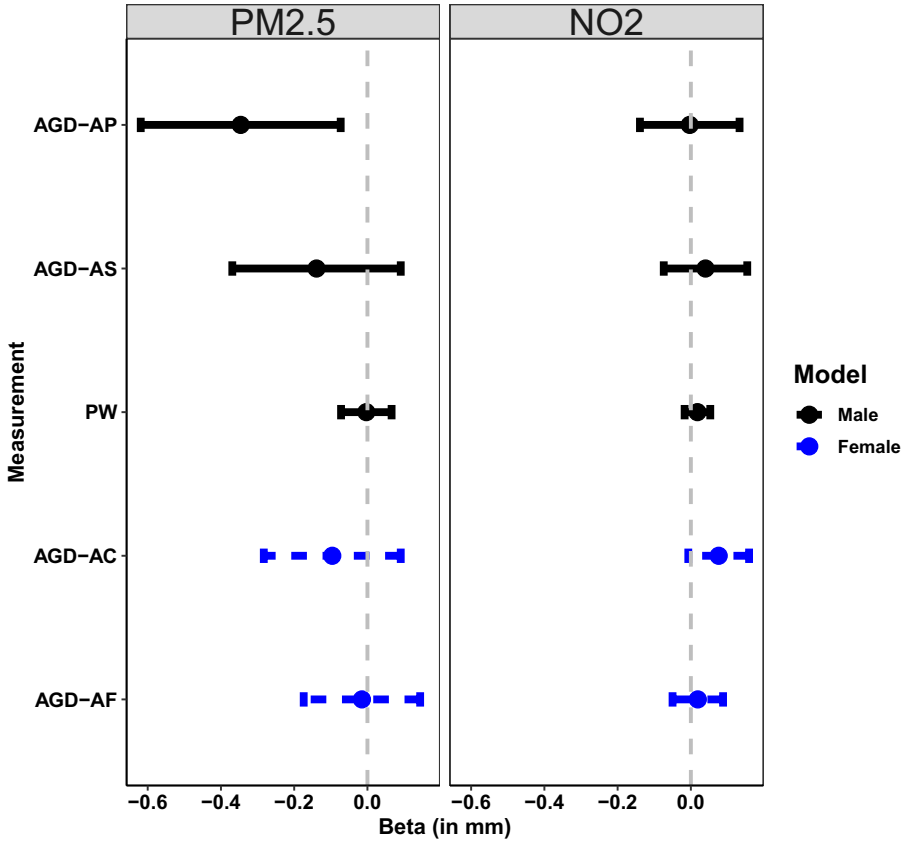

Figure 1. Associations between $PM_{2.5}$ and NO_2 exposures during the male programming window in relation to anogenital distance (AGD) and penile width (PW) (both in mm) in infants at birth in the TIDES cohort, 2010–2012 ($n = 346$–347). Linear regression models were adjusted for center, parity, race/ethnicity, education, smoking, maternal age, postconception age at AGD exam, weight-for-length/height z-score at AGD measurement. Point estimates and 95% confidence intervals are per 1-$\mu g/m^3$ increase in $PM_{2.5}$ or 1-ppb increase in NO_2. Summary data are available in Table S4. Some evidence of nonlinearity between the pollutant and AGD was observed (see Figure S3 for plots). Note: AGD-AC, anogenital distance, anus to clitoris; AGD-AF, anogenital distance, anus to fourchette; AGD-AP, anogenital distance, anus to penis; AGD-AS, anogenital distance, anus to scrotum; mm, millimeters; NO_2, nitrogen dioxide; $PM_{2.5}$, fine particulate matter; PW, penile width; TIDES, The Infant Development and Environment Study.

Additional sensitivity analyses. In models excluding children of mothers reported any smoking during pregnancy ($n = 49$), results were largely unchanged from main models (Table S12). In a final set of sensitivity analyses, we fitted adjusted models examining AGD at 1 year of age in boys without adjustment for the equivalent birth measure. Results were similar to our main models although the association between $PM_{2.5}$ in the MPW and AGD-AS at 1 year of age was weaker ($\beta = 0.23$; 95% CI: −0.18, 0.63; Table S13).

