Author's response to reviews

Title: Personal Endotoxin Exposure in School Children with Asthma

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Author's response to reviews: see over
RE: manuscript MS 7240178905456155 entitled:
"Personal Endotoxin Exposure in a Panel Study of School Children with Asthma."

Dear Editor,

Please find enclosed the above revised manuscript for submission to Environmental Health as an original research article. We have submitted a tracked changes version and clean version of the revised manuscript. The Editor’s requested edits have been made.

The following is a detailed point-by-point response to reviewer concerns. The reviewer’s comments on this paper were helpful.

Sincerely,

Ralph J. Delfino, MD, PhD

Reviewer: Gert Doekes
Reviewer's report:
Main objectives of this study were to assess
(1) relations between personal measurements of airborne endotoxin and simultaneously measured endotoxin in the air at fixed sites in or outside the home, or at a central regional site at max 5-10 km distance;
(2) relations between endotoxin and air pollutants like EC, OC, PM2.5 and NO2 measured in the same samples, or simultaneously at the same sites;
(3) relations between personal endotoxin exposure levels and a number of home characteristics and other possible determinants.
The main answers to the present research questions appear to be that:

(1) personal endotoxin exposure is hardly correlated with ambient levels, and the latter can thus not be reliably used as proxy for personal exposure; the authors claim to have confirmed previous findings of Rabinovitch et al (JACI 2005) but now in a larger population.

(2) there are no clear correlation patterns between airborne endotoxin and other air pollutants – with some exceptions, like high correlations between indoor PM2.5 or EC and endotoxin. Personal exposure data however showed no clear and consistent correlations;

(3) only a few determinants like pet ownership and flooding damage to the home could be identified as significant determinants of personal endotoxin exposure levels.

General and major comments

Comment 1. Why would (1) be a relevant question? Practically all airborne endotoxin exposures in the occupational but also in the indoor home environment have been linked to locally restricted, usually indoor sources and activities. In contrast to traffic-related air pollutants, there is little a priori reason to expect a main impact of regional outdoor background levels on indoor home or school airborne endotoxin levels and on personal exposures. The only exception might be the effect of local sources like concentrated animal feeding operations or other intensive agricultural activities, but those should be only incidentally and locally of relevance, particularly in an urban or sub-urban area.

Response:

We have added several points related to this issue to the Discussion section (paragraphs 1 and 5).

The main study objective concerns determinants of personal exposures, and is a relevant because nearly all previous epidemiologic studies have relied on fixed site measurements of endotoxin (usually indoor) and could thus could be biased due to exposure error. We agree that a conclusion regarding ambient and personal endotoxin exposure is not particularly relevant to research methods concerning endotoxin itself, but it may have some relevance regarding potential impacts of regional sources on personal exposure. Although the finding for ambient endotoxin was expected (weak correlations with personal endotoxin), the analysis is nevertheless novel and fills a data gap. The findings for indoor and personal endotoxin are much more important and suggest that other microenvironments are important to assess.

Although associations for airborne endotoxin exposures have been linked to indoor sources and activities, a considerable amount of the variance remains unexplained, especially with regard to personal exposures among children with asthma. We hold the view that personal endotoxin exposures in our data resulted from multiple sources and complex time-activity-location parameters that are difficult to comprehensively identify, including regional sources in large urban areas such as the one studied. The few identified indoor home sources (dog and cat ownership) did not explain much of the variation in personal endotoxin exposure.

Comment 2. Linked to this: there is extensive experience from decades of organic dust
and endotoxin exposure research in occupational environments, that exposure assessment by personal monitoring gives practically always higher and much more reliable levels than ambient sampling. Apart from reasons mentioned in the MS (generation of ‘personal clouds’ due to someone’s own activities) the main reason is that ambient air sampling does not account for time activity patterns while endotoxin levels show very large spatial variation. The authors’ conclusion that ‘Fixed site measurements ……. do not adequately represent….’ (End abstract and begin Discussion and Conclusions) thus is not a remarkable new message. It seems highly unlikely that anyone with some experience in endotoxin research would seriously consider outdoor measurements at a central monitoring site at 5 km distance from a home or school as a useful proxy for either short- or long-term indoor exposure.

**Response:**
Again, we agree with the view that endotoxin from a central monitoring site would not be considered a useful proxy for either short- or long-term indoor exposure or personal exposure due to large spatial variation. We have tempered our conclusion to reflect that issue as suggested by Joanne Sordillo’s review since when we say fixed site measurements we are also referring to indoor-outdoor home sites (and so have changed the wording to be clearer). We have also moved up our discussion of relations of personal endotoxin to dog and cat ownership and the potential importance of the personal dust cloud. This now comes before the discussion of regional differences.

