Maternal fine particulate matter exposure, polymorphism in xenobiotic-metabolizing genes and offspring birth weight.

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Elsevier Editorial System(tm) for Reproductive Toxicology
Manuscript Draft

Manuscript Number: 2719R1

Title: Maternal Fine Particulate Matter Exposure, Polymorphism in Xenobiotic-Metabolizing Genes and Offspring Birthweight

Article Type: Full Length Article

Keywords: Air pollution; Birth Weight; Epidemiology; Particulate Matter; Passive Smoking; Polymorphism, Genetic; Smoking

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Manuscript Region of Origin: GERMANY

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ABSTRACT

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*Cover Letter

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Grenoble, 25 February 2010

Thomas B. Knudsen
Editor-in-Chief
Reproductive Toxicology

Dear Editor,

We are pleased to submit to Reproductive Toxicology a revised version of our manuscript on gene-environment interactions in the context of the impact of atmospheric pollutants on birth weight.

This revised version takes into account the comments from both reviewers. In particular, the introduction was extented in order to present the motivations of our study and the related literature with more details.

Your journal has published the only study that addressed the question of gene-environment interactions for effects of Particulate Matter on birth weight (Suh et al., *Reprod Toxicol*, 2007). For this reason, we believe that our manuscript is particularly suited to Reproductive Toxicology.

Sincerely,

Rémy Slama, PhD
Corresponding author

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Maternal Fine Particulate Matter Exposure, Polymorphism in Xenobiotic-Metabolizing Genes and Offspring Birthweight.

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Abbreviations:

CI: Confidence interval

CYP: cytochrome P 450

ETS: Environmental Tobacco Smoke

GST: glutathione S-transferase

$PM_{2.5}$: Fine Particulate Matter with an aerodynamical diameter below 2.5 μ

Running head:

Air pollution, genes and birthweight

ABSTRACT

We aimed to describe if polymorphisms in xenobiotics-metabolizing genes modify the effect of maternal exposure to fine particulate matter ($PM_{2.5}$) on offspring birthweight.

Among newborns from LISA cohort, we tested if polymorphisms of *GSTT1*, *GSTP1*, *GSTM1*, and *CYP2D6* genes modified the effect measure of $PM_{2.5}$ on term birthweight. Subsequently, we tested if polymorphisms modified the effect of other exposure factors with similar pathways of action (active or passive smoking).

$PM_{2.5}$ exposure above the median value (reference, below) was associated with birthweight changes by 76g in the *homozygous* wild type genotype (n=161), -90g in the *heterozygous* genotype (n=154) and -168g in children with *GSTP1* *1B/*1B *mutant* genotype (n=39, interaction test, p=0.05). No effect measure modification with $PM_{2.5}$ was detected for *GSTT1*, *GSTM1* or *CYP2D6* polymorphisms (p≥0.12). No effect measure modification with *GSTP1* polymorphism was detected for active (p=0.71) nor for passive smoking effects on birthweight (p=0.13).

Key words: Air pollution; Birth Weight; Particulate Matter; Passive Smoking; Polymorphism, Genetic; Smoking.

1. INTRODUCTION

Several studies reported associations between air pollution levels during pregnancy and measures of foetal size at birth [discussed e.g., by 1, 2, 3, 4]. A few of these studies attempted to identify factors that could entail variations in the estimated sensitivity of foetal size to air pollutants; sensitivity factors considered include sex of the offspring [5, 6, 7], maternal parity [8], diet [9, 10] and genetic polymorphisms [11]. The latter were considered in only one study based on 199 births, in which two polymorphisms of *CYP1A1* gene were assessed. No interaction tests have been reported, but birth weight changes associated with PM_{10} exposure above the 90^{th} percentile (compared to below the 90^{th} percentile) were not clearly in favor of an effect measure modification by any of the polymorphisms considered (see [11] and Table 1 of this paper). Therefore, there is currently very limited direct evidence for genetic polymorphisms modifying the effect measure of air pollutants on foetal growth in humans. Some genes implied in the metabolism of xenobiotics have been shown to modulate the action of drugs in the body [12, 13, 14, 15], and they appear worth being considered in the context of the study of air pollution effects.

The metabolism of xenobiotics can schematically be seen as a process in two phases; phase 1 usually corresponds to a functionalisation of xenobiotics, which makes them more electrophilic or nucleophilic, thus allowing a conjugation step, corresponding to the phase 2 of metabolism, which eventually leads to more hydrophilic compounds that can be more easily eliminated from the body than the parent xenobiotics. Examples of enzymes implied in phase 1 metabolism include the cytochrome P450 (CYP) superfamily. Within this superfamily, *CYP2D6* belongs to the most polymorphic genes [16]. The glutathione transferases (GST) are a family of enzymes implied in phase 2 metabolism of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), which are present in atmospheric pollution [17]. The cytosolic GSTs include 7 classes of polymorphic enzymes, among which *GSTM1*, *GSTT1* and *GSTP1*. *GSTM1* and *GSTT1* null polymorphisms correspond to a lack of expression of the corresponding enzyme [17]. Some of these genes have been considered in

gene-environment interaction studies on human birth weight in relation to maternal active or passive smoking. In addition to the cytochrome P450 (CYP) superfamily and the glutathione transferases (*GST*) family, *NQO1* (NAD(P)H: quinone oxidoreductase 1) and *EPHX1* (epoxide hydrolase 1) polymorphisms have also been considered (see Table 1 for a review). Hong et al. reported an increase in mean birth weight associated with exposure to passive smoking (ETS) in *GSTT1* wild genotype and a decrease in mean birth weight associated with ETS exposure in *GSTT1 null* genotype [18]. In a case-control study, the estimated effect of ETS on mean birth weight also tended to be stronger in the offspring of mothers with *GSTT1 null* genotype than with *GSTT1 wild* genotype [19]. No clear statistical interaction have been described for polymorphisms of *GSTM1* gene, and *GSTP1* and *CYP2D6* have so far very little been considered in this context.

Our aim here was to describe gene-environment interactions for air pollution exposure on birth weight, focusing on the polymorphisms of candidate genes implied in the metabolism of xenobiotics (*GSTT1*, *GSTM1*, *GSTP1* and *CYP2D6*). Our study is based on a birth cohort in which we previously reported an increase in the frequency of birth weights below 3,000 g in association with $PM_{2.5}$ ambient concentrations (particulate matter with an aerodynamical diameter below 2.5 m, or fine PM) and $PM_{2.5}$ absorbance [20]. We used $PM_{2.5}$ ambient concentrations (particulate matter with an aerodynamical diameter below 2.5 μ, or fine PM) as a marker of exposure to atmospheric pollutants. In cities from industrialized countries, $PM_{2.5}$ are mainly derived from combustion processes such as road traffic, heating, biomass burning and specific industrial processes; additionally, a part of $PM_{2.5}$ are derived from the conversion of gaseous precursors such as sulfur or nitrous oxides, ammonia or volatile organic compounds [21]. In the city of Munich, about 60% of the local emissions of the larger PM_{10} stem from (road and train) traffic [22], a proportion that is probably higher for $PM_{2.5}$. $PM_{2.5}$ are a complex mixture composed of air-suspended liquid and solid particles. From a chemical point of view, in urban sites, $PM_{2.5}$ are mainly composed of secondary aerosol (ammonium nitrate and ammonium sulfate), organic matter (including many types of volatile,

semivolatile and non-volatile compounds, such as PAHs and benzene) and elemental carbon [23].

In a first step, we studied modifications of the effect measure of $PM_{2.5}$ exposure on birth weight by genetic polymorphisms. Human exposure to combustion-related air pollution occurs through the same pathway (inhalation) as exposure to tobacco smoke; these two mixtures also share similarities in terms of granulometry of their particulate component [24, 25] and also in terms of chemical composition. Indeed, tobacco smoke particulate matter has an aerodynamic diameter typically in the 50-800 nm range [25], which covers the 10-100 nm range, corresponding to fresh traffic-related PM [24]. Many families of combustions by-products such as volatile organic compounds (e.g., benzene) or polycyclic aromatic hydrocarbons are present in both mixtures. A notable difference is nicotine, which is present in tobacco smoke but not in traffic-related air pollution. In terms of effects on reproductive function, maternal exposure to each of these mixtures has been associated with decrements in term birth weight in humans [3, 26]. Therefore, we hypothesized that, if causal coactions (biological interaction) existed between $PM_{2.5}$ and a specific genetic polymorphism, it may also be present for environmental tobacco smoke (ETS) exposure or active smoking. Accordingly, in a second step, we focused on the polymorphism(s) detected in the analysis implying $PM_{2.5}$ exposure, and studied if this (these) polymorphism(s) also modified the effect measure of active or passive smoking on birth weight. The existence of a coherent pattern of interaction for all three environmental exposures studied was then considered to discuss the plausibility of any effect measure modification observed with atmospheric pollutants.

2. MATERIALS AND METHODS

2.1 Study population

In the LISA (Influences of Lifestyle Related Factors on the Human Immune System and Development of Allergies in Children) birth cohort, women were included after delivery in maternities from obstetrical clinics in the cities of Munich, Leipzig, Wesel and Bad Honef, Germany, between November 1997 and January 1999. Exclusion criteria for the mother were among others immune-related diseases (including diabetes) and being on long-term medication [27]; parents had to be born in Germany and to have the German nationality, which strongly limited the ethnical diversity of the study population. Exclusion criteria for the child were: a birth weight below 2500 g, a gestational duration below 37 completed weeks, congenital malformation, symptomatic neonatal infection, antibiotic medication and hospitalization or intensive medical care during neonatal period [27]. We further excluded twin births.

The study was approved by the ethics commissions of the Landesaerztekammer Bavaria and of the University of Leipzig and was carried out in accordance with the international guidelines for the protection of human subjects. Parents of all subjects gave written informed consent.

Gestational duration and birthweight were collected from the records filled in at birth by the midwife.

2.2 Exposure to cigarette smoke

Women answered a questionnaire shortly after delivery, allowing to collect information on active smoking, ETS, current address, changes of address during pregnancy, height and educational level. Information on active and passive smoking was obtained for each trimester of pregnancy. For active smoking, we considered only active smoking during the third trimester, which was more strongly associated with birth weight in analyses not taking into

account genetic polymorphisms than exposure during either the first, the second or any of the three trimesters. Similarly, we considered as exposed to ETS women who declared to have been exposed to passive smoking during the third trimester of pregnancy.

