Author’s response to reviews

Title: A Prospective Cohort Study of Biomarkers of Prenatal Tobacco Smoke Exposure: The Correlation between Serum and Meconium and Their Association with Infant Birth Weight

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Author’s response to reviews: see over
Author Responses to Teresa Gray’s Review

1. Abstract, Methods – This section seems repetitive and should be clarified.
2. Abstract, Conclusions – The conclusion is too vague for the data presented. The authors should limit the statement to say that meconium is a useful biological matrix for measuring environmental tobacco exposure. Given that meconium has been used to identify tobacco exposure for more than 20 years, it probably should not be considered “promising.”

We appreciate the reviewer’s suggestions on the abstract. We have modified the methods to be less redundant. We have also modified the conclusion to read as:

Conclusions: Meconium is a useful biological matrix for measuring prenatal tobacco smoke exposure and could be used in epidemiological studies that enroll women and infants at birth. Meconium holds promise as a biological matrix for measuring the intensity and duration of environmental toxicant exposure and future studies should validate the utility of meconium using other environmental toxicants.

3. Introduction, paragraph 1 – To my knowledge, there are no data to support the authors claim that meconium is metabolically inert, therefore the authors should add a reference or remove this statement. More importantly, recent prospective data demonstrates that drug exposure during the second trimester (as evidenced by maternal urinalysis) is not reliably reflected in meconium, thus the drug detection window of meconium spans just the third trimester. The authors are referred to Clin Pharmacol Ther. 2008 Nov;84(5):604-12.

We have referenced a citation by Bearer (Ambulatory Pediatrics, 2003) that provides a review of the evidence for meconium’s “inertness.” We have modified the introduction so that our statement about the properties of meconium is not perceived as fact.

Meconium may be metabolically inert and concentrations of drugs and other toxicants are thought to represent cumulative gestational exposure over the latter two-thirds of pregnancy [1].

4. Introduction, paragraph 2 – The authors should specify that the metabolic percentages given are for adult humans as the metabolic capacity of fetuses for nicotine and metabolites are unknown.

We have noted that the percentages are for adults.

5. Methods, Tobacco Smoke Measurements - Why were the self-report periods defined as conception – 20 weeks and 20 weeks – birth? Mid-point of gestation? Also, why were serum specimens collected at 16 weeks, 26 weeks and at birth? What if the women enrolled after 16 weeks; was a serum sample taken at enrollment?

The table below details the collection of exposure information. The periods related to the questionnaires were a function of the timing of questionnaire administration.

Women were enrolled between 13 and 19 weeks gestation at which time a serum sample was taken. Women could not participate if they were more than 19 weeks gestation (see Methods).
<table>
<thead>
<tr>
<th></th>
<th>16 Week Clinic Visit</th>
<th>20 Week Home Visit</th>
<th>26 Week Clinic Visit</th>
<th>Birth Hospital Stay</th>
<th>4 Week Post-Partum Home Visit</th>
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<tr>
<td>Serum Sample</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>Questionnaire</td>
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<tr>
<td>Meconium</td>
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How long was meconium collected (i.e. 48 h or until the appearance of milk stool)?

Meconium samples were collected during the birth hospital stay until a transition stool was seen (this sample was not collected). In most cases, this meant the first 48 hours worth of stools were collected. Samples were collected by nurses in the delivery center. We have added the below text to clarify this.

*In most cases, the first 48 hours worth of stool were collected, otherwise until the first milk stool appeared.*

Kohler et al demonstrated that nicotine and 3HC concentrations in meconium change in successive passages, so were all meconium specimens collected per child?

Meconium samples were pooled for each infant. We could not analyze each individual meconium sample because of logistic constraints.

6. The LOD reported for the serum assay was 0.015 ng/mL, a value approximately 100 times lower than what has been reported in recent literature by multiple investigators. Was this LOD established empirically (i.e. with decreasing concentrations of analytes fortified in matrix)?

