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Prenatal Exposure to Multiple Air Pollutants, Mediating Molecular Mechanisms, and Shifts in Birthweight

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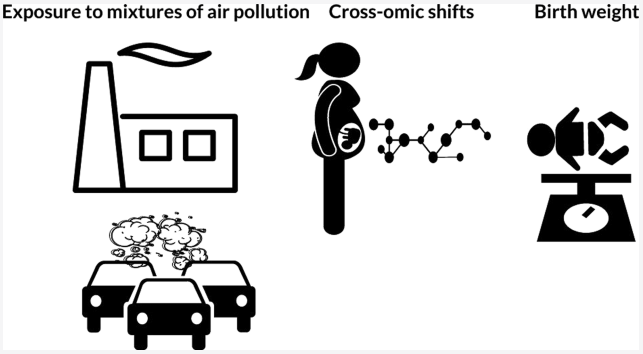
ABSTRACT: Mechanisms underlying adverse birth and later in life health effects from exposure to air pollution during the prenatal period have not been not fully elucidated, especially in the context of mixtures. We assessed the effects of prenatal exposure to mixtures of air pollutants of particulate matter (PM), $PM_{2.5}$, PM_{10}, nitrogen oxides, NO_2, NO_x, ultrafine particles (UFP), and oxidative potential (OP) of $PM_{2.5}$ on infant birthweight in four European birth cohorts and the mechanistic underpinnings through cross-omics of metabolites and inflammatory proteins. The association between mixtures of air pollutants and birthweight z-scores (standardized for gestational age) was assessed for three different mixture models, using Bayesian machine kernel regression (BKMR). We determined the direct effect for $PM_{2.5}$, PM_{10}, NO_2, and mediation by cross-omic signatures (identified using sparse partial least-squares regression) using causal mediation BKMR models. There was a negative association with birthweight z-scores and exposure to mixtures of air pollutants, where up to −0.21 or approximately a 96 g decrease in birthweight, comparing the 75th percentile to the median level of exposure to the air pollutant mixture could occur. Shifts in birthweight z-scores from prenatal exposure to $PM_{2.5}$, PM_{10}, and NO_2 were mediated by molecular mechanisms, represented by cross-omics scores. Interleukin-17 and epidermal growth factor were identified as important inflammatory responses underlyingair pollution-associated shifts in birthweight. Our results signify that by identifying mechanisms through which mixtures of air pollutants operate, the causality of air pollution-associated shifts in birthweight is better supported, substantiating the need for reducing exposure in vulnerable populations.



INTRODUCTION

Mechanisms underlying adverse birth and later in life health effects from exposure to air pollution during the prenatal period have not been fully elucidated, especially in the context of multiple air pollutants and mixtures. Anthropogenic sources of ambient air pollution are composed of complex mixtures, where the main pollutants found in urban areas are particulate matter (PM) with a diameter of 10 μm or less (PM_{10}) and a diameter of 2.5 μm or less ($PM_{2.5}$), ozone (O_3), sulfur dioxide (SO_2), nitrogen oxides (NO_x) composed of nitrogen dioxide (NO_2) and nitric oxide (NO), black carbon (BC), ultrafine particles with a diameter of 0.1 μm or less (UFP), and soot. Despite single and co-pollutant studies on a few of these air pollutants,[1] the cumulative and interactive effects of these mixtures on birth outcomes are not well understood. Identifying the effects of mixtures of air pollution is imperative, as rarely does exposure occur to only a single component and multiple components likely have interactive effects on health. Additionally, considering potential biological mechanisms underlying prenatal air pollution-associated health effects, we have yet to investigate mechanisms as mediators on causal paths leading to adverse birth outcomes. Taken together, there is a need for an exposome approach during this critical and sensitive developmental window. Such an approach accounts for the external exposome of multiple exposures that are assessed simultaneously and the internal exposome, which represents individual's responses to the environmental exposures.[2]

Numerous studies have found substantial impacts of air pollution on infant birthweight, such as low birthweight,

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including evidence from several meta-analyses.[3−7] Importantly, shifts in birthweight are associated with neonatal morbidity and mortality, but are also a main risk factor for developmental problems such as asthma and obesity.[8] Most studies of air pollution-associated shifts in birthweight focus on one or a few pollutants, with a limited number of studies investigating effects from exposure to mixtures of air pollutants,[9−14] and rarely are interactive and nonlinear effects accounted for. Additionally, many previous studies use traditional regression methods to obtain effect estimates, which has limitations due to correlations among different air pollutants, hindering the ability to assess their individual or combined health effects.[15] The application of new multipollutant statistical methods is underutilized.[16] Furthermore, previous research has mostly concentrated on commonly monitored air pollutants of carbon monoxide (CO), NO_2, $PM_{2.5}$, and $PM_{10,}$ where results have been inconsistent in terms of singling out a particular pollutant or combination of pollutants that are consistently inversely associated with birthweight. However, there is emerging evidence that UFP and factors such as oxidative potential (OP) of $PM_{2.5}$, a recent characterization of particulate matter that is likely to reflect toxicity, may have impacts on health. A recent study found that OP may potentially modify the impact of $PM_{2.5}$ on the risk of low birthweight and preterm birth.[17]

Extended, yet inconclusive, research has been conducted regarding the biological mechanisms through which air pollution could influence shifts in birthweight. Inflammation, oxidative stress, placental dysfunction, coagulation, endothelial function, and hemodynamic responses have been put forth as putative mechanisms.[18,19] Prenatal exposure to air pollution leads to potential chronic systematic inflammation; where, maternal inhalation of airborne particles and gaseous pollutants induce an inflammatory reaction in the lungs of the mother, releasing proinflammatory cytokines, and/or via maternal oxidative stress, and/or through ambient particulates that are transported through the placental barrier *in utero*, whereby all paths potentially lead to an inflammatory response of the fetus.[20−22] Likely, the inflammatory responses from prenatal exposure to air pollution are regulated by processes at multiple levels of biological organization, which may be assessed through various 'omics platforms. A few studies have investigated single 'omic responses from prenatal exposure to air pollution, of the epigenome[23−28] and transcriptome.[29−31] Yet, fewer have investigated the metabolome[32,33] and proteome, and no study has examined cross-omics (the relationship of more than one 'omic to one another) responses from prenatal exposure to mixtures of air pollution. This is crucial as single 'omic measures may not capture the full biological complexity, whereas interactive internal responses of cross-omics may be more informative of biological pathways involved in the regulation of cellular processes that underlie air pollution exposure.