2.3 Exposure to $PM_{2.5}$

Exposure to $PM_{2.5}$ was estimated using a previously defined temporally-adjusted land use regression model. The model was based on a measurement campaign of $PM_{2.5}$ in 40 locations across Munich inner city and was initially developed to yield an estimate of a yearly average of air pollution levels [28, 29, 30]. It was thereafter expanded to incorporate a temporal component allowing to estimate an average of exposure during the whole pregnancy [20]. No similar exposure model has been developed for newborns from the other recruitment centers. All analyses implying air pollution levels were conducted excluding women who changed home during pregnancy, as we did not know their previous home address.

2.4 Genotyping

Blood samples were taken from the child at 6 years of age; because of this and because not all parents accepted to give their written consent for genetic analyses, these were only performed on a subgroup of the original birth cohort. Genotyped and non-genotyped cohort members have been compared (see online supplement). DNA was extracted from a thawed whole blood sample by use of a QIAamp Blood Kit (Qiagen) according to the “blood and body fluid protocol” recommended by the manufacturer. We used 600 µl of the blood sample for DNA extraction. The 4 genes considered are implied in the metabolism of xenobiotics. *GSTT1* and *GSTM1 null* genotypes correspond to a loss of glutathione transferase activity [31]. *GSTP1* represents the main glutathione transferase isoenzyme in the lung. The non-

synonymous *GSTP1* polymorphism (*rs1695*, *313A>G*, *Ile105Val*) has been associated with reduced enzyme activity and anticancer drug resistance and toxicity [12, 13]. *CYP2D6* is highly polymorphic, with polymorphism *1846G>A* (*rs1800716*) causing a splicing defect that results in a non-functional protein [32, 33]. This variant is responsible for the majority of the reduced enzyme activity found in Caucasian populations [34]. Genotyping of *GSTM1*, *GSTT1* and *GSTP1* was performed following Bauer et al. [35]: we used a multiplex PCR approach. Briefly, 10 ng genomic DNA was amplified in a 20 µl reaction mixture containing 10 pmol of each of the following primers: GSTM1_for 5'-GTGGAGACAGAAGAAGAGAAGA-3', GSTM1_rev 5'AGAGGCCAGAGCTGA TGAAGG-3', GSTT1_for 5'ACCCTGGCAGAGTTGGATGTGACC-3', GSTT1_rev 5'GTGGAAGACAGGGTGGGGATGGT-3'. As an internal positive control, the *RON* gene was co-amplified with the primers RON_for 5'-CTAGTGGGGAGGTGGAGCAGATA-3' and RON_rev 5'-AAGCAGGTCCAGCCCAAGAACTAA-3'. PCR was performed at 94°C for 5 min, then 38 cycles at 94°C for 30 s, at 60°C for 30 s, at 72°C for 1 min and extension at 72°C for 10 min. The PCR mixture contained 1.25 mM $MgCl_2$, 200 µM each dNTPS, 10 pmol of each primer and 1 U BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany). We used PCR mixture without DNA as a negative control to exclude false-positive data. The multiplex PCR products were then electrophoretically analysed on a 2% ethidium bromide-stained agarose gel (QA-Agarose, Qbiogene, Heidelberg, Germany). The presence or absence of *GSTM1* and *GSTT1* genes was detected by the presence or absence of a 688-bp (corresponding to *GSTT1*) and a 378-bp (corresponding to *GSTM1*) PCR band. *GSTP1 Ile 105 Val* polymorphism was detected by means of restriction fragment length polymorphism approach. Genomic DNA was amplified by PCR with the GSTP1_I4_for 5'-CTGCCCCCGGAGCCCTTTTGTTTA3' and GSTP1_I5_rev 5' CTCGCCCCCATG ACCCGTTACTTG-3' primer pair giving a 655-bp PCR product. The PCR product was consecutively restricted by BsmAI endonuclease (Fermentas, St. Leon-Rot, Germany) for 2 hours at 37°C. The reaction mixture was analysed by agarose gel electrophoresis. A 3-band and 4-band restriction pattern was identified as 105 Ile and Val, respectively.

For *CYP2D6*, we used the approach described by Hersberger et al. [36]. As a quality control procedure, we repeated the genotyping of a random sample of 3% of our study population, which showed a 100% agreement with the original genotyping. Genotyping was performed at Helmholtz Center for Environmental Research (UFZ) Leipzig. We performed a Hardy-Weinberg equilibrium test for *GSTP1* and *CYP2D6* polymorphisms; the test could not be performed for *GSTT1* and *GSTM1* polymorphisms because the genotyping approach used did not differentiate the homozygotic *wild type* variant and the heterozygotic *wild type-missing* variant.

2.5 A priori hypotheses

Based on analogies with previous studies on smoking or air pollutants and birth weight (Table 1) or respiratory health, our a priori hypotheses were that the deleterious effect of exposure would tend to be greater for the *null* genotype of *GSTM1* [18, 37, 38] and *GSTT1* [18, 19, 39], compared to the *present* genotype. We made no a priori hypotheses for the polymorphisms of *GSTP1* and *CYP2D6* because the literature on atmospheric pollutants was more scant for these genes.

2.6 Regression models

Statistical models were implemented using STATA software (version 10, College Station, TX). A statistical interaction cannot generally be considered to correspond to a biological interaction (or causal coaction); however, sufficient conditions for a statistical interaction to correspond to causal coaction have been identified in the case of a departure from additivity of the estimated effects of exposures [40, p.81-82, 41]. Since linear regression allows testing for a departure from additivity and also since the sample size with available $PM_{2.5}$ data was too limited to dichotomize birthweight (below *vs*. above 3000 g), we analysed birthweight as a continuous outcome. A different regression model was estimated for each pair of

environmental factor and genetic polymorphism. Interaction tests corresponded to the test of significance of the product terms between exposure and genotype in the adjusted regression models.

The effect measure modification with ETS was tested only among non-smoking mothers to limit the potential for residual confounding by active smoking. When we considered the effect of active smoking or ETS, we adjusted for maternal parity, education, height, pre-pregnancy weight and center. When we considered the effect of $PM_{2.5}$, we further adjusted for active smoking, as in our previous study [20].

2.7 Sensitivity analysis

We conducted an analysis aiming at quantifying the direction and amplitude of any bias resulting from the fact that birth weights below 2500 g had not been recruited. The approach was slightly modified from a bootstrap approach previously implemented in our study on the same population [20]. This bootstrap approach consisted in adding to the study population an extra population drawn at random from the newborns with a weight between 2500 and 2750 g, but assuming that their weight was 2375 g (the median birth weight among singleton term births weighing less than 2500 g in another cohort [6] in which newborns with a weight below 2500 g had been recruited). We then drew a bootstrap sample from this augmented population sample including 2.2% of birthweights below 2500 g, and estimated the joint effect of the genetic polymorphisms and the considered environmental factor in this sample using the linear regression model presented above. The bootstrap step has been replicated 1,000 times and the parameters associated with environmental factors have been averaged over these replications. The approach has been repeated excluding the offspring of mothers who smoked for the study of effect measure modifications with ETS.

3. RESULTS

3.1 Study population

The LISA cohort included 3038 singleton children, out of which 992 had a blood sample taken that allowed genotyping (Figure 1). Children with genotypic and smoking information had a mean birth weight of 3,495 g (SD, 435 g), similar to that of the remaining 2046 children (mean, 3481 g, SD, 441 g, p=0.4, supplementary material, Table S1). *GSTT1* polymorphism tended to be associated with active smoking status, and *GSTM1* polymorphism with ETS (Table 2); these associations became stronger (p=0.03 and p=0.05, respectively) after exclusion of observations with a birthweight below 3000 g. *CYP2D6* polymorphism was not in Hardy-Weinberg equilibrium (p<0.01), while there was no evidence of a deviation from equilibrium for *GSTP1* (p=0.89).

3.2 Fine particulate matter exposure and birth weight by genetic polymorphisms

A $PM_{2.5}$ level above the median (14.4 $\mu g/m^3$) was associated with a change by -22 g in mean birth weight (95% CI: -99, 55 g, 386 observations), regardless of genetic polymorphisms (Table 3). There was some evidence for an effect measure modification of $PM_{2.5}$ by the *GSTP1* polymorphism: a $PM_{2.5}$ level above the median corresponded to an adjusted change in mean birth weight by 76 g (p=0.18) in the *homozygous wild type* genotype, by -90 g in the *heterozygous *1B/wild type* genotype (p=0.12) and by -168 g in the *homozygous mutant* (**1B*) genotype (p=0.15; interaction test, p=0.05; Table 4 and supplementary material, Figure S1A). Repeating the analysis among non-smoking pregnant women did not weaken the strength of the statistical interaction between $PM_{2.5}$ exposure and *GSTP1* polymorphism (p=0.02). No clear effect measure modification with $PM_{2.5}$ was detected for the other genetic polymorphisms (p≥0.12, Table 4).

3.3 Active smoking and birth weight by genetic polymorphisms

Maternal smoking during pregnancy was associated with an adjusted change by -156 g in birth weight, non-smoking women being taken as a reference (95% CI: -250; -61 g; Table 3). The effect of smoking corresponded to a change in mean birthweight by -173 g for the *GSTP1 homozygous wild type* genotype, by -122 g for the *heterozygous *1B/wild type GSTP1* genotype and by -248 g in the *homozygous mutant (*1B)* genotype (interaction test, p=0.71, Table 5). Results changed little after restriction to the population with available $PM_{2.5}$ exposure (interaction test, p=0.54, not detailed). There was no strong evidence of a departure from additivity for combined effects of polymorphism and active smoking for the three other considered polymorphisms either (interaction tests, p≥0.19, Table 5).

3.4 Passive smoking and birth weight by genetic polymorphisms

ETS was associated with a change by -28 g in mean birth weight in the genotyped population (95% CI, -113, 57 g; Table 3). It was associated with a change by -31 g in the *GSTP1 homozygous wild type* phenotype, by 33 g in the *heterozygous* genotype and by -241 g in the *homozygous mutant (*1B)* genotype (interaction test, p=0.13). After restriction to the subjects with available $PM_{2.5}$ exposure (n=311), ETS was associated with an adjusted birth weight change by -199 g in the *GSTP1 homozygous wild type* genotype (95% CI: -426, 28 g, 10 exposed subjects), by 75 g in the *heterozygous* genotype (95% CI: -147, 297 g, 11 exposed subjects) and by -388 g in the *homozygous mutant* genotype (95% CI: -806, 31 g; 3 exposed subjects; interaction test, p=0.08).

For comparison purposes, we summarize here the results of Tables 4 to 6 in terms of amplitude of association with birth weight: for $PM_{2.5}$, the strongest estimated effects of exposure were seen in the *homozygous mutant (*1B/*1B)* genotype of *GSTP1* (β_{GSTP1}=-168

g) and *homozygous mutant *4/*4* genotype of *CYP2D6* (β_{CYP} =-311 g); this was also the case for ETS (β_{GSTP1} =-241 g, β_{CYP} =-278 g). For active smoking, the strongest estimated effects on birth weight were detected for *GSTP1 homozygous mutant (*1B/*1B)* genotype (β_{GSTP1}=-248 g) and for *CYP2D6 homozygous wild type* genotype (β_{CYP}=-195 g).