The serum cotinine assay used in this study was conducted by the National Center for Environmental Health at the Centers for Disease Control and Prevention (CDC/NCEH) and has been published and is available on the CDC/NCEH website [2]. This method is based on standard analytical criteria and was recently revalidated as part of a published round robin study involving the several laboratories around the world [3]. Numerous other epidemiological studies, including other birth cohorts and the National Health and Nutrition Examination Survey, have used this laboratory method [4-5]. We are not aware what of recent LC/MS/MS methods for serum cotinine that have detection limits of 1.5 ng/mL, which would be of limited utility for SHS measurements.

7. The major limitation of this paper is that the authors do not adequately describe the analytical method used to evaluate meconium. Because the analytical method presumably has not been published, much more detail is necessary. From the given information, it appears that the LC-MS/MS method was not evaluated according to FDA or EU guidelines for bioanalytical methods and has significant deficiencies. The lack of details makes it impossible for the reader to assess the subsequent prevalence findings. The authors should address the following:

1) Which deuterated internal standards were used?
2) More details on the extraction procedure are necessary – particularly the solid phase procedure step. Reference 4 is a review article and does not describe a specific solid phase extraction procedure. Is this the correct reference? Please either give another reference or describe the SPE procedure.
3) What were the conditions for LC-MS/MS analysis, particularly what MS/MS scan mode was employed? Assuming MRM transitions were monitored, how many transitions were analyzed per compound? EU guidelines and most clinical and forensic applications utilize two transitions per analyte and one or two transitions for the internal standard.

4) Because this is a prevalence study, the authors must clearly define how the LOD was determined. Please describe what criteria were required for the LOD (the lowest concentration of an analyte that the bioanalytical procedure can reliably differentiate from background noise) and whether the assay had a separate lower limit of quantification (LOQ, the lowest amount of an analyte in a sample that can be quantitatively determined with suitable precision and accuracy.) If the authors do not have a separate LOQ, please explain why. Was the LOD evaluated empirically with a spiked meconium specimen to determine if a peak was discernible from noise? The reported LODs for cotinine and 3HC are nearly 10 times lower than other published meconium methods; how were the authors able to achieve such low limits and maintain adequate identification criteria?

5) The quantification procedure does not appear to follow normal clinical and forensic standards. Were calibration standards used? Here I am defining calibration standards as a series of samples of the same biological matrix to which a known amount of analyte has been added or spiked that is used to construct calibration curves from which the concentrations of analytes in QCs and in unknown study samples are determined. Was multi-point linear regression quantification curve generated? A single point calibration? A historical curve?

6) How did the authors confirm that their “blank” matrices were, in fact, blank? In our experience, finding negative meconium specimens can be rather challenging.

7) Why were the authentic QC materials treated differently than the unknown specimens? The authors use 0.5 g of meconium per assay for the unknown samples, but only 0.125 g of QC meconium per assay. Using 4 times less meconium for the QCs would affect extraction efficiencies, sensitivity and matrix effects. Also, QC samples were mixed for 2 hours; were unknown samples treated the same way? Were the authors concerned about analyte stability in the extremely alkaline environment?

8) Furthermore, how were the QC concentrations initially determined? Were the low concentrations near the LOD to challenge the accuracy of low concentrations? The RSD for the low nicotine concentration seems high, and while the rest had good precision, what was the accuracy of the concentrations?

We appreciate Dr. Gray’s detailed and thoughtful comments about our meconium assay methodology. We have added some details about the analytic methods to help the reader understand the approach, but refrained from adding all details requested to avoid losing focus on the epidemiological nature of this paper and this journal’s audience. The original purpose of this paper was to validate our meconium assay against serum biomarkers and self-report of tobacco smoke exposures. We are preparing a methodological paper that details the analytic approach of our meconium assay. We would be willing to provide a confidential draft version of this manuscript to address this myriad of questions. We defer to the editor’s judgment on the inclusion of more detail on our meconium assay.