**Comment 3.** Results of Rabinovitch et al. were indeed confirmed, but not in a larger population, since a comparison with indoor and outdoor measurements around the home of the children could only be made for 14 children. In the larger group of 45 only data from the central regional measurement site were used – and as indicated above, it is unlikely that anyone would seriously consider to use these for short-term endotoxin exposure assessment.

**Response:**
Thanks for pointing this out. We have changed the wording in the 4th paragraph of the Background section. We agree again with the view concerning ambient endotoxin and have made that clear in the Discussion.

**Comment 4.** Were any time activity data available? Apart from personal activities affecting the personal exposure the time spent at home, at school, in traffic etc should be major determinants of the daily exposure. It is difficult to imagine that such information was not collected in a panel study like this.

**Response:**
Thanks for suggesting this addition since we did have this information and have added it to the first paragraph of the Discussion. We show the potential importance of other locations we did not monitor for endotoxin and for high levels of physical activity, which mostly occurred away from home.
Comment 5. Where in the indoor home environment was the sampling conducted: the living room, the bedroom, etc? Was there any data how much time the study participant spent in that location during the sampling period?

Response:
The indoor samplers were located in or near the main activity area of the home, usually the living room or family room (now added to Methods). We did not collect data on how much time the study participant spent in that specific indoor location during the sampling period.

Comment 6. Why would a relation be expected between other air pollutants and endotoxin (question 2)? Particularly typical traffic exhaust-related pollutants should not be associated since endotoxin is not a traffic exhaust pollutant. PM2.5 (or more likely, the unfortunately not measured PM10) might show a relation, especially in agricultural areas, where endotoxin may be associated with the non-traffic related fractions in airborne particulate matter.

Response:
We gave the reason in the text, but have added additional rationale from our research on particle size fractions:

“These observations might be attributable to re-suspension of fine and coarse dust laden with bioaerosols along nearby roadways, which also generate higher concentrations of the traffic-related pollutants, especially during periods of air stagnation and cool temperatures. We previously reported moderate correlations between coarse PM mass and PM$_{2.5}$ black carbon in the study region [24]. This potential source of endotoxin could lead to potentially high spatial variability in resuspended dust containing endotoxin between homes and between other locations near vs. far from busy roadways.”

Comment 7. A clear answer to question (3) also requires knowledge of time activity patterns – relations with home characteristics would only be expected if children spent much of their time at home – or more precisely, if most of the personal endotoxin exposure occurred at home.

Response:
We now report in The Discussion section that the percentage of time subjects spent in various locations. Subjects spent on average 73% of their time indoors at home.

Comment 8. The variable numbers of available samples and data are quite confusing and should be better explained. The description of the study design even tends to mask the fact that some analyses could only be done in much smaller subpopulations. This is mentioned in the discussion, but it would be much better to mention the n values also in the tables.

Response:
We have added the N values to the abstract by stating that endotoxin was measured in
“...a subset of 12 indoor and outdoor subject home sites (N=116 and 113 person-days with personal endotoxin, respectively).” We have also added N values to Tables 1 and 3. They are already presented in the Results text, Descriptive analyses, second paragraph.

Comment 9. Many of the descriptive data of Table 1 have already been published in ref. 16 (EHP 2006; 116, table 3 on page 1738) – with slight modifications probably being due to some missing filters in the endotoxin analyses (?). It may indeed be useful to show these values again in the present MS but it should be clearly indicated when they are essentially the same data as published in ref. 16.

Response:
We believe you are referring to the main difference, which is the column with N that is now included in Table 1. One difference is in the missing filters in the endotoxin analyses and the matching of air pollution data to this nonmissing endotoxin data. In addition, the exposure Table 3 in the exhaled NO paper shows Ns and missing observations based on the 372 pairs (83%) of collected exhaled NO measurements that were reliable. The N is the person-days of measurement used in the eNO regression models. Both factors lead to differences of the present with the previous MS. We now present descriptive statistics for the stationary sites based on single measurements that the Ns refer to rather than person-days of measurement (i.e., from merging personal data to the stationary site data).

Comment 10. Endotoxin extraction and analyses were performed with methods specifically developed/adapted for this study, and suggested to be validated (page 7, : ‘a rapid an thorough method ....’). Has this procedure been published? I miss important details like the composition of the extraction fluid, which has been show to be a major factor determining endotoxin yields from filters and also affecting results in subsequent LAL assays (see eg. studies by Douwes, Thorne, Milton, Reynolds and Spaan et al.). Also the extraction volume, dilution factor of extracts in the LAL assay should be reported.

Response:
We added details to the Methods section as requested.