3.5 Sensitivity analysis

The gene-environment patterns corrected for the exclusion of birth weights below 2500 g (Figure 2B) remained qualitatively similar to those not corrected for the exclusion of birth weights below 2500 g (Figure 2A).

4. DISCUSSION

In this birth cohort, *Ile105Val* polymorphism in *GSTP1* gene tended to modify the effect measure of $PM_{2.5}$ levels around the maternal home address averaged during pregnancy on birth weight. There was no strong evidence that this polymorphism modified the effect measure of active or passive smoking on birth weight.

4.1 $PM_{2.5}$ and birth weight

A study in Korea among 199 newborns reported associations between PM_{10} exposure level (as estimated from the permanent monitoring stations network) during the first trimester of pregnancy and birth weight, by genetic polymorphisms of the *CYP1A1* gene [11]. The results (summarized in our Table 1) did not mention interaction tests, but the point estimates did not speak in favour of a clear effect measure modification with PM_{10} exposure, at least for *NcoI* polymorphism of *CYP1A1*. We are not aware of a study that considered the same genetic polymorphisms as those taken into account in our study. $PM_{2.5}$ effects tended to be stronger in the *GSTP1 Ile105Val mutant* genotype than in the *homozygous wild type*. We could control for a variety of potential confounders such as maternal size, pre-pregnancy weight, socio-economic status, active and passive smoking. As an alternative way to control for active smoking, we repeated the analysis among non-smoking pregnant, and the statistical interaction between $PM_{2.5}$ exposure and *GSTP1* was still present ($p=0.02$), confirming that this statistical interaction was unlikely to be explained by factors known to influence birth weight. *GSTP1* codes for a phase-II glutathione transferase enzyme, which has been recognized to detoxify chemicals [31], and *Ile105Val* genotype has been associated with reduced enzyme activity [13]. Therefore, assuming that glutathione-conjugated pollutants are less strongly active than unconjugated pollutants, there is some biological plausibility to the decrease in birth weight associated with $PM_{2.5}$ being strongest for this mutant genotype. The

fact that $PM_{2.5}$ exposure tended to be associated with an increased birth weight (p=0.18) in the *wild type* genotype was unexpected, and might be due to random fluctuation.

We genotyped the newborns and not their mothers. *GSTP1* has been shown to be active in fetus [42]. Some correlation between maternal and foetal heterozygosity can generally be expected [43], so that heterozygosity of the offspring may be a proxy of maternal heterozygosity. In the absence of genotyping of the mothers, and even if the statistical interaction detected corresponded to causal coactions, one should therefore refrain to interpret this interaction as bringing evidence for the offspring genes rather than the maternal ones being implied in the pathway between fine particulate matter exposure and foetal growth. There was no statistical interaction between $PM_{2.5}$ exposure and the three other genes tested; results concerning *CYP2D6* should be interpreted with caution because of a possible deviation from Hardy-Weinberg equilibrium.

4.2 Tobacco smoke and birth weight

The estimated effect of exposure tended to be strongest in the mutant *homozygous* genotypes of *CYP2D6* and *GSTP1* for both ETS and $PM_{2.5}$ exposure; however, there was no clear evidence of an effect measure modification between ETS and *GSTP1* (p=0.13). Since the approach based on the significance level being lower than a given threshold corresponded to our a priori choice, and since this approach might more easily be generalized to studies implying a larger number of polymorphisms than ours, we did not further consider the more qualitative similarity between ETS and $PM_{2.5}$ in terms of the genotypes for which the apparent effect of exposure was strongest. Additionally, the effect of active smoking on birth weight did not clearly differ between the *GSTP1 mutant* and *wild type* genotypes. Therefore, we considered that there was no coherent interaction pattern with genetic polymorphisms for the three exposure variables considered.

Although quite imprecise, our results concerning *GSTM1* were qualitatively in agreement with previous reports in which mothers had been genotyped, in favour of ETS being associated with greater decrements in mean birth weight in *GSTM1 null* genotype, compared to *GSTM1 present* genotype [18, 19].

4.3 Assessment of environmental exposures

The strengths of the approach we used to assess exposure were that we restricted the study to women who had not changed address during pregnancy, that our land-use regression exposure model was based on a measurement campaign in 40 sites of Munich city [20, 30], which allows a finer spatial resolution than approaches relying only on the network of air quality monitoring stations [20]. Limitations were that exposures away from the home address were not taken into account, and that estimated outdoor $PM_{2.5}$ levels were taken as a surrogate for personal exposure at the home address. A longitudinal exposure assessment study in Europe reported median coefficients of correlation between individual exposure and outdoor levels assessed in the vicinity of the home ranging from 0.7 to 0.8 for $PM_{2.5}$ [44], but a meta-analysis based on US studies reported lower correlations [45]. We estimated ETS exposure from a retrospective questionnaire filled by the mother at birth. This approach is generally prone to exposure misclassification [46] but few simple alternatives exist since cotinine, which is generally considered a relevant biomarker of ETS exposure in the general population, has a half-life of only about 9 hours in pregnant women [47].

4.4 Selection of study sample

The LISA birth cohort was primarily designed to study the impact of environmental exposures during early childhood on the occurrence of adverse health outcomes in childhood and adolescence. Preterm birth and low birthweight can be associated with adverse health

outcomes in childhood, which is the reason why premature and low birthweight (<2500 g) newborns had not been recruited. We conducted a sensitivity analysis assuming that low birthweight babies corresponded to 2.2% of singleton live term births, a figure based on the Eden mother-child cohort [6, 48], and close to the figure of 1.9% observed in Germany for the year 2004 [49]. This sensitivity analysis indicated that this exclusion is unlikely to have strongly affected our results. Population stratification bias is another source of error in genetic studies. This term is used to denote confounding by an unmeasured factor (such as ethnicity) associated with the health outcome and the genetic polymorphism under study [50]. If not controlled for, this unmeasured factor may entail a bias in the estimation of the main genetic effect on the health outcome, or of gene-gene or gene-environment interactions [51]. In our cohort, both parents had to be born in Germany and to have the German nationality in order for their child to be eligible in the cohort which, given the rather stringent laws regarding citizenship at the end of the 1990s, strongly limited the proportion of parents whose family immigrated from outside Europe in the recent generations. Therefore, our population was rather homogeneous from an ethnic point of view. This makes a population stratification bias due to ethnic factors unlikely.

4.5 Statistical approach

Under some hypotheses, and at least in the case of binary outcome measures, departure from additivity can allow to infer the presence of causal coactions between the two explanatory variables considered [40, p.81-82, 41]. For this reason, and also because of a more limited sample size, we a priori decided to analyze birth weight using a linear regression model, and not using a multiplicative model with a dichotomized outcome as in our previous study on air pollution effects on birth weight relying on the same cohort [20]. In addition to lack of unmeasured confounding and proper model specification, sufficient hypotheses allowing to infer the presence of causal coactions from departure from additivity

include that neither of the two explanatory factors considered can act as a preventive factor [40, p.81-82, 41]; these hypotheses have been derived in the context of binary (and not continous) outcomes. In our study, *GSTP1* mutant genotype tended to be associated with a decrease in birth weight, but this is not enough to exclude a positive effect of this genotype in specific subgroups of the population. Therefore, sufficient conditions to make inferences on causal coaction of $PM_{2.5}$ exposure and *GSTP1* polymorphisms based on the statistical interaction are not established in our study.

5. CONCLUSION

In a context where very few cohorts simultaneously offer an accurate assessment of exposure to atmospheric pollutants during pregnancy and information on genotype, alternatives to straightforward replication of gene-environment studies are worth being investigated. Our approach consisted in looking for similarities in interaction patterns between various environmental pollutants. It assumed that, because of closeness in chemical nature and pathway of exposure, tobacco smoke and atmospheric pollutants would share common metabolic pathways, which would translate into similar patterns of statistical interaction with xenobiotics-metabolizing genes. Tobacco smoke and traffic-related air pollutants indeed have similarities in terms of chemical nature, with many families of combustions by-products such as volatile organic compounds (e.g., benzene) or polycyclic aromatic hydrocarbons being present in both mixtures. A notable difference is nicotine, which is present in tobacco smoke but not in traffic-related air pollution. In terms of PM, traffic-related fresh pollutants typically have an aerodynamical diameter in the 10-100 nm range [24], while PM from tobacco smoke usually is in the 50-800 nm range [25]. The fact that *GSTP1* polymorphism modified the effect measure of $PM_{2.5}$ on birth weight but not that of active or passive smoking may be due to this statistical interaction not reflecting a biological interaction. For this reason, we warrant against interpreting this statistical interaction as an

evidence of combustion-related atmospheric pollutants acting on foetal growth through a pathway implying glutathione transferase enzymes.

ACKNOWLEDGEMENTS

R.S. benefited from a position of invited researcher funded by Helmholtz Centre Munich and Inserm during part of the project. The team “Environmental Epidemiology applied to Fertility and Reproduction” is funded by an Avenir Grant (2007) from Inserm.

CONFLICT OF INTEREST STATEMENT

Authors declare that they have no conflict of interest.

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The Editor
Reproductive Toxicology

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Manuscript number (if applicable):	Author name:
Article Title: Maternal Fine Particulate Matter Exposure, Polymorphism in Xenobiotic-Metabolizing Genes and Offspring Birthweight	Rémy SLAMA, PhD

Declarations

Reproductive Toxicology requires that all authors sign a declaration of conflicting interests. If you have nothing to declare in any of these categories then this should be stated.

Conflict of Interest

A conflicting interest exists when professional judgement concerning a primary interest (such as patient's welfare or the validity of research) may be influenced by a secondary interest (such as financial gain or personal rivalry). It may arise for the authors when they have financial interest that may influence their interpretation of their results or those of others. Examples of potential conflicts of interest include employment, consultancies, stock ownership, honoraria, paid expert testimony, patent applications/registrations, and grants or other funding.

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None.

Funding Source

All sources of funding should also be acknowledged and you should declare any involvement of study sponsors in the study design; collection, analysis and interpretation of data; the writing of the manuscript; the decision to submit the manuscript for publication. If the study sponsors had no such involvement, this should be stated.