8. Statistical analysis - The statistics section is excessively wordy. It is enough to say, for example, the three serum cotinine measurements were averaged and
women categorized as nonexposed (<LOD), secondhand exposed (LOD-3 ng/mL) and actively exposed (>3 ng/mL).

We appreciate this suggestion and have pared down the statistical analysis section. We have provided a high enough level of detail so that our analyses could be replicated by other investigators.

9. Why did the authors choose to impute values <LOD for geometric mean determinations? What values were imputed (0.5*LOD?).

Imputation was done to produce less biased estimates and standard errors. Simulation studies show that imputation provides better accuracy and precision when there are high proportions (>20%) of left-censored data [6]. The imputation method we used randomly assigns values to left-censored values using the log-transformed distribution of non-censored values and applies a correction factor that accounts for the proportion of censored data. This method is superior to substituting the LOD/2 or LOD/√2 in place of left-censored values.

Also, why did the authors choose to use a geometric mean over the arithmetic mean? Most literature on meconium reports the arithmetic mean or median + range of observed concentrations along with the % positive.

The geometric mean is preferable to the arithmetic mean when concentrations of biomarkers are log-normally distributed, as they were in our data. Arithmetic means will be biased by high values and may result in non-normal residual distributions in regression analysis. Median values will often be similar to the geometric mean, but confidence intervals for medians cannot be easily computed with standard software. We have provided estimates of central tendency (geometric mean) and precision (95% confidence intervals), as well as the proportion of samples detected. We have included an additional table (Table 2) that describes the minimum, maximum, median, and selected percentiles of serum and meconium metabolite concentrations.

10. Results, Paragraph 1/Table 1 –the authors should add statistical support (p-values, etc) to the comparisons between those with complete and incomplete data.

We followed established guidelines for reporting counts and proportions of participants with missing data (STrengthening the Reporting of OBservational studies in Epidemiology [STROBE], http://www.strobe-statement.org/). STROBE guidelines state that “Inferential measures such as standard errors and confidence intervals should not be used to describe the variability of characteristics, and significance tests should be avoided in descriptive tables” (Vandenbroucke et al. 2007). More specifically, p-values for comparisons of measured variables between participants with missing and measured data on other variables are uninformative about the precision loss or the potential for bias from the missing data.

11. Results, Relationship between self-report and biomarkers of tobacco smoke exposure – The prevalence of tobacco metabolites in meconium and serum was surprisingly high and contrary to other literature. In our experience, nicotine, cotinine and OHCOT have similar prevalence, but the authors found nicotine at much higher rates than cotinine and OHCOT. This is particularly interesting given that the LODs of cotinine and OHCOT are approximately 10X less than nicotine’s. Could the author’s please comment on these findings? Also, Kohler et
al. and Gray et al. showed that tobacco biomarkers were not present in meconium of babies whose mothers were nonsmokers or environmentally exposed. While previous research does show higher prevalence and concentrations in non- and passively exposed individuals, the analytical methodology was limited to non-specific immunoassay. Why do the authors think they observed such high rates? The 95% CIs for nicotine, cotinine and 3HC are very small. Do the authors have any theories as to why the concentration ranges were so similar between babies? In paragraph 3, the authors say that SHS-exposed babies have higher meconium concentrations than non-exposed – is this difference statistically significant?

We found nicotine more consistently and at higher concentrations than the other two metabolites. However, the nicotine measurements were also subject to more variance than COT and 3HC. Since our LOD estimates are not derived from simple signal-to-noise estimates but rather are based on a multiple of the observed variance measured for each analyte, the higher LOD for nicotine was not unexpected.”

With the exception of Kohler, the prior literature that has examined meconium tobacco smoke metabolites in SHS exposed infants has relied on self-reported measures. Self-report is notorious for underestimating SHS exposure in pregnant women and most studies examining SHS exposure and meconium metabolite concentrations have had very small number of exclusively SH exposed women. Some meconium metabolite concentrations were significantly higher in categories of SHS exposure relative to unexposed women.