Discussion

In this U.S. cohort, we observed associations between prenatal exposures to air pollutants and AGD measures in both male and female infants. Consistent with our hypotheses, we observed that $PM_{2.5}$ exposure during the MPW was associated with shorter, less masculinized AGD in males at birth. We additionally observed associations with 1-year-of-age genital measures in males that varied by pollutant and timing of exposure. Somewhat surprisingly, in contrast to associations with AGD at birth, $PM_{2.5}$ concentrations in the MPW were associated with longer, more masculinized, AGD-AS at 1 year of age. At the same time, $PM_{2.5}$ exposure during postnatal mini-puberty was associated with shorter, less masculine AGD. These results were robust in sensitivity analyses including models considering both exposure time points contemporaneously. Few associations between NO_2 and AGD in males were observed; however, NO_2 exposures during the MPW and mini-puberty were both associated with smaller PW at 1 year of age. In females, no associations were observed between $PM_{2.5}$ and NO_2 in the MPW and AGD measures at birth, although in secondary analyses, there was some indication of positive associations with exposures in the third trimester. Taken as a whole, these results suggest that exposure to air pollutants during sensitive windows across gestation and infancy may alter the early hormonal milieu and impact development of the human reproductive system, particularly in male infants, with the direction of association varying by timing of exposure and outcome assessment.

Prior research on air pollution and AGD in humans has been limited to the S-MBCS cohort (Shanghai, China; $n = 876$ mother–child dyads), which found that first and third trimester $PM_{2.5}$ exposures were associated with shorter newborn AGD in both sexes (Sun et al.).[56] Similar to that study, we observed inverse associations between newborn AGD in males in relation to $PM_{2.5}$ exposure during early gestation, given that the first trimester largely overlaps with the MPW. However, in our secondary analyses, newborn male AGD was not associated with $PM_{2.5}$ exposure in the third trimester, which is not typically thought of as a critical period for male genital development. In females, our results were in direct contrast with the S-MBCS results as we observed longer AGD-AF in relation to third trimester $PM_{2.5}$ exposure. One important difference between the cohorts was the much higher exposure to $PM_{2.5}$ in the S-MBCS, with median levels nearly 10-fold higher across gestation in