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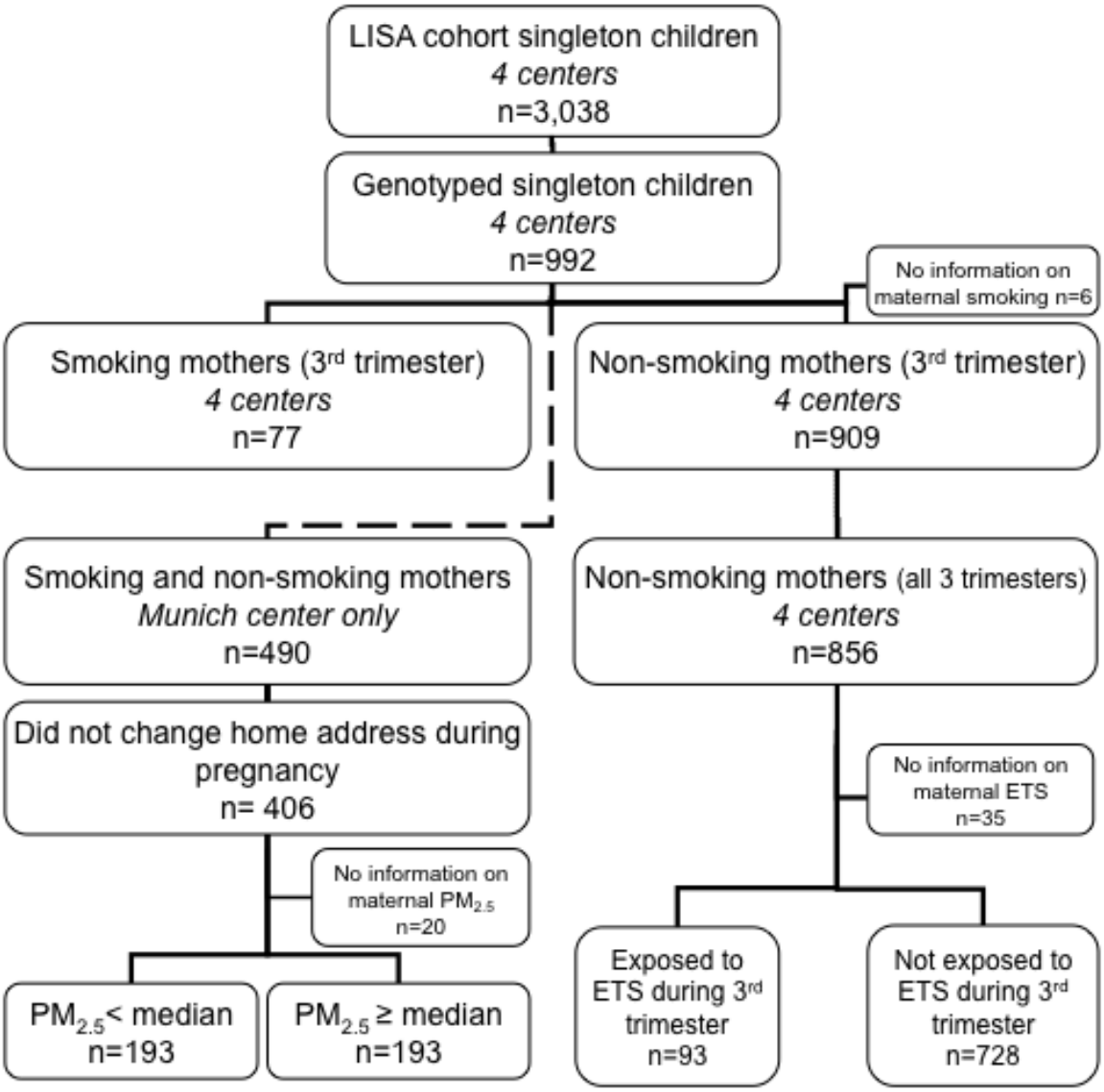
Print name

Rémy SLAMA

Figure

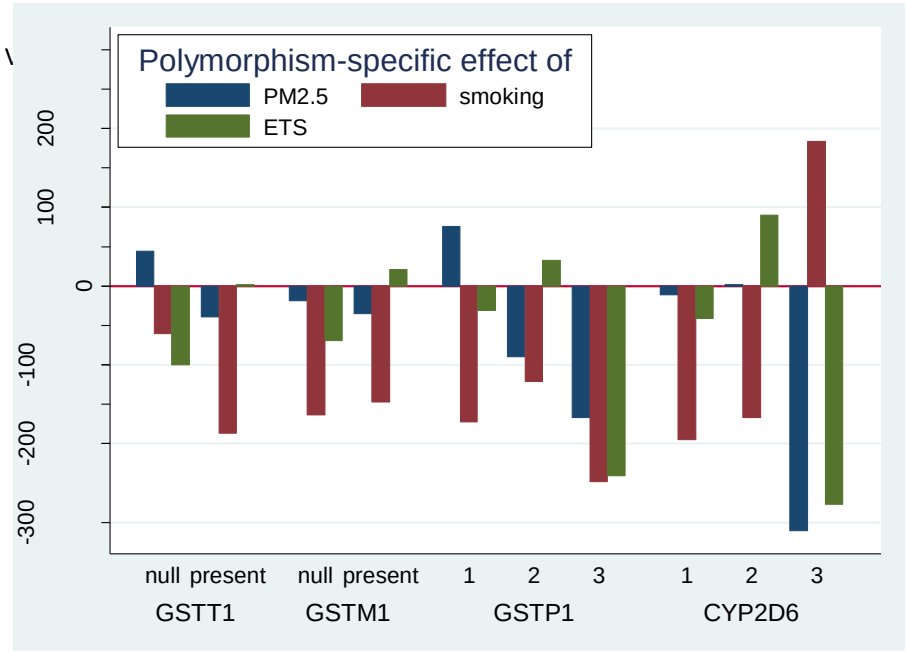
Figure 1: Flow Chart of Study Participants (restricted to singleton births with available birth weight information) of LISA Birth Cohort.



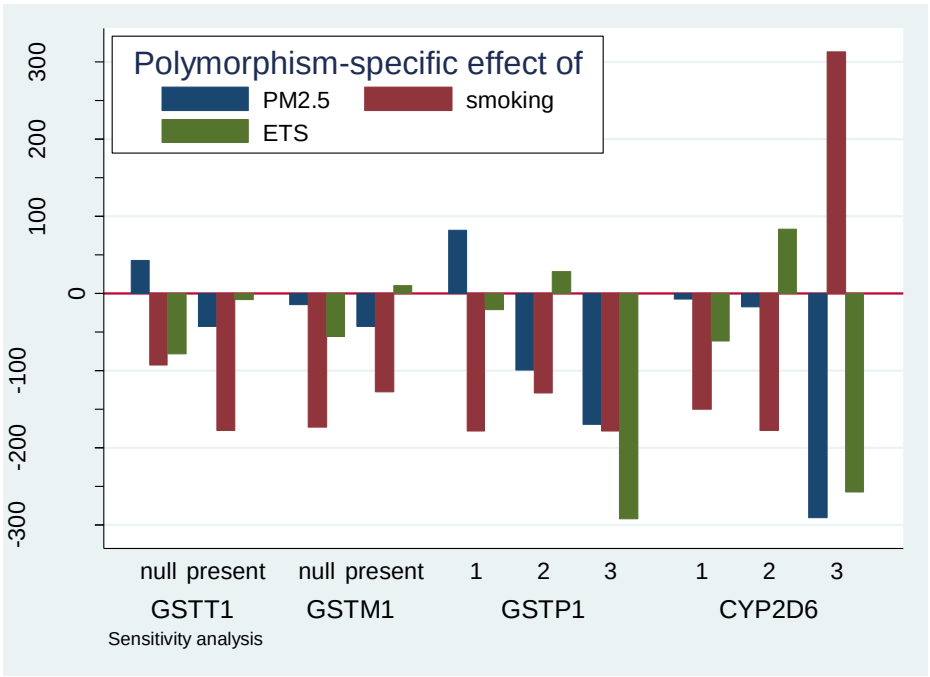
Abbreviations: ETS: Environmental Tobacco Smoke; $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

Figure 2: Sensitivity analysis; polymorphism-specific adjusted association between $PM_{2.5}$ exposure, smoking or ETS and birh weight (linear regression models) in LISA birth cohort (Germany, 1997-99). Each bar corresponds to the adjusted estimated effect of exposure on mean birth weight in the group with the considered polymorphism. A) Original analysis excluding birth weights below 2500 g; B) Sensitivity analysis corrected for the exclusion of birth weights below 2500 g (average of 1,000 bootstrap replications). For *GSTP1* and *CYP2D6*, polymorphism 1 corresponds to the wild type homozygous genotype.

A) Original analysis



B) Sensitivity analysis



Supplement

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TABLES

Table 1: Overview of Previous Gene-Environment Studies of Effects of Atmospheric Pollutants or Active or Passive Smoking on Birth Weight Considering Polymorphisms of Xenobiotics-Metabolizing Genes.