We have added the following sentence to the discussion:

*Improved classification of prenatal tobacco smoke exposure in the later two-thirds of pregnancy using more sensitive laboratory methodology and serial serum cotinine measurements of prenatal exposure may explain why we observed a higher proportion of detectable meconium samples than previous studies.*

We are confident that our data met standard QA/QC criteria and that we have accurately reported our results. It is reasonable to question whether our results are right, but they appear to be internally consistent since meconium metabolite concentrations were positively associated with various serum cotinine characterizations and self-reported tobacco smoke exposure. We have added an additional cautionary limitation to our discussion:

*Finally, meconium biomarkers may require additional validation steps to ensure that they are accurately characterizing prenatal toxicant exposure. This final limitation will be essential in studying emerging toxicants like bisphenol A or phthalates.*

12. Table 2 –It is very difficult to believe that so many unexposed and SHS exposed children had detectable meconium concentration levels, given the targeted population and previous literature demonstrating no tobacco biomarkers in non-exposed and environmentally exposed infants. Also, the geometric means for the non and SHS exposed groups are so close to the LOD and have such narrow CIs (which may be skewed by imputing data), but the authors should comment.
The imputation method we used should provide less biased estimates of the central tendency of meconium and serum metabolite concentrations than other substitution methods (LOD/2 or LOD/√2).

We were also surprised that so many children had detectable meconium metabolite concentrations, especially among infants born to women with serum cotinine concentrations <LOD. However, most prior studies have been limited by self-reported tobacco smoke measures or small samples of secondhand exposed women.

Of course our meconium data could be incorrect. It would be foolish to disregard this possibility, but this is the case for all scientific studies, which makes subsequent replication with our meconium methodology essential to validating or refuting our results. Alternatively, we may have detected more analytes because of our more laborious meconium extraction and sensitive analytic procedure.

13. Table 3 – It is interesting that there appears to be no meaningful difference in birth weight between SHS and active exposure when defined by meconium or serum levels. One would expect more severe deficits among the actively exposed, but all seemed to be relatively healthy (>3200 g).

The magnitude of our point estimates for decrements in birth weight among SH and actively exposed infants are consistent with the prior literature. The 95% CI measures the precision of the estimate and does not reflect the actual distribution of birth weight values among infants in a particular exposure group. Infants born to women with serum cotinine levels consistent with active prenatal tobacco exposure showed greater decrements in birth weight than SHS exposed infants relative to the unexposed (Table 4). Point estimates for the actively exposed infants were very imprecise due to the small sample size.

Why did the authors differentiate SHS and active smokers at a 5 ng/g cutoff for cotinine and 10 ng/g for nicotine and OHCOT?

We based these cutpoints off of a sensitivity and specificity analysis that we conducted. This is stated in the text of the methods:

Several different meconium tobacco smoke metabolite concentrations were used to discriminate secondhand from active tobacco smoke exposure based on sensitivity and specificity analyses.

14. Discussion, paragraph 1 – the authors’ data also suggest that meconium concentrations may also reflect the timing of tobacco use, as concentrations were higher if the mother was deemed SHS-exposed at birth as compared to at 16 weeks.

We have added the following:

Tobacco smoke metabolites may accumulate in meconium differentially across pregnancy since the bulk of meconium is formed later in pregnancy.

15. Discussion, Paragraph 2 – again, the authors should comment on why they found much higher prevalence of tobacco biomarkers in meconium compared to other recent investigations (i.e. Pereg, Kohler and Gray), particularly among babies whose mothers were non-smokers or environmentally exposed.
Please see above.

16. Discussion, Paragraph 4 – The authors cite that Gray et al had similar COT and 3HC prevalence, but overall prevalence in their study was much lower (~35-40%) than the current study (~55-70%). Also, the authors attribute differences in prevalence between studies to analytical parameters, but another important component, namely the targeted study population, should be mentioned.