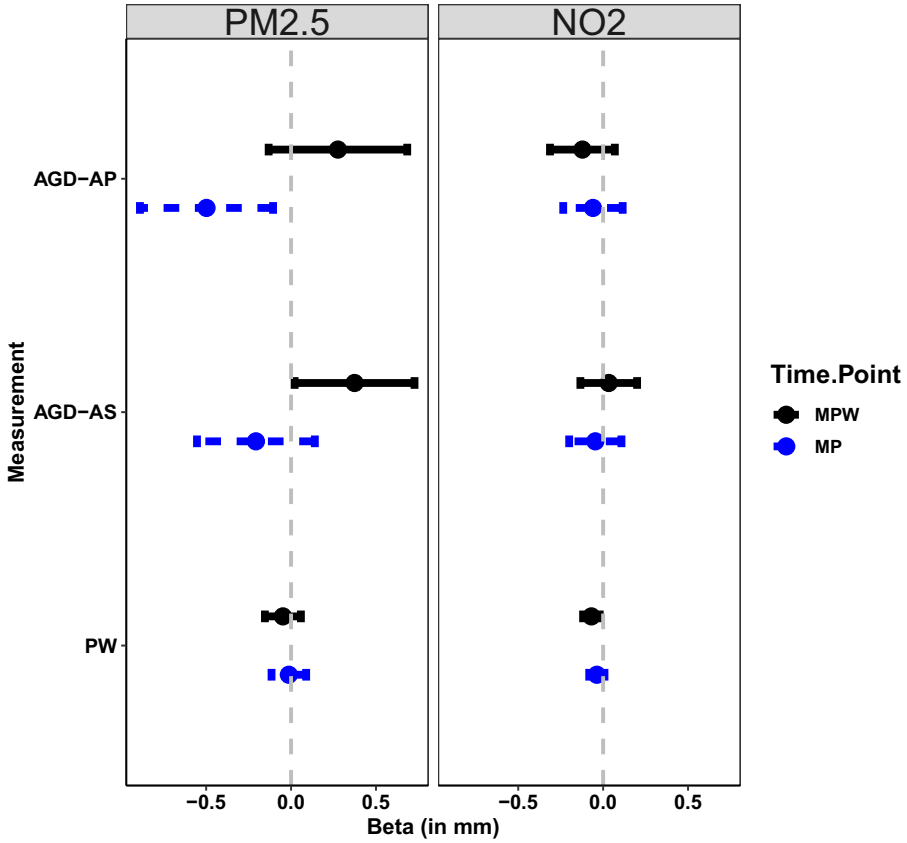

Figure 2. Associations between $PM_{2.5}$ and NO_2 exposures during the male programming window and mini-puberty in relation to anogenital distance (AGD) and penile width (PW) (both in mm) in male infants at 1 year of age in the TIDES cohort, 2010–2012 ($n = 346$–347). Linear regression models were adjusted for center, parity, race/ethnicity, education, smoking, maternal age, postconception age at AGD exam, weight-for-length/height z-score at AGD measurement, and AGD (or PW) at birth. Point estimates and 95% confidence intervals are per 1-$\mu g/m^3$ increase in $PM_{2.5}$ or 1-ppb increase in NO_2. Summary data are available in Table S4. Note: AGD-AC, anogenital distance, anus to clitoris; AGD-AF, anogenital distance, anus to fourchette; AGD-AP, anogenital distance, anus to penis; AGD-AS, anogenital distance, anus to scrotum; mm, millimeters; MP, mini-puberty; MPW, male programming window; NO_2, nitrogen dioxide; $PM_{2.5}$, fine particulate matter; PW, penile width; TIDES, The Infant Development and Environment Study.

their study compared with ours. For example, median first trimester $PM_{2.5}$ was 7.9 $\mu g/m^3$ in our multicenter U.S. study vs. 60.5 $\mu g/m^3$ in Shanghai. Although the dramatic difference in $PM_{2.5}$ exposure may have contributed to some of the differences in results, it is worth noting that our results suggest that even at far lower levels of exposure, air pollutants may still impact reproductive system development. In fact, for some models (such as those examining first trimester $PM_{2.5}$ and newborn AGD-AP), associations were twice as strong in our low-exposure cohort compared with theirs (TIDES: $\beta = -0.49$ mm; 95% CI: -0.83, -0.14; S-MBCS: $\beta = -0.26$ mm; 95% CI: -0.35, -0.17). This may reflect nonmonotonic effects of $PM_{2.5}$ on reproductive development; it may also reflect regional differences in the composition of $PM_{2.5}$ related to endocrine-disrupting potential. In a study of pregnant women in the Northeast Region of the United States with levels of $PM_{2.5}$ exposure similar to that of our participants, early pregnancy $PM_{2.5}$ was associated with higher concentrations of androgenic, pregnenolone, and progestin steroids in late pregnancy, although how this suite of hormonal alterations might collectively impact AGD is unclear and few differences by fetal sex were observed.[62]

The magnitude of the estimates observed in our analyses was small. For instance, the associations observed between $PM_{2.5}$ in the MPW and AGD-AP in males corresponded to ~ 1 mm shorter AGD-AP associated with an interquartile (IQR) range increase in exposure from 6.39 to 9.29 $\mu g/m^3$. That represents 2% smaller AGD in the average male newborn. Similarly, associations between prenatal exposures to air pollutants in females corresponded to ~ 0.5–0.6 mm longer AGD with a 1-IQR increase in exposure, a difference of $\sim 2\%$–3% for the average female newborn. Although these differences are small, they may be important on a population level and are in line with prior research on environmental pollutants and newborn AGD. For instance, in our prior research on phthalates, an increase in di(2-ethylhexyl) phthalate metabolite concentrations from the 10th to 90th percentile of exposure was associated with 2%–5% smaller in AGD measures in male newborns.[39] Importantly, on a population level, even small decrements in reproductive health measures may have a considerable public health impact.

We believe this to be the first study to examine gestational NO_2 exposure in relation to reproductive development in offspring. Although no significant associations between gestational NO_2 and newborn AGD were observed in males, higher NO_2 exposure during the MPW and mini-puberty were both associated with smaller PW at 1 year of age. Furthermore, in secondary analyses, NO_2 exposures in late pregnancy were associated with longer AGD at birth in females. It is believed that longer female AGD may reflect higher exposure to prenatal androgens; however, to date, there have been few epidemiological or basic science studies examining NO_2 exposure in relation to sex steroid activity. In a Hong Kong birth cohort, prenatal exposure to NO_2 was negatively associated with pubertal staging in sons at 11 years of age; however, no associations were observed in daughters.[73] Similar work in Polish adolescent females showed earlier ages of menarche following prenatal