We basically applied the method described by Mueller-Anneling (2004, ref 25) with a few modifications: Instead of vortexing, 37 mm Whatman quartz filters were transferred into pyrogen-free extraction tubes with 4 mL PFW. The tubes were loaded into a FastPrep® instrument (MP Biomedicals). The processing of the sample tubes at 6.5m/second for 60 seconds efficiently homogenizes the filter membrane. After rotating the extraction tubes for 30 min (Dynal Biotech®, speed 36) followed by 15 minute sonication (and clearing the PFW extracts of quartz fibers and particles by centrifugation), the undiluted supernatants were then directly used for endotoxin analysis. Instead of using PFW with 0.05% Tween, we decided to use PFW only: We are aware of previous studies that Tween 20 may improve the extraction efficiency for endotoxin in dust samples. However, the actual extraction efficiency of airborne endotoxins from filters after sampling is still unknown (Spaan 2008). Furthermore,
Tween 20 has been reported to interfere with the LAL assay reagents and may decrease its sensitivity and reproducibility (Spaan 2008).


Minor and specific

Comment 11. Background: P 3 line 4. : ‘Includes endotoxin’ suggests that endotoxin is a particle. Better might be: “may carry’ or ‘may contain' endotoxin.

Response:
This was changed to ‘may contain.’

Comment 12. P 3, line 8-10: It is not clear what this sentence exactly means: that Ryan et al showed (plural) that early life house dust endotoxin and traffic-related air pollutants showed a (positive?) interaction in their relations with the risk of persistent wheeze at age 3?

Response:
This was reworded as suggested.

Comment 13. P 3, line 12-13: ‘Between-home’ variation is exactly what the ‘many other studies’ wished to assess, and should thus not be included in this sentence starting with ‘However’ and mentioning objections against settled dust analyses.

Response:
Yes, that’s right. Between-home was deleted from this sentence.

Comment 14. P 4, line 10-11: the actual present panel with complete data was not larger but smaller than that of Rabinovitch et al (see point 3).

Response:
We have changed the wording in the 4th paragraph of the Background section.

Comment 15. M&M, page 6, line 6: These data were not used. (see point 8: the authors should in general more clearly explain the variable numbers in their data analyses)

Response:
That’s correct and we have clarified this point. We now say:
“Lack of motion at expected times (e.g. during known school periods), resulted in no monetary compensation to the subject for that day and resulted in the exclusion of data. This occurred on < 6% of person-days of follow-up. Additional filters were not assayed for endotoxin due to air sampler malfunction or problems with filters (10%). Out of 450 expected samples (45 subjects, 10 days per subject), we obtained 376 valid personal endotoxin measurements (83.6%).”

**Comment** 16. P 8, line 5: Correlations were different for Riverside and Whittier; these differed also in time of study. Were correlations also studied separately for the various study periods?

**Response:**
Riverside (2003) was studied in a different year than Whittier (2004) and for both sites, the study period was similar and encompassed the end of summer and early fall period, which in southern California, is still primarily a warm period. Subjects were studied in blocks of 10 contiguous days so a seasonal effect in this limited time period would not be expected in this data.

**Comment** 17. P 8, line 8: “data” (not the ‘distribution’) were log-normally distributed. Was a 10-log or a ln-transformation performed (this would be of relevance for interpretation of the axes of Figs 1 and 2).

**Response:**
We revised the sentence and now say natural log transformation was performed.

**Comment** 18. P 9: what is meant by ‘most nonsignificant’ predictors?

**Response:**
We now say “predictors with the largest p-value over 0.05.”

**Comment** 19. Results ; page 9, line 2: Is 0.58 indeed the median for the combined two populations? How could then the GM of Riverside be 0.58 and that in Whittier 0.28 (Table 1)?

**Response:**
Thanks for pointing this error out, which for the overall observations is 0.57. This overall median is dominated by Whittier, which has 282 observations (median 0.48) as compared with 94 in Riverside (median 1.01). We now present medians in Table 1 instead of GMs. The previous GMs were calculated without first adding 1.0. Nevertheless, adding 1.0 before LN transformation and then calculating the GM is less representative of the central tendency of the data than the median since a majority of endotoxin values are < 1.0.

**Comment** 20. It would be better to present either GM’s and GSD’s (which would fit for log-normally distributed data and analyses of ln-transformed data with parametric methods as done in the GLM analyses), or median and IQRs – suitable for
non-parametric analyses like Spearman correlation.

**Response:**
We now present medians and IQRs in Table 1 instead of GMs and GSDs.

**Reviewer:** Joanne Sordillo

**Reviewer's report:**

**Summary:**
Overall, this article would be a nice addition to the endotoxin exposure assessment literature. Revisions for this manuscript are outlined below.

