Author's response to reviews

Title: Effects of Endotoxin Exposure on Childhood Asthma Risk are Modified by a Genetic Polymorphism in ACAA1

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Responses to Reviewer Comments:

REVIEWER 1

Reviewer #1, Comment #1: Sample size must be considered a rather limited factor since only 95 asthma cases and 196 ctrls (from the two studies) are included in the final analyses. This is also reflected by the fact that despite a very significant p=0.003 for interaction between exposure and rs156265 for asthma, the OR fails to be significant in individuals with at least one copy of the minor allel (95%CI 0.15-1.04). Sample size has discussed to some extent, but it needs to be underlined that this is a rather small study after all.

Response: We agree that sample size is a significant limitation, and have added the following to the limitations paragraph at the end of the discussion (pg. 13):

“Even after combining two separate cohorts, the total number of children with complete genotype, endotoxin and health outcome data was relatively small. Although we were able to detect a significant gene by environment interaction, sample size may have reduced our power to detect additional interactions between endotoxin and other genetic polymorphisms.”

Reviewer #1, Comment #2: How were the 95 cases and 196 ctrls selected from the original 505 infants in the Boston study and 1002 families in the Yale study? This is a very small subset of the original study participants and the selection process must be described in more detail. Risk of selection bias? Also, it might be worthwhile to point of that these studies are “high-risk” since inclusion criteria included one parent or one older sibling with asthma or allergy. Does this affect the possibility to generalize the results to a broader population?

Response: We selected all children with complete data on endotoxin exposure, genotype and health outcomes as subjects for our analyses (this included 95 asthma cases and 196 controls).

In the methods section, on pg. 5, we state:
“There were 291 children with endotoxin exposure assessment, SNP genotyping data, and health outcome data.”

Then, in the results, on pg. 8, we have added the following:
“Of the 291 children with available data on endotoxin exposure, genotyping and health outcomes, 95 were identified as asthma cases, and 196 were controls who did not have asthma.”

We agree that selection bias is an important issue to investigate in this study. We were most concerned about potential bias resulting from genotyping only a subset of each cohort. To examine this further we created a supplemental table which compared two subsets of children (white children without genotyping vs. white children with genotyping). These comparisons were done for both the Home Allergens and Yale cohorts.

The supplemental tables show no differences between those with and without genotyping. These tables (supplementary table 3 and 4) have been added to the manuscript’s supplemental file.

We added the following to the Results section (under the population characteristics section, pg. 8) to address the issue of selection bias.

“Although a subset of white subjects had genotyping, the characteristics of this subset (sex, maternal/paternal history of asthma and eczema, day care attendance, and pet ownership) were similar to those for the total number of white subjects enrolled in each cohort (see online repository, tables 3 and 4).”
We were less concerned with selection based on endotoxin exposure assessment (88% of subjects with genotyping had endotoxin exposure assessment also). We did not detect any differences between sex, maternal asthma/eczema, paternal asthma/eczema, day care, or pet ownership for those with vs. without endotoxin exposure assessment.

Lastly, we added the following statement to the discussion (pg. 13) to reflect the fact that we have used high risk cohorts in these analyses.

“The use of high risk cohorts in this study may limit the generalizability of our results to populations that are not high risk.”

Reviewer #1, Comment #3: Asthma was defined as parental report of doctor’s diagnosis during first 6 y of life. This is a well established definition, albeit rather unspecific (many early wheezers may be included). Total IgE was also measured but no interaction was found related to this outcome. Since endotoxin exposure also has been associated with reduced risk of atopy, it would be valuable to see interaction results also for specific allergic sensitization (I assume these data are available in the studies).

Response: We agree that examining specific allergic sensitization would be valuable. Although the Home Allergens study does have specific allergic sensitization data, the Yale Cohort study unfortunately does not. The sample size for examining gene by environment interactions and allergic sensitization in Home Allergens only is simply too small.

Reviewer #1, Comment #4: Adjustments were done for maternal asthma, day care, income and breastfeeding. How were these potential confounders selected? Adjustment for exposure to passive smoking? Sex? Boys seem to have higher asthma risk.