exposures to a number of air pollutants including nitric oxide (NO),[74] but NO_2 was not studied. Studies using large administrative databases in Taiwan have additionally reported that among adult women, higher exposure to NO_2 is associated with elevated risks of polycystic ovary syndrome, a disorder characterized by hyperandrogenism,[75] as well as dysmenorrhea.[76] Some studies have noted associations between adult NO_2 exposures and breast cancer, a hormone-sensitive condition, whereas others have not.[77–79] Given the paucity of work in this area, our analyses on NO_2 and AGD need to be replicated in other cohorts and supported by complementary animal studies to better understand potential mechanisms.

A novel aspect of our study is the longitudinal assessment of AGD in males at both birth and at 1 year of age, which allowed us to examine exposures during the MPW as well as during mini-puberty, a potentially endocrine-sensitive postnatal window. Very few epidemiological studies have directly studied the impact of exposure to endocrine disruptors during mini-puberty on subsequent health outcomes. A small body of work, for example, has examined the use of soy formula, which has estrogenic potential, in relation to measures of reproductive organ size across childhood with few associations observed.[80,81] A separate study reported positive associations between urinary BPA and urinary estradiol in both sexes at multiple time points across early infancy.[82] Plasticity of reproductive development across infancy is suggested by our results showing that $PM_{2.5}$ exposure during mini-puberty is associated with smaller AGD at 1 year of age. We additionally observed evidence of weak associations between NO_2 exposure in mini-puberty and smaller PW. If reproductive development were fully canalized at birth, we would expect there to be no association between postnatal exposure to endocrine disruptors and 1-year-of-age reproductive measures, particularly after adjusting for potential confounding by prenatal exposures.

Some animal literature supports the possibility of overall stability of reproductive development over time while allowing for plasticity. For example, in a rat model, Kita et al. observed that exposures to the antiandrogens di(2-ethylhexyl) phthalate and flutamide on postnatal days 23–53 resulted in reductions in male AGD that were smaller than those observed following prenatal exposure, but statistically significant nonetheless.[32] Similarly, in an adult male rat model, AGD decreased by 17% after postnatal castration, and administration of the estrogen diethylstilbestrol (DES) similarly reduced AGD by 11%, but AGD returned to "normal" after DES treatment cessation.[83] At present, temporal changes in (or stability of) human AGD over time are not well characterized. A recent analysis of longitudinal AGD data from 3,705 children 0–24 months of age showed consistent increases in AGD from birth to 6 months of age followed by a plateau; the observed changes appeared to be largely reflective of overall increases in infant body size.[41] However, to our knowledge, no prior epidemiological study has examined endocrine-disrupting exposures during multiple potentially sensitive pre- and postnatal windows in relation to AGD. Even if AGD is largely stable over time, with changes only reflecting increased body size with growth, our results suggest that insults during sensitive prenatal and early postnatal windows may nevertheless alter AGD trajectories. Future research in animal models is needed to selectively target exposures during different critical windows to help clarify the plasticity of reproductive development. Better understanding this plasticity is also important for future work on adult AGD and reproductive health outcomes. To date, much of the cross-sectional epidemiological research in this area has been conducted under the premise that adult AGD is a marker of the fetal hormone milieu[45,46,52,53,84–87]; however, if AGD is also responsive to exposures at other sensitive postnatal time points as our results suggest, the interpretation of adult AGD as an indicator of early exposures may be more challenging. Additional longitudinal studies of human AGD that extend beyond early childhood and include reproductive transitions (e.g., puberty) are needed.

Unexpectedly, we observed that whereas prenatal $PM_{2.5}$ concentrations were associated with shorter AGD at birth in males, associations with AGD at 1 year of age were in the positive direction (and of similar magnitude with or without adjusting for AGD at birth). This apparent paradox suggests a possible prenatal programming effect where endocrine-disrupting exposures during critical early programming windows (e.g., MPW, mini-puberty) may have both androgenic and anti-androgenic effects. Theoretically, maintenance of homeostasis and compensation following endocrine disruption could lead to nonmonotonic effects on androgenic activity. For example, research in rodent models demonstrates that early androgen exposure during mini-puberty triggers negative feedback signals[88] and that pharmacologically elevated androgens during mini-puberty can disrupt subsequent androgen production during adolescence and adulthood.[89] It is also possible that postnatal exposures are a proxy for prenatal exposures; however, the low-to-moderate correlation between prenatal and postnatal estimated exposures, as well as the opposite directions of association in both individual time point and mutually temporally adjusted models, suggest other potential explanations. We cannot rule out the possibility that these unexpected findings are due to chance. More research is needed on the dynamics of this endocrine physiology, including consideration of dose, to understand the effects of air pollution as a disruptor of the hypothalamic–pituitary–gonadal axis.