**Major Revisions**

**Comment 1.** The conclusion in the abstract states “Fixed site measurements of endotoxin do not adequately represent personal exposure, including measurements in the home environment.” Although the authors do make a comparison between personal exposure measurements and home samples, the actual number of subjects in this comparison is relatively small, even if repeated measures were used (n=14 subjects with home samples). I recommend tempering the language in the conclusion a little bit, given the fairly small group of individuals used in the analysis.

**Response:**
We agree and have done so as discussed under Gert Doekes’ comments. We have tempered the language in the abstract, Discussion (first paragraph) and Conclusion.

**Minor Revisions**

**Comment 1.** The type of buffer used to extract the filters should be mentioned in the methods section. A number of published reports have shown that extraction buffer can have a significant impact on endotoxin measurements; therefore, readers may want to know exactly what was used here.

**Response:**
We decided to use PFW only (now added to the Methods text): We are aware of previous studies that Tween 20 may improve the extraction efficiency for endotoxin in dust samples. However, the actual extraction efficiency of airborne endotoxins from filters after sampling is still unknown (Spaan 2008). Furthermore, Tween 20 has been reported to interfere with the LAL assay reagents and may decrease its sensitivity and reproducibility (Spaan 2008).


**Comment 2.** Numerous variables were tested in the model building stage, using the backwards stepwise elimination technique. One of these was age of the child. It appears that age was dichotomized (13-18 years old vs. 9-12 years old). Why
was age dichotomized this way? It is worth mentioning why this cut-point was chosen.

Response:
We have added that we expected activities and thus exposures to differ between teenagers and younger children.

Comment 3. For tables 4 and 5, the meaning of the coefficients isn’t immediately clear at first glance. Adding a more descriptive label to the “adjusted coefficient” column (or maybe even to the title of the table?) may help clarify things a bit, so that readers understand what the coefficients mean right away.

Response:
Thanks, we added a more descriptive label (proportional change) to the title of the tables.

Comment 4. Dog and cat ownership reportedly confounded some of the other home characteristic estimates in models with personal endotoxin exposure as the outcome. (This was mentioned in the top paragraph on pg. 15, discussion section). However, models shown were adjusted only for personal temperature, relative humidity and study region. If cat and dog ownership were confounders, the authors may want to consider adjusting for these characteristics as well, with the results in a table in the appendix or supplementary file.

Response:
The results section on Table 4 explain that results were for crude models and presented results for the final selected model in the text for only cat and dog numbers, which were adjusted as in crude models for personal temperature, personal relative humidity and region. We added to the text the confounding effects of dog and cat numbers on the only other two variables that were significant in crude models (flooding damage and sex).

Comment 5. Did season influence the relationship between indoor and outdoor endotoxin levels?

Response:
See response to Gert Doekes’ comment 16 above.

Comment 6. Is it possible that wearing the personal exposure monitor backpack altered subjects’ activities, potentially affecting the endotoxin exposure levels? If so, the authors should mention this as a potential limitation in the discussion.

Response:
Yes, this is possible, especially when playing sports makes it impossible to safely carry the backpack. We have added this limitation.
Comment 7. What was the within subject variability in personal endotoxin exposure measures over the 10 day period of sampling? It would be a good idea to include some sort of estimate of within-subject variability in the results. (Readers may be interested to know how constant personal endotoxin exposures are from day to day).

Response:
This is a very good suggestion. We now state in the beginning of the Results section: “Within-subject coefficients of variation for personal endotoxin ranged from 69% to 224% (median 116%).”

Comment 8. Typo- Pg. 4 “In the present study we tested the consistency of the personal exposure assessment findings of Rabinovitch et al [14] using a larger cohort panel of 45 children with asthma followed over up to 10 days, and using home rather than school endotoxin measurements.” Should read “followed up over 10 days”

Response:
Thanks, the phrase was changed to “… followed over a period of up to 10 days.”

Comment 9. Typo pg. 4. Second sentence should read “decrease in expiratory lung function” (it is written as decreased).

Response:
The typo was edited.

Comment 10. Typo pg. 6. “There were a pair sibling subjects”….need to add “of” in sentence.

Response:
The typo was edited.

Discretionary Revisions
1. Did the authors have data on allergens? Although it is certainly interesting to study the correlation between air pollutant exposures (PM2.5, EC, OC, etc) and endotoxin, allergens have a greater potential to confound associations between endotoxin and health in epidemiological studies. (Some sources of allergens are also sources of endotoxin; dogs and cats, for example. Also, damp conditions can increase dust mite populations, and have also been linked to increased microbial levels). If the authors do have allergen data for personal exposure measurements, it would be a good idea to add it to the manuscript.

Response:
We do not have airborne allergen data.