Response: We chose to adjust for common early life exposures and environmental variables that are associated with asthma (day care, breast feeding and income), as well as one of the strongest predictors of childhood asthma (maternal history of asthma). We agree that sex is an important risk factor for childhood asthma, and have updated the models to include this variable as well (see new estimates and revised legend in tables 2, 3 and 4). Sex is also a potential confounder, as male gender is associated with increased endotoxin exposure (Sordillo et al Environ Health Perspect. 2011 Feb;119(2):189-95). In response to a suggestion from Reviewer #2 we also adjusted for cohort in the models.

The statistical analyses section of the Methods (pg. 7 to 8) has been updated to reflect the addition of sex and cohort to the models. Tables 2, 3, and 4 have also been updated to show additional adjustment for sex and cohort.

Passive smoke exposure was relatively rare in both cohorts (less than 10%), and did not confound our estimates in our models (estimates were exactly the same). Therefore, we did not include this covariate in the final models. (For comparison between Odds ratios for asthma both with and without adjustment for passive smoke, see table on next page).
**Interaction between Rs156265 and Endotoxin in Logistic Regression Models for Asthma**  
(Unadjusted model vs. Model Adjusted for Passive Smoke Exposure)

| Gene   | SNP † | Genotype | OR (95% CI) for quartile increase in endotoxin  
|   |   |   (Unadjusted) | p-value for Interaction  
| (Unadjusted) | OR (95% CI) for quartile increase in endotoxin  
|   |   |   (Adjusted for Passive Smoke Exp.) | p-value for Interaction  
| (Adjusted for Passive Smoke Exp.) |
|---|---|---|---|---|---|---|
| ACAAl | Rs156265 | CC | 1.21 (0.94 to 1.56) | 0.003 | 1.21 (0.94 to 1.56) | 0.003 |
| | | CG/GG | 0.43 (0.17 to 1.08) | | 0.43 (0.17 to 1.08) | |

**Reviewer #1, Comment #5:** I assume CD14 genotypes are available in these studies as reported in Litonjua, JACI 2005? Are CD14-exodoxin interaction analyses significant in these studies? Although the CD14 SNPs fall outside the inclusion criteria for SNP selection here (associated with asthma or eczema as presented in Sharma et al, submitted), it would be valuable to see if one of the most replicated GxE interactions in the asthma-allergy field (CD14-endotoxin) can be observed also in this study. Replication must be considered gold standard not only in genetic association studies, but also in interaction studies. Thus, replication and validation of GxE interaction results, as well as meta-analyses, are needed in this field.

**Response:** Thank you for this suggestion. It is true that CD14 genotypes were associated with eczema in early life (diagnosis by age 2), in a prior analysis of these two cohorts. Interestingly, this association did not emerge in the most recent genetic analysis that considered a different eczema phenotype (eczema by age 6). Based on reviewer #1’s recommendation to examine the CD14 SNPs further, we did some exploratory analyses, looking at how endotoxin exposure interacts with CD14 genotype to alter eczema or asthma risk by age six. The results of these exploratory analyses are shown below. We did not find any significant interaction for endotoxin and the CD14 SNPs. However, we may be underpowered to examine these additional SNPs (which is why our main focus was to look at any SNP with a significant main genetic effect).

**Early life endotoxin exposure and asthma by age 6: Effect modification by genetic polymorphisms in CD14† (P values for interaction term shown)**

| Gene | SNP † | Genotype | OR (95% CI) for quartile increase in endotoxin | p-value for Interaction  
| (SNP*Endotoxin Quartile) |
|---|---|---|---|---|
| CD14 | rs2569190 | CC | 1.12 (0.69 to 1.80) | 0.73 |
| | | CT/TT | 1.01 (0.37 to 2.81) | |
| CD14 | rs5744455 | CC | 0.93 (0.69 to 1.26) | 0.32 |
| | | CT/TT | 1.18 (0.55 to 2.52) | |
| CD14 | rs2569193 | CC | 1.17 (0.86 to 1.59) | 0.31 |
| | | CT/TT | 0.87 (0.40 to 1.85) | |
| CD14 | rs5744441 | CC | 0.93 (0.69 to 1.25) | 0.27 |
| | | CT/TT | 1.19 (0.56 to 2.51) | |
Early life endotoxin exposure and eczema by age 6: Effect modification by genetic polymorphisms in CD14† (P values for interaction term shown)*