We note several strengths of this work. First, AGD was measured in a highly standardized manner by trained examiners and with extensive quality controls in place.[67] By measuring infants shortly after birth (usually before leaving the birth hospital), we were able to minimize the potential effects of any postnatal exposures on our birth AGD measure. Second, we capitalized on advanced, national spatiotemporal models for air pollutants, and advanced beyond the conventional approach of studying exposures by trimester to focus on the most salient developmental periods for early reproductive development, the MPW and mini-puberty. Characterizing exposures across a longer period is also an advance over much work on endocrine-disrupting chemicals that relies upon single spot biospecimens collected opportunistically as a proxy for exposures over longer periods. Finally, because we followed participants through infancy and collected AGD data in males at 1 year of age, we were also able to look at the impact of exposures to air pollutants during postnatal mini-puberty on male reproductive development. Mini-puberty has received relatively little attention in environmental epidemiology thus far, but given our results and the known hormonal activation during this period, it may be critical for subsequent reproductive development.

We additionally note several limitations. Air pollution estimates were based on home addresses at the time of consent in early pregnancy, and it is likely that some families relocated prior to outcome assessment, which could have led to exposure measurement error. This would potentially lead to greater exposure misclassification at the later exposure time points (e.g., mini-puberty) and would likely bias our estimates toward the null. In addition, we did not capture addresses of other places where participants spend time, such as work, nor did we query the use of air filters and other air pollution mitigation efforts. We acknowledge, furthermore, that our sample was restricted to live births, which has the potential to induce bias.[90] Although we characterized levels of $PM_{2.5}$ exposure across gestation, we do not have data on the chemical composition of that PM; therefore, we cannot speak to the specific endocrine-disrupting properties of the individual components. Fine and ultrafine PM may act as a Trojan horse,

distributing numerous endocrine-disrupting chemicals to distal tissues through direct and indirect mechanisms.[91] In the future, understanding the chemical constituents of $PM_{2.5}$ (and how they vary temporally and geographically) will be critical to predicting endocrine-disrupting effects across difference critical windows of development. Similarly, we did not evaluate the role of other endocrine disruptors (e.g., phthalates, phenols, soy, alcohol) in these relationships, and future work may consider more complex, environmentally relevant mixtures. Relatedly, there is the possibility of residual confounding by factors including sociodemographic characteristics (beyond maternal education and race/ethnicity); however, this concern is somewhat tempered by a lack of prior research showing strong associations between sociodemographic factors and AGD measures. The critical windows of reproductive development in females are poorly understood at present moreover, and therefore we examined the same time points in both sexes. As additional work emerges to elucidate the timing of female prenatal reproductive development, we can revisit our data and update models as appropriate. Owing to funding limitations, only male infants participated in 1-year-of-age visits; thus, we were unable to study perinatal air pollutant exposures in relation to AGD in 1-y-old females. Finally, given our interests in multiple exposures during multiple sensitive windows of development in both sexes, we fit a large number of models and it is possible that some results were due to chance.

Our results indicate that in addition to the many cardiovascular and pulmonary sequelae of exposure to air pollutants, there may be impacts on the developing human reproductive system that may occur through disruption of typical hormone activity during gestation and infancy. Changes in infant AGD may be an early signal of altered reproductive development and, although epidemiological evidence linking developmental alterations in AGD to adult reproductive health outcomes is currently lacking, a growing body of cross-sectional research suggests that adult AGD may be a marker for a wider array of reproductive health concerns, including endometriosis[53–55] and polycystic ovary syndrome[52] in females, as well as reduced semen quality and fertility in males.[45–51] Moving forward, longitudinal studies are needed to examine the long-term sexually dimorphic reproductive sequelae of early life alterations in AGD related to air pollution or other endocrine-disrupting exposures.

Acknowledgments

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