In the present study, we take an exposome approach to determine the effects of prenatal exposure to multiple air pollutants of $PM_{2.5}$, PM_{10}, NO_2, NO_x, UFP, and OP on infant birthweight in four European birth cohorts, by incorporating multipollutants into models and by assessing simutaneously multiple molecular signatures. We have two aims in this work: the first is exploratory, to assess the external exposome, namely, to identify the effects (single, joint, and interactive) of mixtures of multiple air pollutants on birthweight, and the second is to determine the molecular internal responses from exposure to multiple air pollutants, represented by the interactive cross-talk of metabolomic signatures and targeted inflammatory proteins, to determine if they act as mediating mechanisms between prenatal exposure to multiple air pollutants and infant birthweight. These aims build off of previous work, where we identified epigenetic shifts from prenatal air pollution.[26] Exposure to atmospheric air pollution is highly prevalent, and yet a controllable risk factor for shifts in birthweight and other adverse birth outcomes. This work may aid in determining the underlying mechanisms of air pollution-driven birth outcomes, substantiating further evidence to mitigate exposures for prevention of air pollution-associated birth outcomes.

MATERIALS AND METHODS

Cohort Description. Participants in the study were from mother−child cohorts within the EXPOsOMICS consortium[34] comprising a subset of four population-based cohorts with measurements of the internal and external exposome from representative subjects from a Belgium cohort, ENVIRonmental influence ON AGEing (ENVIRONAGE);[35] a Spanish cohort, INfancia y Medio Ambiente (Environment and Childhood) (INMA);[36] a Greek cohort, Rhea;[37] and an Italian cohort, the Turin center of the Piccolipiù study.[38] Detailed information about the selection of these participants and methods has been previously described,[39] and is presented in the Supporting Information. Briefly, selection for the subset was conducted randomly from each cohort (N = 199 for ENVIRONAGE, N = 99 for Piccolipiù, N = 100 for INMA and N = 100 for Rhea) for those that had sufficient cord blood sample volume and quality, and available covariate data. Demographic and pregnancy information was harmonized across the cohorts and includes maternal and paternal education (trichotomized into primary school, secondary school, or university degree and higher), maternal age at delivery, maternal pre-pregnancy and paternal body mass index (BMI (kg/m^2)), exposure to tobacco smoke during the prenatal period (dichotomized as yes/no) measured for both active maternal smoking, and maternal exposure to passive smoke. Birth characteristics were derived from medical records and include gestational age in weeks measured by the last menstrual period or via ultrasound, sex of infant, and parity (trichotomized as primiparas for mothers who had not previously had a live birth, or multiparas if this was their second birth or multiparas of more than two for those who had experienced more than two births) and season of conception, which may represent factors such as temperature and humidity, based on an estimated date of conception (categorized into four seasons: January−March, April−June, July−September, October−December). Birthweight in grams was standardized using sex-specific gestational age z-scores using international growth standards.[40] The outcome of standardized birthweight by gestational age z-scores, versus assessing raw birthweight and adjusting for gestational age, was chosen for several reasons. The first was for the potential role of gestational age as a mediator, whereby adjusting could result in biased estimates.[41] Second, birthweight rises in a nonlinear pattern as gestational age increases; thus, adjustment may result in bias.[42] However, the use of z-scores accounts for differences across the gestational period in a precise manner, as it considers nonlinear growth, which may reduce both bias and potential residual confounding[42] and has previously been examined in prenatal studies for the effects of air pollution on birthweight.[43,44]

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External Exposome: Exposure Assessment of Air Pollutants. We assessed prenatal exposure to air pollution for seven different air pollutants including $PM_{2.5}$, PM_{10}, NO_2, NO_x, OP, a measure of the capacity of PM to deplete certain antioxidant molecules for ascorbic acid (OP_{AA}) and for glutathione (OP_{GSH}), and UFP as the annual average for the year before birth. Exposure assessment has been previously described for all EXPOsOMICS cohorts[39,45,46] and is further detailed in the Supporting Information.

To address mixtures of air pollutants, we first consider all pollutants that were assessed in each cohort (n = 498), a three-pollutant mixture of $PM_{2.5}$, PM_{10}, and NO_2. NO_x, OP_{AA}, OP_{GSH}, and UFP were not measured in all cohorts, where in ENVIRONAGE, NO_x was not measured and UFP readings were not reliable, and in Rhea, OP was not measured. Therefore, we assessed a five-pollutant mixture of $PM_{2.5}$, PM_{10}, NO_2, NO_x, and UFP in all cohorts except ENVIRONAGE (n = 298) and a five-pollutant mixture of $PM_{2.5}$, PM_{10}, NO_2, OP_{AA}, and OP_{GSH} in all cohorts except Rhea (n = 398). The average annual exposure levels were also categorized as above and below the EU Ambient Air Quality Directives (EU_{AQ}) and the WHO recommended guidelines (WHO_{AQ}) to determine the amount of women who were exposed to above or below the annual limit values. For $PM_{2.5}$, the limit value for EU_{AQ} is 25 $\mu g/m^3$, and for WHO_{AQ}, it is 10 $\mu g/m^3$; for PM_{10}, the EU_{AQ} is set to 40 and 20 $\mu g/m^3$ for WHO_{AQ}; and for NO_2, they are both set to not exceed 40 $\mu g/m^3$. There are no current limit values set for the other pollutants.

Internal Exposome: Multi-Omics Measurements. The internal exposome, of 'omics representing the metabolome and a set of targeted proteins representing a targeted inflammatory proteome, was measured using cord blood collected at birth that was stored at each cohort center at −80 °C. Full details of the omic's assessments are presented in the Supporting Information. Briefly, untargeted metabolomics analyses were carried out using high-resolution mass spectrometry coupled to ultraperformance liquid chromatography. Samples were analyzed in a randomized order as a single uninterrupted batch; after quality control, this resulted in 4712 identified features, with tentative annotations assigned by the Mummichog program to 1629 features.[47] Proteins were analyzed in stored cord blood, and those included in the present study were among those that were detectable and/or imputed using a targeted inflammatory protein panel, which included 16 proteins. These proteins were chosen across several different EXPOsOMICS cohorts for their association with air pollutants.[39] Proteins were analyzed using Luminex panels A and B and ELISA. We measured Tumor Necrosis Factor α (*TNF-α*), Interferon-γ−inducible protein 10 (*IP-10*), Interleukin 1 receptor Antagonist (*IL-1rA*), Granulocyte-colony stimulating factor (*GCSF*), Vascular Endothelial Growth Factor (*VEGF*), Human Macrophage-derived Chemokine (*MDC*), Periostin, Interleukin 8 (*IL-8*), Interleukin 17 (*IL-17*), Epidermal Growth Factor (*EGF*), Macrophage Inflammatory protein 1 (*MIP1*), Growth-regulated protein (*GRO*), Interleukin 6 (*IL-6*), Myeloperoxidase (*MPO*), C-reactive protein (*CRP*), and Monocyte Chemoattractant protein (*MCP-1*).