Reference	Environmental factor	Genetic polymorphisms	Origin of DNA	Outcome	Type of model	Main results
Suh, 2007 [10]	PM_{10} (above 90th centile vs. below 90th centile)	*CYP1A1 MspI* *CYP1A1 NcoI*	Maternal (n=199)	Birth weight	Additive	Birth weight change associated with PM_{10}: *-MspI*: -349 g for *TT* genotype of *CYP1A1 MspI* polymorphism and -394 g for *TC/CC* genotype; *-NcoI:* -261 g for *IleIle* genotype of *CYP1A1 NcoI* polymorphism and -489 g for *IleVal/ValVal* genotype.
Wang, 2002 [30]	Maternal smoking during pregnancy	*CYP1A1* (AA: homozygous wild type; aa: homozygous variant type) *GSTT1*	Maternal (n=741)	Birth weight	Additive (with a case-control sampling)	Birth weight change associated with smoking: *CYP1A1*: -252 g for *AA* polymorphism, -520 g for *Aa/aa* genotype (interaction test, P=0.06). *GSTT1*: -285 g for *present* genotype, -642 g for *absent* genotype (interaction test, P=0.04).
Grazuleviciene 2009 [39]	Maternal smoking during pregnancy	*GSTT1* *GSTM1*	Maternal (n=646)	Birth weight<2,500 g	Multiplicative	Interaction test between smoking and genetic polymorphisms: P>0.5 for *GSTT1* and *GSTM1*.
Infante-Rivard, 2006 [31]	Maternal smoking during 3rd trimester of pregnancy	*GSTT1* *GSTM1* *CYP1A1**2A, 2B, 4 *XRCC3*	Newborn (n=465)	Small-for gestational age (SGA) birth	Multiplicative	*GSTT1*: OR of SGA associated with smoking =0.85 for +/+ genotype, 2.77 for -/+ and 1.42 for -/- genotypes (statistical interaction, P=0.01). P for statistical interaction=0.18 for *XRCC3*, P=0.66 for *GSTM1*, P=0.98 for *CYP1A1*2B*, P=0.06 for *CYP1A1*4*.
Infante-Rivard, 2006 [31]	Maternal smoking during 3rd trimester of pregnancy	*GSTT1* *GSTM1* *CYP1A1**2A, 2B, 4 *XRCC3*	Maternal (n=465)	Small-for gestational age (SGA) birth	Multiplicative	*GSTT1*: OR of SGA associated with smoking =1.39 for +/+ genotype, 1.93 for -/+ and 2.63 for -/- genotype (P for interaction, 0.46). *GSTM1*: P for interaction, 0.18. *CYP1A1*2B:* P=0.84. *CYP1A1*4:* P=0.84. *XRCC3*: P=0.03.
Sasaki, 2008 [40]	Maternal smoking during 2nd or 3rd trimester of pregnancy	*NQO1,* *CYP2E1*	Maternal (n=460)	Birth weight (length and head circumference also considered)	Additive	Birth weight change associated with smoking: *NQO1*: beta=-77g for *Pro*/Ser** or *Ser/Ser* genotypes; beta= -199 g for *Pro/Pro* genotype (P for interaction, 0.05). *CYP2E1*: beta=-170 g for *c1/c2* or *c2/c2* genotypes and -195 g for *c1/c1* genotype (P for interaction, 0.63).
Wu, 2007 [41]	Passive smoking at home during pregnancy (ETS)	*CYP1A1 MspI* *EPHX1* (*Tyr113His*)	Maternal (n=680)	Birth weight	Additive	Birth weight change associated with ETS: *CYP1A1*: beta=15 g for *T/T* genotype, -11 g for *T/C* genotype and 14 g for *C/C* genotype. *EPHX1*: beta=2g for *Tyr/Tyr* genotype, -104g for *Tyr/His* genotype and -687g for *His/His* genotype
Hong, 2003 [28]	Passive smoking (ETS)	*GSTT1* *GSTM1*	Maternal (n=266)	Birth weight	Additive	Birth weight change associated with ETS: *GSTM1*: -11 g for *wild* genotype and -158 g for *null* genotype

						(interaction test, P=0.17). *GSTT1*: 203 g for *wild* genotype and -236 g for *null* genotype (interaction test, P<0.01).
This study	$PM_{2.5}$ (above *versus* below median)	*GSTT1 GSTM1 GSTP1 Ile105Val CYP2D6 G>A*	Newborn (n=386)	Birth weight	Additive	Birth weight change associated with $PM_{2.5}$ exposure: *GSTP1*: beta=-168 g in birth weight in children with *GSTP1 *1B/*1B* mutant genotype, -90 g in the *heterozygous* genotype and +76 g in the *homozygous wild type* genotype (interaction test, P=0.05). No effect measure modification with $PM_{2.5}$ was detected for the other genetic polymorphisms (P≥0.12).
This study	Active smoking	*GSTT1 GSTM1 GSTP1 Ile105Val CYP2D6 G>A*	Newborn (n=986)	Birth weight	Additive	P for interaction=0.25 for *GSTT1,* P=0.86 for *GSTM1*, P=0.71 for *GSTP1* and P=0.19 for *CYP2D6*.
This study	Passive smoking (ETS)	*GSTT1 GSTM1 GSTP1 Ile105Val CYP2D6 G>A*	Newborn (n=821)	Birth weight	Additive	P for interaction=0.38 for *GSTT1,* P=0.30 for *GSTM1*, P=0.13 for *GSTP1* and P=0.06 for *CYP2D6*.

Abbreviation: PM_{10}: particulate matter with an aerodynamical diameter lower than 10 μ.

Table 2: Characteristics of the LISA birth cohort with Information on Genotypes, Stratified According to Environmental Tobacco Smoke (ETS, Restricted to Non-Smoking Women), Pregnancy $PM_{2.5}$ Average and Active Smoking.

Characteristic	**ETS (n=821)**					**$PM_{2.5}$ (n=386)**					**Active smoking (n=986)**				
	No (n=728)		Yes (n=93)		p	<median (n=193)		≥median (n=193)		p	No (n=909)		Yes (n=77)		p
	n	%	n	%		n	%	n	%		n	%	n	%	
Genetic polymorphisms															
GSTT1 null					0.73					0.52					0.07
present	589	81	76	83		157	82	152	79		735	81	56	73	
null	137	19	16	17		35	18	40	21		171	19	21	27	
GSTM1					0.07					0.72					0.25
present	369	51	56	61		102	53	98	51		461	51	34	44	
null	356	49	36	39		90	47	93	49		444	49	43	56	
GSTP1 Ile105Val					0.80					0.84					0.48
wt/wt	322	44	38	41		88	46	83	43		398	44	30	39	
*wt/*1B*	318	44	44	47		83	43	89	46		407	45	40	52	
**1B/*1B*	86	12	11	12		21	11	21	11		102	11	7	9	
CYP2D6 G>A					0.33					0.34					0.86
wt/wt	447	62	50	55		117	62	112	60		552	61	48	63	
*wt/*4*	226	31	32	35		54	29	64	34		286	32	24	32	
**4/*4*	48	7	9	10		18	10	12	6		62	7	4	5	
Characteristics of pregnancy															
Gestational duration (weeks)					0.58					0.68					0.58
37	24	3	4	4		3	2	8	4		31	3	4	5	
38	68	9	8	9		19	10	15	8		86	9	6	8	
39	149	20	18	19		36	19	33	17		189	21	17	22	
40	266	37	32	34		69	36	73	38		325	36	21	27	
41	161	22	18	19		43	22	40	21		195	21	25	19	
≥ 42	60	8	13	14		23	12	24	12		83	9	10	13	
Sex of the child					0.29					0.84					0.87
Female	340	47	38	41		85	44	87	45		416	46	36	47	
Male	388	53	55	59		108	56	106	55		493	54	41	53	
Birth weight					0.95					0.40					0.05
<3000 g	80	11	11	12		24	12	30	16		102	11	17	22	
3000-3249 g	140	19	15	16		38	20	37	19		171	19	15	19	
3250-3499 g	165	23	20	22		43	22	36	19		199	22	17	22	
3500-3999 g	251	34	34	37		62	32	73	38		316	35	22	29	
≥ 4000 g	92	13	13	14		26	13	17	9		121	13	6	8	

Mean (g)	3502		3515		0.78[a]	3480		3450		0.42[a]	3508		3370		0.008[a]
Period of conception					0.97					0.001					0.76
January to March	162	22	21	23		45	23	33	17		204	22	17	22	
April to June	188	26	26	28		55	29	54	29		238	26	24	31	
July to September	169	23	20	22		33	17	66	34		213	23	15	19	
October to December	209	29	26	28		60	31	40	21		254	28	21	27	
Maternal age					0.01					0.81					0.001
<25 years	34	5	5	5		2	1.0	5	3		47	5	13	17	
25-29 years	209	29	41	44		38	20	37	19		272	30	22	29	
30-34 years	356	49	29	31		102	53	105	55		420	46	25	32	
35-39 years	113	16	16	17		46	24	40	21		149	16	13	17	
≥ 40 years	15	2	2	2		5	3	5	3		19	2	4	5	
Maternal parity					0.01					0.002					0.24
0	384	53	61	67		85	44	115	60		490	54	47	61	
≥ 1	343	47	30	33		108	56	78	40		416	46	30	39	
Maternal tobacco smoking (3rd trimester)					N.A.					0.99					N.A.
No	728	100	93	100		177	92	178	92		909	100	0	0	
Yes	0	0	0	0		15	8	15	8		0	0	77	100	
Maternal ETS (3rd trimester)					N.A.					0.22					<0.001
No	694	100	0	0		168	91	157	87		764	88	29	40	
Yes	0	0	132	100		17	9	24	13		106	12	43	60	
Maternal education					0.001					0.005					<0.001
≤9 years of school	34	5	7	8		19	10	8	4		55	6	16	21	
10 years degree	240	33	48	52		61	32	50	26		328	36	36	47	
Vocational school	50	7	6	7		17	9	9	5		65	7	7	9	
High school ("Abitur")	400	55	31	34		94	49	126	65		456	50	17	22	
Maternal height (cm)					0.37					0.04					0.90
≤ 160	69	10	12	13		24	13	12	6		96	11	7	9	
161-170	399	55	55	60		110	58	102	53		503	56	44	58	
171-180	240	33	23	25		55	29	74	39		284	32	23	30	
> 180	12	2	2	2		1	1	3	2		15	2	2	3	
Maternal pre-pregnancy weight					0.22					0.26					0.46
≤ 50 kg	29	4	4	4		11	6	9	5		40	4	6	8	
51-60 kg	269	38	29	31		68	36	84	44		339	38	26	35	
61-70 kg	262	37	30	32		76	40	59	31		310	35	22	30	
71-80 kg	94	13	16	17		22	12	21	11		127	14	14	19	
> 80 kg	63	9	14	15		11	6	17	9		81	9	6	8	
Maternal pre-pregnancy BMI					0.02					0.10					<0.001

≤ 18 kg/m^2	14	2	1	1		2	1	7	4		17	2	8	10	
18< BMI ≤ 20 kg/m^2	135	19	5	5		33	17	43	22		157	17	11	14	
20< BMI ≤ 22.5 kg/m^2	294	40	41	44		74	38	84	44		368	40	20	26	
22.5 < BMI ≤ 25 kg/m^2	140	19	22	24		42	22	27	14		179	20	16	21	
25 < BMI ≤ 30 kg/m^2	93	13	16	17		29	15	22	11		122	13	12	16	
30 < BMI (kg/m^2)	52	7	8	9		13	7	10	5		66	7	10	13	
Centre					0.003					N.A.					0.99
Munich	377	52	32	34		193	100	193	100		450	50	39	51	
Leipzig	184	25	37	40		0	0	0	0		250	28	20	26	
Wesel	97	13	10	11		0	0	0	0		118	13	10	13	
Bad Honef	70	10	14	15		0	0	0	0		91	10	8	10	

Abbreviations: BMI: Body mass index; ETS: Environmental Tobacco Smoke; $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

[a] p-value of Student's t test. All other p-values correspond to χ^2 tests.

Table 3: Estimated Effect of Genetic Polymorphisms, $PM_{2.5}$ Exposure, Active Smoking or ETS on Birth Weight among Genotyped Children of the LISA Cohort. The effect of each factor was estimated in a separate regression model not adjusted for the other exposure factors but adjusted for potential confounders.