We appreciate this comment and have added the following:

Variations in study results could be due to differences in meconium digestion/extraction, analytical chemistry methods, or exposure characteristics of the targeted study population.

17. Discussion, Paragraph 5 – there are other factors which may influence meconium metabolite concentrations including the amount of meconium deposited in the gut, the time of meconium collection relative to birth (Kohler et al showed that concentrations of NIC and OHCOT varied over sequential meconium passages), extracorporeal urine contamination within the diaper. Also, the authors should provide a reference for the last sentence and summarize what other investigators have found and if this differs from what they observed.

The timing of collection and extracorporeal urine contamination should not play a role in the metabolite concentrations in our sample since all meconium stools were pooled. Urine contamination should be non-differential with respect to “true” exposure, so this will just add additional variability to our measurements. We have modified our statement in this paragraph to address concerns about meconium deposition in the gut.

We are not sure what reference to provide in the last sentence since we are referencing our own results.

18. Discussion, Paragraph 6 – why did the authors choose serum over another less invasive matrix such as maternal urine? Also, the cited references seem to support the previous paragraph, not the use of serum to identify and classify smoking status.

Maternal urine was available in our study, but we have not analyzed it for tobacco smoke metabolites. Serum measurements have been routinely used in the prior literature studying SHS exposure and birth outcomes, making our results more comparable with other studies.

19. Discussion, Paragraph 7 – The authors conclude that meconium is an adequate matrix for identifying prenatal tobacco exposure, but suggest using serum cotinine concentrations for quantifying prenatal exposures because meconium samples require additional resources. Could the authors please elaborate on the additional requirements? Meconium is more easily and less invasively collected than serum and tobacco biomarkers can be easily extracted from meconium with a simple solid phase extraction procedure without significant specimen pretreatment. The initial meconium digestion and initial liquid liquid
extraction performed by the authors could be eliminated without severely compromising detection of tobacco exposed neonates as Gray et al showed that hydrolysis of meconium with enzyme (analogous to the basic digestion performed by the authors) only identified one additional neonate.

We appreciate Dr. Gray’s comments on the matter of meconium collection. While the analytic methodology for meconium may be comparable to serum, the logistics of collecting meconium may impose additional burdens on large prospective epidemiological studies. Meconium collection will require that hospital staff is willing and able to collect and store samples properly. In addition, this will require that there be adequate freezer storage space on wards where infants are born. This may be particularly important to the National Children’s Study where samples will be collected in non-academic hospitals that do not routinely engage in research. We have added the following to our discussion:

Investigators may wish to use serum cotinine measurements to quantify prenatal exposure since collecting meconium samples will require hospital staff be able and willing to properly collect and store meconium samples.

20. Discussion, Paragraph 8 – What are the authors referring to by “associations among active smokers?” Relationships between dose-serum/meconium concentration, dose-infant outcome, concentration-outcome? Please elaborate.

We have modified the first sentence of that paragraph as follows:

The small number of actively smoking women in our sample limited our ability to precisely estimate relationships between prenatal tobacco smoke exposure and meconium tobacco smoke metabolites among active smokers.

21. Discussion, Paragraph 10 – Since the study population was of relatively high SES, which has lower active and SHS exposure, are the authors surprised at the nearly universal detection of cotinine in at least one serum sample?

We are not surprised by these results since tobacco smoke exposure is still prevalent in the US. Ohio did not enact a ban on smoking in public places until November 2006, around the same time our last mother gave birth. In addition to household exposures, women could have had tobacco exposure at work or in restaurants. Furthermore, our median serum cotinine concentrations are consistent with concentrations in non-smoking adults from the 2001-2002 NHANES (Pirkle 2006).

22. Discussion, Paragraph 11 – Are the authors suggesting that meconium concentrations can be used to predict the number of cigarettes (i.e. dose) the mother smoked? Because of the analytical deficiencies in the meconium method – namely the determination of LOD - it is very difficult to agree with the authors that nicotine in meconium indicates transient tobacco exposure.