Statistical Analyses. All analyses were performed with the R statistical software (version 3.4; R Development Core Team). The correlation between air pollutants was assessed using Pearson's correlation coefficient (r^2). Summary statistics were calculated for the exposures, covariates, and outcome. Because $PM_{2.5}$, PM_{10}, and NO_2 have recommended limit values set by the EU and WHO, data are graphically presented as above and below these thresholds.

To assess the effects of prenatal exposure to mixtures of air pollutants on birthweight, we used Bayesian kernel machine regression (BKMR).[48] This approach allowed for flexible modeling for potential joint effects of exposures, linear/nonlinear relationships between the exposures and outcome, and has recently been extended into mediation models, facilitating our incorporation of 'omics as a mediator. Another advantage of the BKMR method, as previously outlined, is that it was developed to study environmental mixtures, and in its application, we can determine: (1) if exposure to the mixture is associated with the outcome of interest (the direct effect of the mixture); (2) what the exposure−response relationships are between individual chemical exposures and outcome (the direct effects of individual pollutants); and (3) whether the components of the mixture interacts (the direct effect of interactions on an outcome).[49] Because birthweight may depend on a subset of the mixture components, we conduct a component-wise variable selection, allowing each of the individual air pollutants to enter the model. Fitting the models with variable selection allowed us to estimate the posterior inclusion probability (PIP) for each of the exposures, where we set the threshold of 0.5 for the PIP variable selection. Specifically, we model birthweight z-scores as a smooth function h, represented using a kernel function, on the logged exposure variables, adjusting for potential confounders that were scaled. We apply BKMR to the three different mixture models of air pollutants described above: three-pollutant model of $PM_{2.5}$, PM_{10}, and NO_2; five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2, NO_x, and UFP; and five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2, OP_{AA}, and OP_{GSH}. The combinations of air pollutants in each model were chosen based on available data for each cohort. Cohorts were pooled and models were adjusted for a set of potential confounders of season of conception (categorical), sex (categorical), parity (categorical), maternal age (continuous), education of the mother and father (categorical), active and passive smoking during pregnancy (categorical), and maternal and paternal BMI (continuous), and a fixed effect for cohort, selected based on a directed acyclic graph for their association with the exposures, outcome, and not on the causal path (Figure S1). For each model, linearity/nonlinearity was assessed by estimating the univariate exposure−response functions and the overall effect of the mixture (estimates and 95% confidence intervals). We also estimated the change in birthweight z-scores comparing exposures at a particular percentile to exposures at their median value. The median value, within our data, was selected to facilitate comparability to other air pollution studies and because there are not yet environmental standards/cutoff values developed for air pollution mixtures (limiting our assessments based on regulatory levels).

Internal Exposome: Cross-Omics Assessment. To assess the internal exposome, we determined relationships between cord blood metabolomic features and targeted inflammatory proteins. Cross-omics variable selection was conducted between cord blood inflammatory proteins (n = 16) and the cord blood metabolome (n = 4712). Variable selection and the correlation structure of each 'omic to one another, was determined by identifying which components were linear combinations of predictors and responses by maximizing their variance-covariance, using sparse partial least-squares regres-

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Table 1. Demographic Characteristics of the Cohort[a]

	Pooled cohort (n = 498)	ENVIRONAGE (n = 199)	INMA (n = 100)	Rhea (n = 100)	Piccolipiù (n = 99)
Maternal Age (years)	31 (18−43)	30 (18−43)	31 (24−41)	30 (20−42)	33 (20−43)
		Maternal Education			
primary school	63 (12)	29 (15)	18 (18)	8 (8)	8 (8)
secondary school	213 (43)	68 (34)	47 (47)	57 (57)	41 (41)
≥university degree	222 (45)	102 (51)	35 (35)	35 (35)	50 (51)
		Paternal Education			
primary school	105 (21)	39 (20)	28 (28)	22 (22)	16 (16)
secondary school	238 (48)	87 (43)	48 (48)	57 (57)	46 (47)
≥university degree	155 (31)	73 (37)	24 (24)	21 (21)	37 (37)
		Maternal Active Smoking			
nonsmoker	408 (82)	173 (87)	77 (77)	80 (80)	78 (79)
smoker	90 (18)	26 (13)	23 (23)	20 (20)	21 (21)
		Maternal Passive Smoking Exposure			
nonexposed	321 (64)	178 (90)	51 (51)	16 (16)	76 (77)
exposed	177 (36)	21 (10)	49 (49)	84 (84)	23 (23)
Maternal BMI	24 (16−47)	24 (16−42)	24 (17−36)	25 (18−47)	23 (17−34)
Paternal BMI	26 (18−60)	26 (18−60)	26 (18−35)	27 (19−40)	25 (19−38)
		Season of Conception			
January−March	122 (25)	45 (23)	17 (17)	28 (28)	32 (32)
April−June	129 (26)	44 (22)	27 (27)	24 (24)	34 (35)
July−September	161 (32)	79 (40)	31 (31)	29 (29)	22 (22)
October−December	86 (17)	31 (15)	25 (25)	19 (19)	11 (11)
Gestational Age (weeks)	39 (37−45)	39 (37−41)	40 (37−45)	38 (37−41)	40 (37−45)
		Sex of Newborn			
female	240 (48)	97 (49)	51 (51)	47 (47)	45 (46)
male	258 (52)	102 (51)	49 (49)	53 (53)	54 (54)
		Parity			
primiparas	130 (26)	0	55 (55)	29 (29)	46 (47)
multiparas: 2 births	239 (48)	109 (55)	37 (37)	48 (48)	45 (45)
multiparas: >2 births	129 (26)	90 (45)	8 (8)	23 (23)	8 (8)
Newborn Birthweight (g)	3309 (1920−4910)	3385 (1920−4530)	3298 (2010 −4400)	3259 (2370−4910)	3221 (2110−4440)
Newborn Birthweight (z-scores)	0.26 (−2.2−3.5)	0.47 (−2.0−2.7)	0.04 (−2.2−2.4)	0.39 (−2.1−3.5)	−0.08 (−2.1−2.9)