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP †</th>
<th>Genotype</th>
<th>OR (95%CI) for quartile increase in endotoxin</th>
<th>p-value for Interaction (SNP*Endotoxin Quartile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD14</td>
<td>rs2569190</td>
<td>CC</td>
<td>1.01 (0.65 to 1.54)</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT/TT</td>
<td>1.04 (0.41 to 2.62)</td>
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</tr>
<tr>
<td>CD14</td>
<td>rs5744455</td>
<td>CC</td>
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<tr>
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<td></td>
<td>CT/TT</td>
<td>1.07 (0.51 to 2.24)</td>
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<tr>
<td>CD14</td>
<td>rs2569193</td>
<td>CC</td>
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<td>0.54</td>
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<td></td>
<td>CT/TT</td>
<td>1.08 (0.52 to 2.25)</td>
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<tr>
<td>CD14</td>
<td>rs5744441</td>
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<tr>
<td></td>
<td></td>
<td>CT/TT</td>
<td>1.06 (0.51 to 2.18)</td>
<td></td>
</tr>
</tbody>
</table>

**Reviewer #1, Comment #6:** Figure 2 and 3, please add 95% CI to the asthma frequency numbers to get an estimation of the precision of each point estimate.

**Response:** The 95% CI for the asthma frequency estimates have been added to the figures.

**Reviewer #1, Comment #7:** In the Abstract, Results, I recommend to delete “term”, leaving only “(p=0.003 for interaction)”

We have deleted the “term” in phrase interaction term in the abstract.

**REVIEWER #2**

**Reviewer #2, Comment #1:** Methods: Multivariate regression was adjusted for several covariates. However "cohort" was not one of the covariates listed in the adjusted models, and should be included. Authors state in their results that "the effect of cohort did not alter the observed interaction"; therefore including it in the models should not alter the results.

**Response:** We agree. We have now added “cohort” as one of the covariates in the models. Please see updated Tables 2, 3, and 4. Selection of model covariates is also addressed in response to Reviewer # 1, comment # 4. Since cohort was added to the adjusted models within the manuscript, we removed the analysis stratified by cohort at in the supplemental file of the manuscript.

**Reviewer #2, Comment #2:** Figures should be numbered in the order they appear in the text. Currently fig.2 is referenced first and should become Figure 1. The current figure 1 should become Figure 2 (furthermore, figures 1 and 3 could become Figure 2A and 2B, since both depict LD plots for the same two SNP).
Response: Thank you for catching this. We have taken this suggestion, and re-ordered the figures as recommended above. (Figure 1, Figure 2A+2B to show LD plots, followed by Figure 3).

Reviewer #2, Comment #3: Table 1: While subjects were all enrolled within a relatively narrow time period and should therefore be all of similar age, "Age" should be included in the baseline characteristics of the cohort.

Response: We agree that age is typically an important baseline characteristic listed in many prospective cohort studies. However, for this analysis, children were followed from birth until age 6, and disease incidence was captured some time before the age 6 time point. Since the dataset used was anonymized, we were unable to calculate exact ages (plus or minus 3 months within age 6) of follow-up for the children.

Reviewer #2, Comment #4: I did not see mentioned how many cases and controls come from each of the cohorts. It should be stated. This could explain why (in the stratified analysis in Supplemental Table 4) the Yale cohort shows a much more pronounced "protective" OR and a significant interaction value, whereas the Home Allergens cohort does not.

Response: Thank you for this recommendation. We have added the following lines to the results section on pg.8.

“Approximately half of the asthma cases and controls came from each cohort (48 asthma cases and 100 controls from the Yale cohort; 47 asthma cases and 96 controls from Home Allergens). In the Yale cohort, there were 89 eczema cases and 51 controls; in the Home Allergens cohort, 73 cases and 67 controls. The percentage of eczema cases (of total eczema cases) from the Yale cohort was higher (55%) than the percentage of eczema cases (45%) from Home Allergens (p= 0.053 for Chi Square).”