We are not suggesting that meconium can be used to calculate the number of cigarettes a woman smoked or was exposed to. The number of cigarettes are still an exposure since there are additional factors that impact the received dose of active (depth of inhalation, nicotine content of cigarette, genetic/metabolic factors, etc.) and secondhand (proximity to smoker,
ventilation, nicotine content of cigarette, genetic/metabolic factors, breathing rate, etc.) tobacco smoke exposures. Meconium tobacco smoke metabolites are a better reflection of that dose than other measures like serum cotinine, number of smokers, or number of cigarettes since the meconium metabolite concentration reflects what actually made it into the fetal compartment.

23. Conclusion – “Additional research should determine meconium’s ability to measure gestational exposure to other environmental toxicants.” Please remove as it is not germane to this manuscript.

We respectfully disagree with Dr. Gray’s comment. There has been interest in using meconium in the field of environmental epidemiology for the last decade. Additional research with other toxicants is necessary, given that the National Children’s Study is proposing to use meconium to measure prenatal pesticide and tobacco exposures.

Minor Essential Revisions
1. Please change gm to g throughout.

Done.

2. Abstract, Results – the authors should specify that meconium tobacco smoke metabolite concentrations were inversely related with birth weight. As the sentence is currently constructed it is not clear what is inversely related.

Done.

3. Introduction, paragraph 2 – Why do the authors describe active smoking as environmental?

Some environmental epidemiologists define environmental factors as any non-genetic factor (Chapter 30 of [7]). To the fetus, toxicants that pass through the placenta are environmental since they are not endogenously produced.

4. Introduction, paragraph– The first sentence requires a reference.

Done.

5. Introduction, Paragraph 4, sentence 2 – please change the word “women” to “babies.”

Thank you. Done.

6. Introduction, Paragraph 5 – please move reference 17 to after National Children’s Study, as it seems as though the National Children’s Study is suggesting that the validation be conducted.

Done.

7. Methods, study sample – some inclusion criteria are listed twice. Please revise.
We have modified this.

8. Methods, Tobacco Smoke Measurements – Please clarify if the number of cigarettes smoked per day in the home was for the mother or her housemates. Why switch between ng/mL and pg/mL when discussing the %CV of the serum assay?

We have clarified the sentence about the number of cigarettes smoked by others in the home. We also switched to ng.

9. Table 1 – the marital status data does not add up correctly – 217+31=248 not 249. Also please add p-values.

Thank you for catching our math error. Please see above comment regarding p-values.
Author Responses to Theo de Kok Review

Minor comments/discretionary revisions
1. Although it is good to have an elaborate description of the data analysis procedures, these could be condensed to some extent.

We appreciate the reviewer’s suggestion and have pared down the statistical analysis section. We provided a high level of detail so that other investigators could replicate our statistical approach.

2. The authors indicate the relevance of taking genetic factors such as CYP2A6 into consideration, but did not perform these analyses. As this would not have been a major effort, it now appears as a shortcoming that could have been fixed quite easily.

Samples from our cohort have not been genotyped for budgetary reasons. Future work will examine the role of genetic polymorphisms in susceptibility to environmental toxicant exposure.

3. The authors conclude that meconium is a promising biological matrix for exposure assessment in future studies; do they advise to collect meconium samples in newly planned mother-child cohort studies, or do they still consider this to be ‘premature’?

We have reworded our discussion to be more forthcoming with our conclusion about meconium a biomarker. We believe that meconium is a useful biological matrix for tobacco smoke exposure. Additional validation will be required for other environmental toxicants, especially those with short half-lives. We believe meconium can be used successfully in studies that enroll women-infant pairs at birth, but may not be worth the additional effort in studies already collecting serum or urine biomarkers. We have modified our discussion to reflect this.
References