[a]Data are presented as mean and range for continuous variables and number (percentage) for categorical variables.

sion (sPLS).[50] Variable selection was achieved by LASSO, and dimension reduction was achieved by imposing sparsity in the loading coefficients. Each of the latent components is a linear combination of the original variables. We ran sPLS for variable selection between two 'omics at a time. We ran sPLS on X ($sPLS_X$), sPLS on Y ($sPLS_Y$), and sPLS on X and Y ($sPLS_{XY}$). For example to compute, $sPLS_X$, the proteins (X) loading coefficients are decreased to 0 for those proteins that are least informative to the metabolomic features, whereas the Y loading coefficients for $sPLS_Y$ correspond to shrinking the metabolomic features that are least informative for the proteins. Together, these models aid in identifying the most relevant 'omic respective to one another. Calibration of the sPLS models was done using fivefold cross-validation, which was independently repeated 1000 times. More details are provided in the Supporting Information. Scores from the $sPLS_{XY}$ model were derived from the variates of X (proteins that best predicted metabolomic features) and the variates of Y (metabolomic features that best predicted proteins) to use in mediation analysis (described below). Pathway analysis of the metabolomic features identified through cross-omics was carried out using the Mummichog program.[51]

Mechanistic Assessment: Cross-Omics as a Mediator of Multi-Air Pollutants and Infant Birthweight. To identify potential molecular mechanisms, representing cross-omics relationships, that may mediate the relationship between exposure to multiple air pollutants and birthweight, we ran causal mediation analysis models. We estimated the natural direct effect (NDE), natural indirect effect (NIE), and controlled direct effects (CDEs), using BKMR-causal mediation analysis (BKMR-CMA) methods developed recently.[52] The BKMR-CMA method allows for all possible interactions and nonlinear effects of the co-exposures on the mediator and the co-exposures and mediator on the outcome. Multipollutants of the three air pollutant mixtures of $PM_{2.5}$, PM_{10}, and NO_2 were assessed as the exposures (A) for mediation analyses, as these were measured for all participants, whereas other mixture models were not explored due to small sample sizes. The mediator considered was a cross-omics score (M) derived from $sPLS_{XY}$, of the metabolomic features that best predicted proteins and vice versa. The outcome was birthweight z-scores (Y). All models were adjusted for the same set of potential confounders listed above, considering that these may also be potential confounders of the exposure−mediator relationship and mediator−outcome relationship. Our mediation notation is the same as developed and described in ref 52. Briefly, Y_{am} denotes the counterfactual outcome Y if the exposure level A was set to a and mediator level M was set to m. Let Ma be the counterfactual mediator level M that would have been observed if the exposure A was set to a. Accordingly,

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Y_{aMa*} represents the counterfactual outcome Y if the exposure level A was set to a and the mediator M was set to the level it would have taken if the exposure level A was a^*. We estimated the NDE, NIE, and total effect (TE) for a change of the three-pollutant mixture from a^*, where the exposures set equal to their 25th percentile, to a, where the exposures set is equal to their 50th percentile. We also estimated the controlled direct effect (CDE), where the mediator was held at the 25th, 50th, and 75th percentiles. The CDE represents potential to alter the mediator.

Sensitivity Analyses. Several sensitivity analyses were run to assess the robustness of our findings. We consider that multiple air pollutants measured in the present study represent overlapping exposure assessments, and thus it may be important to account for overlapping values of pollutants in the mixture models. To address this for the pollutants of PM_{10} and $PM_{2.5}$, where PM_{10} contains $PM_{2.5}$, we estimate the aerodynamic diameter between 2.5 and 10 μm (PM_{coarse}) calculated as $PM_{10}-PM_{2.5}$. Additionally, NO_x contains NO_2, and therefore in the only model containing NO_x, we exclude NO_2. Thus, the sensitivity models for overlapping exposure assessments are (1) a two-pollutant model of PM_{coarse} and NO_2; (2) a three-pollutant model of PM_{coarse}, NO_x, and UFP; and (3) a four-pollutant model of PM_{coarse}, NO_2, OP_{AA}, and OP_{GSH} for the direct effects of mixtures on birthweight.

RESULTS

Population Characteristics and Exposure to Multiple Air Pollutants. Participants of the study were largely similar among the cohorts with respect to demographic characteristics (Table 1). The average maternal age was 31 (range: 18−43). Several (45%) mothers had a university degree or higher, while many (48%) fathers completed only secondary school. Majority of women did not report smoking or exposure to passive smoking during pregnancy, though this did differ by cohort. The average birthweight was 3309 grams (g) (range: 1920−4910 g). Exposure to multiple air pollutants varied slightly by geographic area of the cohort (Figure S2). All pollutants were correlated, where most pairwise correlations had $r^2 \geq 0.5$ (Figure S3). The average levels of exposure, representing the year before birth, was 19 $\mu g/m^3$ (range: 9−45 $\mu g/m^3$) for $PM_{2.5}$, 35 $\mu g/m^3$ (range: 10−45 $\mu g/m^3$) for PM_{10}, 30 $\mu g/m^3$ (range: 8−98 $\mu g/m^3$) for NO_2, 73 $\mu g/m^3$ (range: 12−236 $\mu g/m^3$) for NO_x, 62 OP/m^3 (range: −18−131 OP/m^3) for OP_{AA}, 5 OP/m^3 (range: 0.6−14 OP/m^3) for OP_{GSH}, 13393 n/cm^3 (range: 1465−30 644 n/cm^3) for UFP, and 15 $\mu g/m^3$ (range: 1−35 $\mu g/m^3$) for PM_{coarse} (Table S1). Ninety-seven percent of pregnant women were exposed to levels of $PM_{2.5}$ above the WHO guideline, 63% of pregnant women were exposed to levels of PM_{10} above the WHO guideline, and 26% of pregnant women were exposed to levels of NO_2 above the WHO guideline (Figure 1).