Characteristic	n	Mean birth weight (SD) (g)		Adjusted β [a] (g)	95% CI	p
Genetic polymorphisms						
GSTT1 null	*983*					
present	791	3506	438	0		
null	192	3465	423	-9	-73, 55	0.78
GSTM1 null	*982*					
present	495	3502	425	0		
null	487	3493	445	17	-33, 67	0.51
GSTP1 Ile105Val	*984*					*0.41*
wt/wt	428	3521	429	0		
*wt/*1B*	447	3481	433	-25	-79, 28	0.36
**1B/*1B*	109	3471	468	-51	-134, 31	0.22
CYP2D6 G>A	*976*					*0.92*
wt/wt	600	3510	440	0		
*wt/*4*	310	3482	417	-4	-54, 55	0.87
**4/*4*	66	3469	473	-22	-115, 92	0.68
$PM_{2.5}$ exposure [b]	*386*					
< median	193	3479	417	0		
≥ median	193	3446	402	-22	-99, 55	0.57
Active smoking [c]	*955*					
No	883	3508	434	0		
Yes	72	3355	451	-156	-250, -61	<0.001
ETS [d]	*798*					
No	709	3503	426	0		
Yes	89	3499	440	-28	-113, 57	0.52

Abbreviations: ETS: Environmental Tobacco Smoke; $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

[a] Change in mean birthweight adjusted for gestational duration, infant sex, parity, duration of education, size, pre-pregnancy weight, number of cigarettes smoked daily (excepted for ETS exposure), ETS exposure and centre (excepted for $PM_{2.5}$ exposure).

[b] Restricted to genotyped newborns recruited in the Munich area with an estimate of maternal $PM_{2.5}$ exposure.

[c] Among the whole genotyped population.

[d] Among the genotyped population restricted to non-smokers.

Table 4: Association Between Maternal $PM_{2.5}$ Exposure (- indicates subjects below $PM_{2.5}$ median level and + those above $PM_{2.5}$ median level) and Mean Birth Weight according to Infant Genetic Polymorphisms, among 386 Newborns from Munich Center of the LISA Cohort.

Polymorphism	$PM_{2.5}$	n	Mean birth weight (SD), g	β [a] (g)	p	Effect of $PM_{2.5}$			Interaction [d]
						β [a,b] (g)	95% CI	p	
GSTT1 null					*0.24* [c]				0.40
present	-	144	3480 (419)	0 (ref)		0 (ref)			
	+	140	3420 (414)	-39	0.38	-39	-127, 48	0.38	
null	-	32	3510 (450)	42	0.55	0 (ref)			
	+	37	3550 (327)	86	0.20	44	-126, 213	0.61	
GSTM1 null					*0.43* [c]				0.83
present	-	96	3470 (344)	0 (ref)		0 (ref)			
	+	92	3430 (425)	-35	0.51	-35	-139, 69	0.51	
null	-	80	3500 (505)	44	0.42	0 (ref)			
	+	84	3450 (374)	26	0.63	-19	-131, 94	0.75	
GSTP1 Ile105Val					*0.14* [c]				0.05
wt/wt	-	81	3470 (439)	0 (ref)		0 (ref)			
	+	80	3540 (378)	76	0.18	76	-36, 188	0.18	
*wt/*1B*	-	75	3480 (422)	32	0.57	0 (ref)			
	+	79	3380 (399)	-58	0.31	-90	-203, 24	0.12	
**1B/*1B*	-	20	3600 (373)	54	0.54	0 (ref)			
	+	19	3290 (414)	-114	0.21	-168	-395, 59	0.15	
CYP2D6 G>A					*0.38* [c]				0.12
wt/wt	-	108	3500 (432)	0 (ref)		0 (ref)			
	+	106	3460 (430)	-12	0.85	-12	-109, 85	0.85	
*wt/*4*	-	50	3460 (400)	-26	0.78	0 (ref)			
	+	57	3470 (350)	-24	0.74	2	-134, 137	0.98	
**4/*4*	-	15	3520 (384)	74	0.39	0 (ref)			
	+	10	3180 (302)	-237	0.05	-311	-590, -31	0.03	

Abbreviation: $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

[a] Parameter (grams) of the linear regression models adjusted for gestational duration, infant sex, maternal active (number of cigarettes) and passive smoking, parity, duration of education, size, pre-pregnancy weight.

[b] Effect of $PM_{2.5}$ exposure (above (+) *versus* below (-) median) within each polymorphism.

[c] Heterogeneity test across all categories (Wald test).

[d] Interaction tests were performed through a Wald test of the significance of the interaction terms in the model.

Table 5: Association between Maternal Active Smoking during Third Trimester of Pregnancy and Mean Birth Weight according to Infant Genetic Polymorphisms (986 Singleton Births from Munich and Leipzig Centers of LISA Cohort).

Polymorphism	Smoking	n	Mean birth weight (SD) (g)	β [a] (g)	p	Effect of smoking			
						β [a,b] (g)	95% CI	p	Interaction [d]
GSTT1 null					*0.007* [c]				0.25
present	**No**	716	3517 (434)	0 (ref)		0 (ref)			
	Yes	53	3324 (462)	-187	0.001	-187	-296, -78	0.001	
null	**No**	164	3475 (428)	-29	0.39	0 (ref)			
	Yes	19	3440 (418)	-90	0.32	-61	-245, 123	0.52	
GSTM1 null					*0.01* [c]				0.86
present	**No**	449	3509 (421)	0 (ref)		0 (ref)			
	Yes	30	3361 (466)	-147	0.04	-147	-289, -5	0.04	
null	**No**	430	3508 (447)	16	0.53	0 (ref)			
	Yes	42	3350 (445)	-148	0.02	-164	-288,-40	0.01	
GSTP1 Ile105Val					*0.03* [c]				0.71
wt/wt	**No**	383	3528 (427)	0 (ref)		0 (ref)			
	Yes	29	3392 (439)	-173	0.02	-173	-321, -26	0.02	
*wt/*1B*	**No**	397	3494 (429)	-26	0.36	0 (ref)			
	Yes	36	3352 (494)	-148	0.03	-122	-254, 10	0.07	
**1B/*1B*	**No**	102	3489 (475)	-38	0.38	0 (ref)			
	Yes	7	3215 (219)	-286	0.05	-248	-544, 48	0.10	
CYP2D6 G>A					*0.01* [c]				0.19
wt/wt	**No**	535	3523 (438)	0 (ref)		0 (ref)			
	Yes	45	3354 (454)	-195	0.001	-195	-313, -76	0.001	
*wt/*4*	**No**	280	3497 (413)	-5	0.85	0 (ref)			
	Yes	22	3289 (464)	-173	0.04	-168	-335, -1	0.05	
**4/*4*	**No**	59	3451 (477)	-36	0.49	0 (ref)			
	Yes	4	3635 (289)	148	0.45	184	-207, 575	0.36	

Abbreviation: $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

[a] Parameter (grams) of the linear regression models adjusted for gestational duration, infant sex, parity, duration of education, size, pre-pregnancy weight and centre.

[b] Effect of active smoking (reference, non-smoking women) within each polymorphism.

[c] Heterogeneity test across all categories (Wald test).

[d] Interaction tests were performed through a Wald test of the significance of the interaction terms in the model.

Table 6: Association between Maternal Passive Smoking and mean Birthweight According to Genetic Polymorphisms, among 826 Non-Smoking Women from Munich and Leipzig Centers of LISA Cohort.

Polymorphism	ETS	n	Mean birth weight (SD) (g)	β [a] (g)	p	Effect of ETS			
						β [a,b] (g)	95% CI	p	Interaction [d]
GSTT1 null		*795*			*0.62* [c]				0.38
present	**No**	575	3507 (428)	0 (ref)		0 (ref)			
	Yes	72	3526 (455)	-2	0.98	2	-96, 93	0.98	
null	**No**	132	3483 (418)	-20	0.59	0 (ref)			
	Yes	16	3431 (314)	-120	0.22	-100	-298, 98	0.32	
GSTM1 null		*794*			*0.45* [c]				0.30
Present	**No**	359	3490 (407)	0 (ref)		0 (ref)			
	Yes	54	3512 (424)	21	0.70	21	-89, 131	0.70	
null	**No**	347	3515 (446)	41	0.15	0 (ref)			
	Yes	34	3502 (452)	-29	0.67	-70	-205, 64	0.31	
GSTP1 Ile105Val		*797*			*0.29* [c]				0.13
wt/wt	**No**	313	3524 (408)	0 (ref)		0 (ref)			
	Yes	35	3491 (393)	-31	0.65	-31	-164, 103	0.65	
*wt/*1B*	**No**	309	3485 (434)	-36	0.24	0 (ref)			
	Yes	43	3531 (425)	-3	0.96	33	-90, 156	0.60	
**1B/*1B*	**No**	86	3490 (461)	-25	0.59	0 (ref)			
	Yes	11	3398 (632)	-267	0.02	-241	-481, -2	0.05	
CYP2D6 G>A		*789*			*0.25* [c]				0.06
wt/wt	**No**	435	3521 (429)	0 (ref)		0 (ref)			
	Yes	38	3505 (439)	-41	0.48	-41	-155, 73	0.48	
*wt/*4*	**No**	221	3476 (400)	-21	0.51	0 (ref)			
	Yes	31	3595 (478)	69	0.33	90	-54, 234	0.22	
**4/*4*	**No**	46	3449 (398)	-16	0.78	0 (ref)			
	Yes	8	3414 (604)	-294	0.03	-278	-562, 7	0.06	

Abbreviation: ETS: Environmental Tobacco Smoke; $PM_{2.5}$: Particulate matter with an aerodynamical diameter below 2.5 μ.

[a] Parameter (grams) of the linear regression models adjusted for gestational duration, infant sex, parity, duration of education, size, pre-pregnancy weight and centre.

[b] Adjusted effect of ETS on mean birthweight within each polymorphism.

[c] Heterogeneity test across all categories (Wald test).

[d] Interaction tests were performed through a Wald test of the significance of the interaction terms in the model.

*Cover Letter

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Grenoble, November 11, 2009

The Editor
Reproductive Toxicology

Dear Editor,

We are pleased to submit to Reproductive Toxicology an original contribution entitled

”Maternal Fine Particulate Matter Exposure, Polymorphism in Xenobiotic-Metabolizing Genes and Offspring Birthweight”

This study is one of the first to explore effect-measure modifications between maternal exposure to atmospheric pollutants and genetic polymorphisms, for effects on birth weight. It follows a previous publication in which we reported associations between maternal exposure to fine particulate matter and birth weight, without consideration of genetic polymorphisms (Slama et al., EHP, 2007).