Effects of Prenatal Exposure to Different Mixtures of Air Pollutants on Birthweight. To identify the effects of mixtures of air pollutants on infant birthweight, we assessed three different mixtures models. For our main models, all air pollutants had a posterior inclusion probability equal to or greater than 0.5 (Table S1). NO_2 had a particularly high probability of inclusion compared to the other components of the mixtures, with an estimated posterior inclusion probability of 0.78, 0.68, and 0.76 for the three-pollutant model of $PM_{2.5}$, PM_{10}, and NO_2; five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2 NO_x, and UFP; and five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2, OP_{AA}, and OP_{GSH}, respectively (Table S1). Based on the cross-sectional univariate assessments between each air pollutant and birthweight z-scores, there are potential nonlinear effects of the air pollutants on birthweight (Figures S4 and S7). Additionally, based on a cross-sectional univariate relationship between each air pollutant and birthweight, where the other exposures are fixed to their median value, there was a negative association with birthweight z-scores with increasing levels of NO_2 and PM_{10} in the three-pollutant model (Figure S4A), with increasing levels of NO_2 and NO_x for the five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2, NO_x, and UFP (Figure S4B), and with increasing levels NO_2 and OP_{AA} for the five-pollutant model of $PM_{2.5}$, PM_{10}, NO_2, OP_{AA}, and OP_{GSH} (Figure S4C). The overall effects of mixtures of air pollutants on infant birthweight z-scores are presented in Figure 2. Estimates and

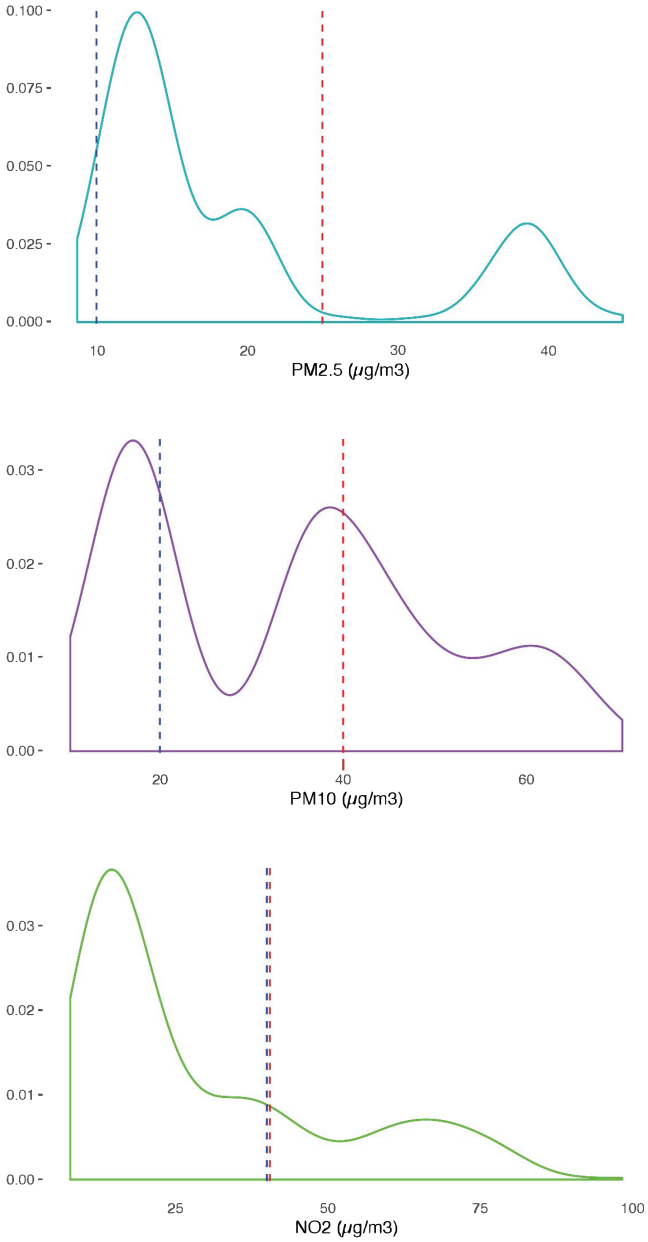

Figure 1. Pregnant women's average annual exposure levels to PM2.5, PM10, and NO_2 presented as above and below the European Union Ambient Air Quality Standards (red lines) and the World Health Organization recommended guidelines (blue lines) for annual limits.

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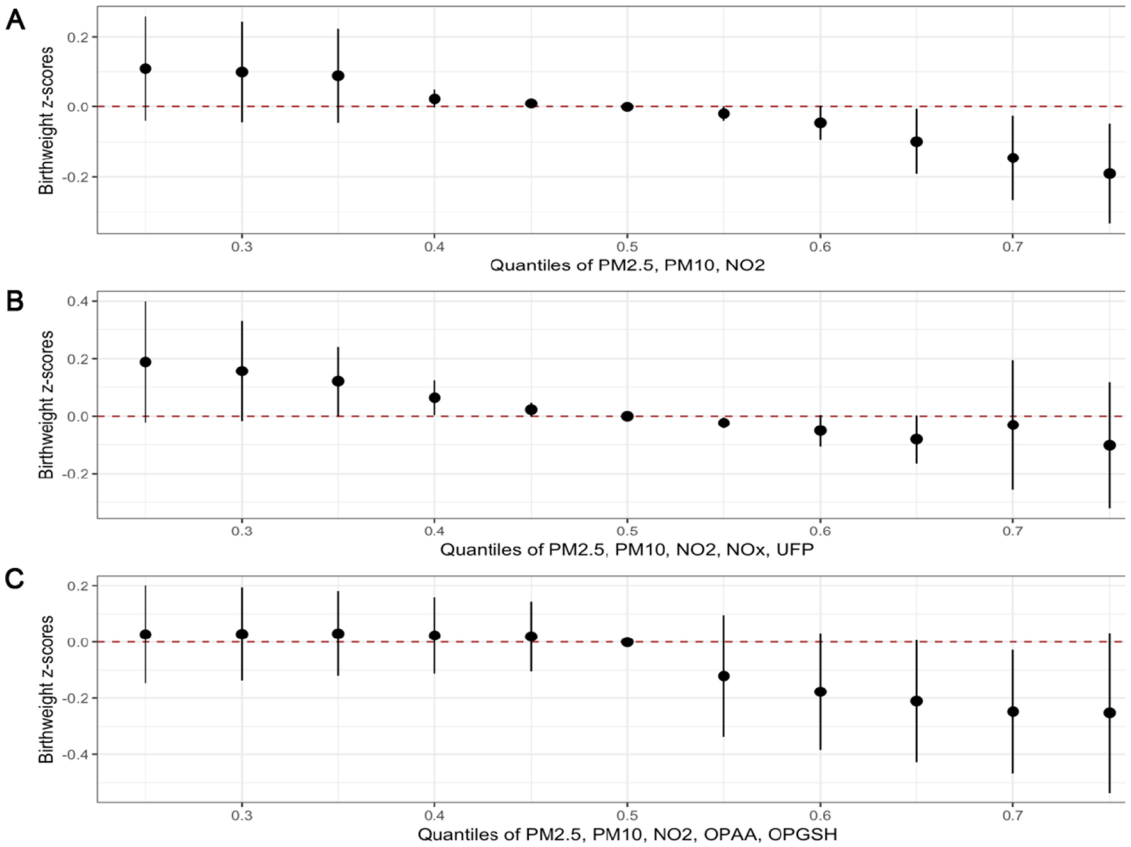

Figure 2. Overall effects of mixtures of air pollutants on infant birthweight z-scores. Estimates represent the predicted birthweight z-scores based on the overall effect of the mixture (estimates and 95% confidence intervals), comparing birthweight z-scores when all exposures are at a particular quantile compared to the median value. Models adjusted for season of conception, sex, parity, maternal age, education of the mother and father, active and passive smoking during pregnancy, and maternal and paternal BMI and cohort.

Table 2. Number of Components (N) and Explained Variance of X (Proteins) and Y (Metabolomic Features) from the Sparse Partial Least-Squares Regression Models for Proteins and Metabolomic Features

	$sPLS_X$ Proteins			$sPLS_Y$ Metabolomic Features			$sPLS_{XY}$ Proteins and Metabolomic Features			
	Number of Components and Explained Variance									
	N	X	Y	N	X	Y	NX	$N\,Y$	X	Y
Component 1	4	0.13	0.092	353	0.18	0.078	2	665	0.13	0.08
Component 2	3	0.083	0.068	23	0.16	0.051				
Component 3	6	0.065	0.12	279	0.076	0.020				
Total	13	0.28	0.19	655	42	0.15				

95% CI are presented and represent the comparison of air-pollutant-associated birthweight z-scores when all exposures are at a particular quantile compared to the median value. For the three-pollutant model, estimates where the CI did not cross the null signify that birthweight z-scores are −0.020 when air pollutants are set at the 0.55 quantile to −0.19 at the 0.75 quantile of exposure compared to their median value (Figure 2A). For the five-pollutant model, including NO_x and UFP, estimates where the CI did not cross the null signify that birthweight z-scores are −0.024 when air pollutants are set at the 0.55 quantile to −0.081 at the 0.65 quantile compared to their median value (Figure 2B). For the five-pollutant model, that includes OP, estimates where the CI did not cross the null signify that birthweight z-scores are −0.21 when air pollutants are set at the 0.70 quantile compared to their median value (Figure 2C).

Cross-Omics Relationships. To assess the relationship of targeted inflammatory proteins to metabolomic features, cross-omics assessments were carried out. We aimed to select the most influential proteins and the most affected metabolomic features and vice versa using sPLS models with penalization of the loading coefficients applied to proteins and metabolomic features, respectively. When performing variable selection on proteins, the calibrated $sPLS_X$ model included three components, in which *EGF*, *VEGF*, *IL-17*, *MPO*, *IP10*, and *Perios* were selected (Table 2). This model explained 28% of the variance in proteins and 19% of the variance in metabolomic features. The loading coefficients are presented in Table S2. The calibrated $sPLS_Y$ models for metabolomic features included three components, and in those components, 4336 metabolomic features were excluded and 376 were selected (Table 2). These three components of Y explained 15% of the variance in the metabolomic features and 42% of

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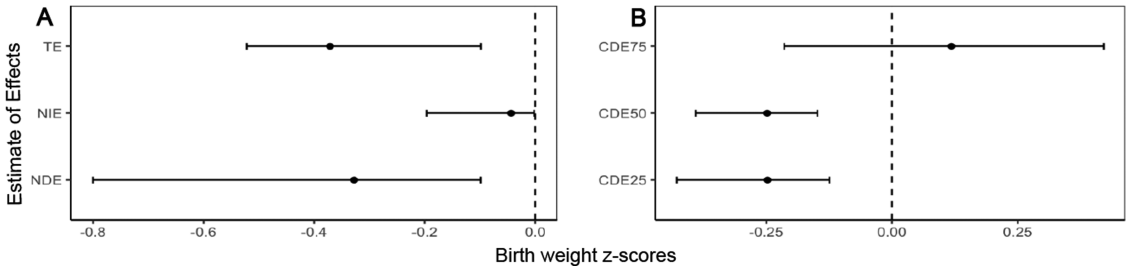

Figure 3. Mediated effects of the three-pollutant mixture of $PM_{2.5}$ and PM_{10} and NO_2 on birthweight z-scores for cross-omics scores for metabolomic features that best predicted proteins. Estimates (Figure 3A) represent the total effect (TE), the natural indirect effect (NIE). Estimates (Figure 3B) represent the controlled direct effects of the mediator set to their 75th percentile (CDE75), 50th percentile (CDE50), and 25th percentile (CDE25).

the variance of the proteins. Variable selection was also performed on both *X* and *Y*. In the calibrated $sPLS_{XY}$ (Figure S5), there was a single component included, where 14 proteins were not selected, and *IL-17* and *MPO-5* had positive loading coefficients of 0.49 and 0.87, respectively (Figure S6). Of the 4712 metabolomic features, 4047 were not selected, with the remaining 665 metabolomic features having both positive and negative loading coefficients (Figure S6). Through pathway analysis of those 665 metabolomic features identified through cross-omics ($sPLS_{XY}$), we found enrichment for several pathways. The top 10 were aspartate and asparagine metabolism, arginine and proline metabolism, tyrosine metabolism, urea cycle/amino group metabolism, glycerophospholipid metabolism, fatty acid activation, tryptophan metabolism, arachidonic acid metabolism, vitamin A (retinol) metabolism, and prostaglandin formation from arachidonate (Table S3).