One strength of our study is the land-use regression approach based on a measurement campaign of air pollution at 40 sites used to assess exposure to fine particulate matter, which has a fine spatial resolution. In contrast, the only previous study on this topic (Suh, Reprod Toxicol, 2007) relied on air quality monitoring stations to assess exposure, an approach limited by the poor spatial density of air quality monitoring networks. Another originality of our approach lies in the consideration of gene-environment interactions with several environmental factors with similar pathways of action; indeed, we tried to “replicate” the effect measure modification between air pollution exposure and *GSTP1* polymorphism with two additional exposure variables: passive and active smoking. This approach might prove useful in the context of gene-environment (and possibly gene-environment-wide) interaction studies when several exposure factors with similar mechanisms of action have been assessed in one population, but no independent population with similar information is available for replication purposes.

Your journal has published the only study that addressed the question of gene-environment interactions for effects of Particulate Matter on birth weight (Suh et al., Reprod Toxicol, 2007). For this reason, we believe that our manuscript is particularly suited to your journal.

Sincerely,

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Ref.: Ms. No. 2719
Maternal Fine Particulate Matter Exposure, Polymorphism in Xenobiotic-Metabolizing Genes and Offspring Birthweight
Reproductive Toxicology

Reply to the reviewers
25 Feb. 2010
Reproductive Toxicology

Reviewer #1:

GENERAL COMMENTS:

1.1) The paper is well presented and the objectives of the report are of interest. Nevertheless, the conclusions are weakened by numbers that are either small or very small when assessing heterogeneity of association according to genotype. The conclusions are appropriately prudent, but this hardly compensates for the fact that the limited sample size probably cannot support the demonstration of the hypotheses. Given also that the same mechanisms of action are postulated for the 3 studied exposure variables, it becomes difficult to isolate the effect of any, in particular, ETS and active smoking.

Reply: An originality of the article is indeed the fact that we considered gene-environment interactions with 3 different exposure variables (fine particulate matter, passive smoking (ETS) and active smoking). The reviewer rightly points that effects of ETS and active smoking may be difficult to disentangle one from another. Indeed, women living with a smoking husband (and hence exposed to ETS) may more often smoke than partners of non-smoking men. This issue has been addressed in our analyses: analyses of ETS effects are restricted to non-smoking women. The potential for ETS to confound the association between active smoking and birth weight is much weaker because doses of tobacco smoke stemming from ETS are much lower than those due to passive smoking (see also point 1.6 below).

1.2) Unfortunately, I was not able to access the supplemental material and tables 4 and 5 were truncated. The Editorial Assistant confirmed this but could not provide new tables or materials for evaluation.

R: The right part of Tables 4 and 5 were indeed missing, probably as a result of the conversion in pdf format, and we apologize for any mistake in manuscript uploading from our side. We made sure that these Tables appear in totality in the revised version. Concerning the supplemental material, it was accessible by double-clicking on the sentence "Click here to download supplemental material" on the last page of the document, at least in the version that we uploaded from the journal web site.

SPECIFIC COMMENTS:

1.3) Test for Hardy-Weinberg (in the 3 category genotypes).

R: We now report the results of Hardy-Weinberg disequilibrium test for the 2 genotypes with 3 categories in the results section (section 3.1); the gene for which an interaction has been highlighted (*GSTP1*) was in Hardy-Weinberg equilibrium:

"CYP2D6 polymorphism was not in Hardy-Weinberg equilibrium (p<0.01), while there was no evidence of a deviation from equilibrium for *GSTP1* (p=0.89)." (3.1, p.12, l.20)

1.4) Discuss the potential for population stratification bias.

R: This point has been added in the discussion section:
"Population stratification bias is another source of error in genetic studies. This term is used to denote confounding by an unmeasured factor (such as ethnicity) associated with the health outcome and the genetic polymorphism under study [1]. If not controlled for, this unmeasured factor may entail a bias in the estimation of the main genetic effect on the health outcome, or of gene-gene or gene-environment interactions [2]. In our cohort, both parents had to be born in Germany and to have the German nationality in order for their child to be eligible in the cohort which, given the rather stringent laws regarding citizenship at the end of the 1990s, strongly limited the proportion of parents whose family immigrated from outside Europe in the recent generations. Therefore, our population was rather homogeneous from an ethnic point of view. This makes a population stratification bias due to ethnic factors unlikely." (4.4, p.18, l.11)

1.5) The paper is exceedingly short on DNA extraction and genotyping methods. Please provide more information.

R: References on genotyping methods are provided, and we have now expanded this section as requested:
"DNA was extracted from thawed whole blood sample by use of a QIAamp Blood Kit (Qiagen) according to the "blood and body fluid protocol" recommended by the manufacturer. We used 600 µl of the blood sample for DNA extraction. The 4 genes considered are implied in the metabolism of xenobiotics. *GSTT1* and *GSTM1 null* genotypes correspond to a loss of glutathione S-transferase activity [3]. *GSTP1* represents the main glutathione S-transferase isoenzyme in the lung. The non-synonymous *GSTP1* polymorphism (*rs1695*, *313A>G*, *Ile105Val*) has been associated with reduced enzyme activity and anticancer drug resistance and toxicity [4, 5]. *CYP2D6* is highly polymorphic, with polymorphism *1846G>A* (*rs1800716*) causing a splicing defect that results in a non-functional protein [6, 7]. This variant is responsible for the majority of the reduced enzyme activity found in Caucasian populations [8]. Genotyping of *GSTM1*, *GSTT1* and *GSTP1* was performed following Bauer et al. [9]: we used a multiplex PCR approach. Briefly, 10 ng genomic DNA was amplified in a 20 µl reaction mixture containing 10 pmol of each of the following primers: GSTM1_for 5'-GTGGAGACAGAAGAAGAGAAGA-3', GSTM1_rev 5'AGAGGCCAGAGCTGA TGAAGG-3', GSTT1_for 5'ACCCTGGCAGAGTTGGATGTGACC-3', GSTT1_rev 5'GTGGAAGACAGGGTGGGGATGGT-3'. As an internal positive control, the *RON* gene was co-amplified with the primers RON_for 5'-CTAGTGGGGAGGTGGAGCAGATA-3' and RON_rev 5'-AAGCAGGTCCAGCCCAAGAACTAA-3'. PCR was performed at 94°C for 5 min, then 38 cycles at 94°C for 30 s, at 60°C for 30 s, at 72°C for 1 min and extension at 72°C for 10 min. The PCR mixture contained 1.25 mM $MgCl_2$, 200 µM each dNTPS, 10 pmol of each primer and 1 U BIOTAQ DNA polymerase (Bioline, Luckenwalde, Germany). We used PCR mixture without DNA as a negative control to exclude false-positive data. The multiplex PCR products were then electrophoretically analysed on a 2% ethidium bromide-stained agarose gel (QA-Agarose, Qbiogene, Heidelberg, Germany). The presence or absence of *GSTM1* and *GSTT1* genes was detected by the presence or absence of a 688-bp (corresponding to GSTT1) and a 378-bp (corresponding to

GSTM1) PCR band. GSTP1 Ile 105 Val polymorphism was detected by means of restriction fragment length polymorphism approach. Genomic DNA was amplified by PCR with the GSTP1_I4_for 5'- CTGCCCCCGGAGCCCTTTTGTTTA3' and GSTP1_I5_rev 5' CTCGCCCCCATG ACCCGTTACTTG-3' primer pair giving a 655-bp PCR product. The PCR product was consecutively restricted by BsmAI endonuclease (Fermentas, St. Leon-Rot, Germany) for 2h at 37°C. The reaction mixture was analysed by agarose gel electrophoresis. A 3-band and 4-band restriction pattern was identified as 105 Ile and Val, respectively. For *CYP2D6*, we used the approach described by Hersberger et al. [10]. As a quality control procedure, we repeated the genotyping of a random sample of 3% of our study population, which showed a 100% agreement with the original genotyping." (2.4, p.8)

1.6) As mentioned above, it seems difficult to attribute to a particular exposure what could be due to the others that are postulated to have the same mechanisms. This issue needs to be addressed.

R: This issue has been addressed in our analyses: analyses of ETS effects are restricted to non-smoking women, so that the reported parameters quantifying ETS effects on birth weight directly or in interaction with genotypes cannot be influenced by active smoking. The potential for ETS to confound the association between active smoking and birth weight (or any interaction between active smoking and genotypes) is much weaker because doses of tobacco smoke stemming from ETS are much lower than those due to passive smoking, and because of the weak reported effect of ETS on birthweight in our population. Concerning the interactions with exposure to fine particulate matter, analyses are adjusted for active and passive smoking, as reported in the methods section so that, again, the pattern of interaction between PM2.5 and genotypes cannot be explained by active or passive smoking. If we exclude active smokers (thus reducing the population size from n=354 to n=328), the statistical interaction between PM2.5 and GSTP1 polymorphism is still present (p=0.02).
We now mention this issue in the discussion section:
"We could control for a variety of potential confounders such as maternal size, pre-pregnancy weight, socio-economic status, active and passive smoking. As an alternative way to control for active smoking, we repeated the analysis among non-smoking pregnant, and the statistical interaction between $PM_{2.5}$ exposure and GSTP1 was still present (p=0.02), confirming that this statistical interaction was unlikely to be explained by factors known to influence birth weight." (4.1, p.15, l.35)

1.7) Page 6 states that authors investigated change of address but on page 7 they excluded those who did change address. Why was it not possible to assign exposures to all addresses?

Reply: It was not possible to assign exposure to women who moved out during pregnancy because only the maternal address at the time of birth has been recorded upon inclusion in the cohort; for women who changed address during pregnancy, the former address has not been recorded. Using the birth address for these women would create exposure misclassification, which is the reason why we preferred to exclude them. Note that in contrast to our study, most former studies on the subject usually had no information on changes in the home address during pregnancy, and therefore had to assume that all women did not move out during pregnancy, entailing a possible bias due to exposure misclassification. This issue has been discussed in our previous publication based on this cohort (Slama et al., *Env Health Perspect*, 2007).

Reviewer #2:

2.1) The manuscript by Slama et al. presents results from a population-based study in which gene-environment interactions for air pollution exposure on infant birthweight were examined. The strengths of the study reside in the examination of a robust and well-defined study population - the LISA cohort. The premise and study design are straightforward and the manuscript is well written overall, with conservative conclusions evolved from the study findings. The principle limitations center on the need for more informative Introduction and Discussion sections, and more detailed elaboration of the results (Tables 2 through 6).

The Introduction is exceedingly abbreviated and provides only a superficial overview of key concepts that the authors attempt to integrate in their study. The manuscript would benefit from more substantive introductory remarks on:
Air pollutants; definition, composition, forms serving as exposure sources in the LISA birth cohort - the study population under investigation; Fine particulate matter definition, sizes, relationship between environmental particulate matter and smoke related particulate matter.