Mediation of Cross-Omics for Prenatal Exposure to Mixtures of Air Pollutants and Birthweight. To determine if the cross-omics score, representing the relationship between metabolites and proteins, is a mediator on the causal path of air pollution driven shifts in infant birthweight, we ran mediation models for the three-pollutant mixture of $PM_{2.5}$, PM_{10}, and NO_2. Mediated effects for cross-omics scores were assessed using the score from the sPL_{XY} model (Figure 3). The total effect (TE) of the three-pollutant model of $PM_{2.5}$, PM_{10}, and NO_2 was −0.37 (95% CI of −0.52, −0.098), corresponding to the natural direct effect (NDE), the effect of the mixtures not through 'omics, of −0.33 (95% CI of −0.80, −0.099) and a natural indirect effect (NIE), representing the effect that goes through the path of the 'omics, of −0.044 (95% CI of −0.20, −0.0020) (Figure 3A). The controlled direct effect (CDE), of setting the 'omics to different levels, of the three-pollutant model of $PM_{2.5}$, PM_{10}, and NO_2 was estimated at three different quantiles, the CDE at the 25th was −0.25 (95% CI of −0.43, −0.12), at the 50th was −0.25 (95% CI of −0.39, −0.15), and at the 75th was 0.12 (95% CI of −0.21, 0.42) (Figure 3B).

Sensitivity Analysis. There was a negative association with increasing levels of mixtures of air pollutants and birthweight z-scores in all sensitivity analyses (Figures S7 and S8), and the magnitude of effects was similar to our main analyses.

DISCUSSION

Most of the pregnant women in the present study, representing four European countries, were exposed to levels of $PM_{2.5}$ and PM_{10} above the WHO guidelines, 97% and 63%, respectively. While less (26%) were exposed to levels of NO_2 above the WHO guideline, exposure to NO_2 was negatively associated with birthweight. Additionally, exposure to mixtures of air pollutants, from three different mixture pollutant models, was negatively associated with infant birthweight. Our results suggest that cross-omics scores, representing inflammatory proteins and metabolomic features that are related to each other, mediate the relationship between prenatal exposure to air pollution and birthweight. These findings imply that molecular shifts from prenatal exposure to mixtures of air pollution may lie on the causal path to infant birthweight.

The effects of prenatal exposure to air pollution on birthweight have been previously established, with varying results. Most studies have examined a single pollutant at a time, finding a negatvie association with birthweight with increasing levels of exposure. For example, in a meta-analysis, the pooled effect per 10 $\mu g/m^3$ of prenatal expoure to ambient $PM_{2.5}$ was a reduction in birthweight by 23.4 g (95% CI: −45.5, −1.4).[6] For comparison, in the present study, in the three-pollutant mixture model of $PM_{2.5}$, PM_{10}, and NO_2, we estimated an association of −0.21 z-score, which could lead to an approximate 96 g decrease in birthweight, comparing the 75 percentile to the median level of exposure to the air pollutant mixture. This signifies a substantial decrease in birthweight, which could lead to early life and later in life health effects. Only a few studies have investigated the effects of mixtures on birthweight,[10−14] and fewer have investigated the cumulative/interactive effects of the different components of air pollution. A previous study using Bayesian mixture models found that mixtures of NO_2, NO, and $PM_{2.5}$ concentrations (averaged over census block groups) was associated with an increase in log odds of low birthweight.[53] Importantly, these air-pollution-related shifts in birthweight could lead to increases in neonatal morbidity and mortality, but are also a main risk factor for developmental problems such as asthma and obesity.[8] These results signify the need to assess multipollutant exposures, as exposure to combinations of air pollutants may lead to greater shifts in infant birthweight that may be underrepresented by single-pollutant assessments.

We identified cross-omics relationships between inflammatory proteins and the blood metabolome, indicating an association between two different 'omics measurements. Our study is the first to assess cross-omics of the metabolome and targeted inflammatory proteins representing a targeted proteome *in utero*, and to perform mediation analyses, representing a potential causal link for molecular mechanisms underlying air-pollution-associated shifts in birthweight. Results from the sparse partial least-squares regression model selecting both proteins and metabolomic features suggest an

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immune response of decreased levels of *IL-17* and *MPO*. Interestingly, *IL-17* was selected as an important protein in two different assessments. This protein was identified in cross-omics scores of proteins to metabolomic features and was represented in the mediation models, indicating its role in air-pollution-associated shifts in birthweight. *EGF* was selected in the $sPLS_X$ model. These findings suggest *IL-17* and *EGF* as important inflammatory responses in association with prenatal air pollution. We have previously identified *IL-17* in association with air pollution and cardiovascular disease in adults.[54] However, previous studies have identified other inflammatory responses from air pollution that were not identified through our cross-omics assessments. For example, $PM_{2.5}$ exposure has been associated with increased C-reactive protein concentrations in early pregnancy, which is indicatative that inflammation that could impact gestation.[55] The targeted inflammatory protein responses identified in the present study play an important role in air-pollution-associated shifts in birthweight and will need to be considered in future studies.

We identified 665 metabolomic features that mediate air-pollution-associated shifts in birthweight. Metabolomic signatures have been associated with air pollution in a targeted assessment of oxylipins from umbilical cord blood plasma[33] and lung lavage fluid.[32] The metabolome associated with birthweight has been described in a previous study from EXPOSOMICS.[47] Of the 665 metabolomic features selected from the cross-omics assessment in the present study, seven were associated with birthweight in a previous study from EXPOSOMICS, and included the following lipids: diacylglycerol (C34:2), diacylglycerol (C36:3), lysoPC (C20:2), lysoPC (C22:5), and retinol, and two unknown metabolomic features.[47] Pathway analysis of the 665 metabolomic features identified through cross-omics indicates enrichment for several metabolomic features that are involved in fetal development and growth. In particular, arginine metabolism plays an important role in reproduction, fetal and postnatal development, wound healing, immune function, and tissue integrity, as well as prevention and treatment of endothelial dysfunction.[56] Additionally, concentrations of arginine in plasma are reduced in response to infection or inflammation.[57] Tyrosine metabolism and other amino acid metabolism have been previously associated with fetal growth.[58] Interestingly, tyrosine metabolism has previously been associated with exposure to air pollution in adults in two separate studies,[59,60] indicating a potential important pathway targeted by air pollutants.

This study is not without limitations. We measured exposure to air pollution only at the participants' residence, and at one time during pregnancy, which may underestimate their exposure and miss critical windows of gestation. Given that there are many inconsistencies in findings from studies assessing critical and/or sensitive exposure windows (e.g., trimester-specific exposures),[7] the determination of the most susceptible exposure periods, in particular for mixtures, needs further investigation. Additionally, our mixtures assessments were based on the available combinations of air pollutants previously measured in each cohort, limiting the sample sizes for estimating the direct effects of all mixtures on birthweight, and only facilitating the application of a mediation model for the mixture of PM_{10}, $PM_{2.5}$, and NO_2. This also limited our ability to assess potential effect modifiers. The OP_{GSH} estimates were from weak performing models and may not be as reliable as the other estimates, and therefore results of models including this pollutant should be interpreted with caution.[54] We were limited in our assessment of potential effect modifiers and/or confounders, including known factors such as nutrition,[19] joint effects of air pollutants and social determinants, or preexisting chronic health conditions, such as maternal stress.[61] We did not have information on pregnancy-related complications such as gestational diabetes or hypertension that could be potential confounders or that are more likely to be on the causal path, though the prevalence of these complications is likely low. If such complications are on the causal path, they would represent other mediating mechanisms not accounted for in our assessments and should be considered for future studies. Furthermore, we cannot completely rule out reverse causality of the outcome with the 'omics signatures as the 'omics were collected at birth. Unfortunately, this is a prominent issue that continues to affect perinatal molecular epidemiological studies, and future studies will need to consider alternative tissues that can capture molecular profiles across gestation, such as amniotic fluid (though this can be considered an invasive process). While we had a comprehensive 'omics assessment, representing more than one 'omic, other regulators of transcription factors, such as epigenetics, will need to be considered in future studies. Additionally, we measured a limited number of targeted inflammatory proteins, and other studies have identified other inflammatory proteins, not measured in the present study, associated with prenatal exposure to air pollution.[62] Future studies should consider a larger proteomic profile of inflammatory and noninflammatory proteins, even though inflammation has been highlighted as a key pathway for air-pollution-associated shifts in birthweight.[21,22] Finally, we only consider cross-omics scores in our mediation model, whereas other 'omics not selected from our sPLS models may mediate the relationship between air pollutants and birthweight. However, our aim was to target cross-omics to better represent correlation (potentially at the biological level) of one 'omic to another; therefore, the cross-omics scores were our primary focus in the present study.

The strengths of this study include the representation of multiple birth cohorts across Europe, increasing the generalizability of our findings to other European and similar populations, unique exposure assessments for multiple air pollutants, and the combination of multi-omic methodologies along with causal mediation assessments. Our study found shifts in birthweight with mixtures, not commonly assessed, such as oxidative potential and UFP. According to a recent review,[1] the most commonly studied ambient air pollutants in association with birth outcomes are PM, NO_2, ozone, and carbon monoxide. A strength of our exposure assessment approach is in the application of land use regression models, representing personal exposure. We applied several advanced techniques for our statistical approach that address previous limitations, including the assessment of mixtures allowing for their interactions and nonlinear relationships, the functional assessment of cross-omics in terms of two 'omics to one another, and their causal mediating role in air-pollution-associated birthweight. Multipollutant modeling may also reduce co-exposure confounding.[63] Cross-omics may better characterize genes in the context of the molecular pathophysiology of the disease and its interacting genes and pathways.

In conclusion, we find that shifts in birthweight are associated with prenatal exposure to mixtures of air pollution that are mediated by cross-omics signatures of targeted inflammatory proteins and metabolomic features, highlighting

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the role of potential biological pathways and supporting the causal role of air pollution's effect on birthweight. Additionally, our study demonstrates the complexities in understanding the biological intricacies of environmental exposures and associated health effects, and why it is crucial that we begin to take an exposome approach in the field of environmental epidemiology to fully capture multiple exposures and mediating molecular mechanisms. By investigating molecular mechanisms, we can further support associations between exposure to air pollutants and adverse health. Our findings support that measures should be taken to reduce prenatal exposure to air pollutants.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.0c02657.

Further detailed descriptions of methods including, detailed cohort description, exposure assessment, 'omics assessments, cross-omics analysis; average level of exposure of each pollutant the year before birth, and posterior inclusion probability from the Bayesian machine kernel regression models (Table S1); loadings coefficients for proteins identified from the $SPLS_X$ models (Table S2); pathways and number of metabolites represented in each pathway identified from the 665 metabolites selected from the cross-omics assessments (Table S3); directed acyclic graph to determine the set of potential confounders (Figure S1); levels of air pollutant by cohort (Figure S2); correlation of air pollutants to one another, assessed using Pearson's correlation coefficient (Figure S3); cross-sectional univariate relationships between each air pollutant and birthweight z-scores, where other exposures are fixed to their median value (Figure S4); calibration plot for the $SPLS_{XY}$ model for metabolomic features and proteins (Figure S5); loading coefficients for the $SPLS_{XY}$ model (Figure S6); sensitivity results of cross-sectional univariate relationships between each air pollutant and birthweight z-scores, where other exposures are fixed to their median value (Figure S7); and results from the sensitivity analyses of the overall effects of mixtures of air pollutants on infant birthweight z-score (Figure S8) (PDF)

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Author Contributions

All authors provided comments and feedback during the project proposal and writing of the manuscript. J.E.L.

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conducted the statistical analyses, as well as wrote and edited the manuscript. B.B. assisted with the statistical analyses. O.R. was responsible for data cleaning and harmonization of cohort data and metabolomics support. M.P. was responsible for data cleaning and harmonization for certain 'omics and is a representative of the ENVIRONAGE cohort. A.S. carried out metabolomics assessments. P.K.R. carried out metabolomics assessments. N.R. carried out metabolomics assessments. R.V. contributed to air pollution modeling, particularly for UFP. C.P. and F.A. represent the Piccolipiù cohort, contributing to data collection and processing. T.N. is PI of ENVIRONAGE and was responsible for data collection. J.G. carried out air pollution assessments. L.C. worked on multiple aspects of the birth cohorts, including data cleaning and harmonization. M.K. represents the Rhea cohort, responsible for data collection. M.N. contributed to air pollution modeling. J.S. represents the Rhea cohort. M.V. is the principal investigator of INMA cohort and is responsible for data collection. M.C.-H. provided statistical support for cross-omics assessments. P.V. is the principal investigator of the EXPOsOMICS Consortium and also contributed to writing the manuscript.

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Notes

Where authors are identified as personnel of the International Agency for Research on Cancer/World Health Organization, the authors alone are responsible for the views expressed in this article, and they do not necessarily represent the decisions, policy, or views of the International Agency for Research on Cancer/World Health Organization.

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