R: We now expanded the introduction regarding fine particulate matter and its sources in an urban setting:
"In cities from industrialized countries, $PM_{2.5}$ are mainly derived from combustion processes such as road traffic, heating, biomass burning and specific industrial processes; additionally, a part of $PM_{2.5}$ are derived from the conversion of gaseous precursors such as sulphur or nitrous oxides, ammonia or volatile organic compounds [11]. In the city of Munich, about 60% of the local emissions of the larger PM_{10} stem from (road and train) traffic [12], a proportion that is probably higher for $PM_{2.5}$. $PM_{2.5}$ are a complex mixture composed of air-suspended liquid and solid particles. From a chemical point of view, in urban sites, $PM_{2.5}$ are mainly composed of secondary aerosol (ammonium nitrate and ammonium sulfate), organic matter (including many types of volatile, semivolatile and non-volatile compounds) and elemental carbon [13]." (p.5, l.41)

Regarding the study population, it is described in the methods section, which is, we believe, a more appropriate location than the introduction. Please see point 2.2) below regarding the relation between environmental $PM_{2.5}$ and smoke-related PM.

2.2) What is the relationship between air pollution related fine particulate matter and particulate matter associated with active smoking or environmental tobacco smoke exposure? Authors indicate "human exposure to air pollution occurs through the same pathway as exposure to tobacco smoke [active and passive smoke exposure]"..it is unclear what they authors mean by "same pathway". What are the commonalities..as well as the differences?

R: We have now detailed this part of the introduction:
"Human exposure to combustion-related air pollution occurs through the same pathway (inhalation) as exposure to tobacco smoke; these two mixtures also share similarities in terms of granulometry of their particulate component [14, 15] and also in terms of chemical composition. Indeed, tobacco smoke particulate matter has an aerodynamic diameter typically in the 50-800 nm range [15], which covers the 10-100 nm range, corresponding to fresh traffic-related PM [14]. Many families of combustions by-products such as volatile organic compounds (e.g., benzene) or polycyclic aromatic hydrocarbons are present in both mixtures. A notable difference is nicotine, which is present in tobacco smoke but not in traffic-related air pollution. In terms of effects on

reproductive function, maternal exposure to each of these mixtures has been associated with decrements in term birth weight in humans [16, 17]." (p.6, l.8)

2.3) What is the relationship between atmospheric particulate matter and xenobiotic metabolizing enzymes? Some perspective should be provided. What is the justification for the choice of the four genes investigated..are they of particular relevance to the type of particulate matter exposures incurred by the LISA cohort?

R: We have now expanded the introduction on this issue:
"The metabolism of xenobiotics can schematically be seen as a process in two phases; phase 1 usually corresponds to a functionalisation of xenobiotics, which makes them more electrophilic or nucleophilic, thus allowing a conjugation step, corresponding to the phase 2 of metabolism, which eventually leads to more hydrophilic compounds that can be more easily eliminated from the body than the parent xenobiotics. Examples of enzymes implied in phase 1 metabolism include the cytochrome P450 (CYP) superfamily. Within this superfamily, CYP2D6 belongs to the most polymorphic genes [18]. The Glutathione transferases (GST) are a family of enzymes implied in phase 2 metabolism of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), which are present in atmospheric pollution [19]. The cytosolic GSTs include 7 classes of polymorphic enzymes, among which GSTM1, GSTT1 and GSTP1. GSTM1 and GSTT1 null polymorphisms correspond to a lack of expression of the corresponding enzyme [19]." (p.4, l.35)

2.4) The authors note that only the offspring were genotyped in the present investigation. No information or discussion regarding the contribution of maternal genotype to detoxification mechanisms of air pollution/cigarette smoke is provided. Additional discussion of the role of maternal versus foetal genotype (xenobiotic metabolizing enzymes) on environmentally-induced adverse birth outcomes - particularly low birth weight - is warranted. How, if any, does the lack of maternal genotype information limit interpretation of the four genes modifying the effect of maternal exposure to particulate matter on offspring low birthweight? The authors need to clarify whether the metabolizing enzyme genotypes were determined in newborns or in six-year olds..there is an apparent discrepancy in the text.

R: Concerning the last point, genotyping was performed in blood samples collected when the children were 6 year old, as stated in the method section (section 2.4). This is unlikely to have induced bias compared to a situation in which genotyping would have been performed on biological samples collected at birth, because genetic characteristics vary little during short time periods.
So far, to our knowledge, only one gene-environment study on birth weight has simultaneously considered the genotypes of the offspring and of the mother, as shown in our table (Table 1) reviewing the gene-environment studies published so far. A correlation between the presence of heterozygosity in mothers and their children is expected, and for several of the considered genes, there is some evidence of an activity in the placenta or the foetus. Therefore, the current body of evidence does not allow to discuss the relative roles of maternal and foetal polymorphisms in xenobiotic-metabolizing genes. This issue is presented in the discussion section:
"We genotyped the newborns and not their mothers. *GSTP1* has been shown to be active in foetus [20]. Some correlation between maternal and foetal heterozygosity can generally be expected [21], so that heterozygosity of the offspring may be a proxy of maternal heterozygosity. In the absence of genotyping of the mothers, and even if the statistical interaction detected

corresponded to causal coactions, one should therefore refrain to interpret this interaction as bringing evidence for the offspring genes rather than the maternal ones being implied in the pathway between fine particulate matter exposure on foetal growth." (4.1, p.16, l.5)

The paragraph above also indicates that *GSTP1* (the gene for which a statistical interaction with $PM_{2.5}$ exposure has been highlighted) is active in the foetus [20], which means that not only maternal but also foetal enzymes could be implied in the detoxification of tobacco smoke or atmospheric pollutants.

2.5) There is a wealth of information in Table I relating to the premise and interpretation of the current study. However little of this information regarding prior findings on gene- environment interactions modifying risk of birthweight alterations following particulate matter exposure is discussed in the manuscript - begging the question as to why this has been included. Certainly the information is rich for the Introductory remarks or Discussion.

R: There is actually only one previous study describing gene-environment interactions in the context of particulate matter exposure for effects on birth weight (Suh et al., Reprod Toxicol, 2007). Our original idea was to present this study and other gene-environment studies on birth weight dealing with tobacco smoke exposure in Table 1 in order to maintain the text short. We have now expanded the introduction, detailing some of the results contained in the Table, without integrally duplicating this information:

"The latter were considered in only one study based on 199 births, in which two polymorphisms of *CYP1A1* gene were assessed. No interaction tests have been reported, but birth weight changes associated with PM_{10} exposure above the 90^{th} percentile (compared to below the 90^{th} percentile) were not clearly in favor of an effect measure modification by any of the polymorphisms considered (see [22] and Table 1 of this paper). Therefore, there is currently very limited direct evidence for genetic polymorphisms modifying the effect measure of air pollutants on foetal growth in humans. Some genes implied in the metabolism of xenobiotics have been shown to modulate the action of drugs in the body [4, 5, 23, 24], and they appear worth being considered in the context of the study of air pollution effects. (…) Some of these genes have been considered in gene-environment interaction studies on human birth weight in relation to maternal active or passive smoking. In addition to the cytochrome P450 (CYP) superfamily and the glutathione transferases (*GST*) family, *NQO1* (NAD(P)H: quinone oxidoreductase 1) and *EPHX1* (epoxide hydrolase 1) polymorphisms have also been considered (see Table 1 for a review). Hong et al. reported an increase in mean birth weight associated with exposure to passive smoking (ETS) in *GSTT1* wild genotype and a decrease in mean birth weight associated with ETS exposure in *GSTT1 null* genotype [25]. In a case-control study, the estimated effect of ETS on mean birth weight also tended to be stronger in the offspring of mothers with *GSTT1 null* genotype than with *GSTT1 wild* genotype [26]. No clear statistical interaction have been described for polymorphisms of *GSTM1* gene, and *GSTP1* and *CYP2D6* have so far very little been considered in this context." (p.4, l.11)

We also expanded the discussion, indicating that:

"Although quite imprecise, our results concerning GSTM1 were qualitatively in agreement with previous reports in which mothers had been genotyped in favor of ETS being associated with greater decrements in mean birth weight in *GSTM1 null* genotype, compared to *GSTM1 present* genotype [25, 26]." (4.2, p.16, l.1)

2.6) The manuscript itself needs a more substantive discussion regarding the fact that the LISA cohort is biased in that the cohort excludes birth outcomes wherein the birthweights are below 2500g (the accepted weight for low birthweight outcomes)..i.e. the linkage between particulate matter exposure/genotype and all low birthweight and very low birthweight outcomes would not have been assessed in the present study.

R: The exclusion of children born at term with a birth weight below 2,500 g is in principle an issue. We have discussed this point in our previous publication based on the same population (Slama et al., EHP, 2007), and developed a sensitivity analysis to correct for this exclusion (Slama et al., EHP, 2007). We used a similar approach in this new manuscript, and the result of the sensitivity analysis (presented in the appendix) again indicates that the exclusion of the children with a birthweight below 2,500 g is unlikely to have strongly biased our results. This may be explained by the fact that they only represent about 2.2% of all term newborns (see 2.6) below). In order to make these results more visible, we moved them from the online supplement to the main text.

2.7) The authors should address an apparent discrepancy in Section 4.4 wherein they cite the low birthweight incidence in the German population as less than 2.5% of term births. Incidence appears quite low; varied published statistics for the geographic area in Germany during the comparable time period of study suggest low birthweight rates approaching 6.5% of term births. Could this discrepancy affect their sensitivity analysis? In summary, the authors provide unique observations - garnered from a robust and informative study cohort - regarding the potential role of xenobiotic metabolizing gene polymorphisms in modifying risk of gestational particulate matter exposure on birth weight outcomes. The manuscript would make a significant contribution to the knowledge base in reproductive toxicology and environmentally based pregnancy risks, and would benefit from the revisions as noted.

R: We are not aware of publications indicating a low birthweight (<2500 g) rate of 6.5% among term births in Germany. This value is actually very close to the low birthweight rate among *all* births (including multiple births and preterm births); indeed, in Germany in 2004, the rate of low birthweight was 7.1% among all births, 5.2% among singleton births and 1.9% among live term singleton births, a population corresponding to the inclusion criteria in our cohort. Therefore, the rate of 2.2% of low birthweight babies among live term births assumed for our simulation appears very close to the national statistics (European Perinatal Health Report, appendix B, table C4, http://www.europeristat.com/bm.doc/appendix-b-data-tables.pdf). We now quote this source in the article:

"We conducted a sensitivity analysis assuming that low birthweight babies corresponded to 2.2% of singleton live term births, a figure based on the Eden mother-child cohort [27, 28], and close to the figure of 1.9% observed in Germany for the year 2004 [29]." (4.4, p.18, l.2)

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