Author's response to reviews

Title: Early life microbial exposure and fractional exhaled nitric oxide in school-age children: a prospective birth cohort study.

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Author's response to reviews: see over
Dear reviewers,

Please find enclosed the revised version of our paper entitled "Early life microbial exposure and fractional exhaled nitric oxide in school-age children: a prospective birth cohort study".

In accordance with your comments, we have thoroughly revised our paper. The changes are marked in the manuscript. In this document you will also find a detailed report of the actions that have been taken in response to each of your comments.

We would like to thank you for your valuable suggestions. These comments have surely enhanced the quality of our manuscript.

Yours sincerely,

Lidia Casas
Answer to the reviewers’ comments

Reviewer 1

This manuscript communicates an interesting and topical study on the effects of early-life indoor microbial exposure on FeNO levels later in childhood. FeNO is considered a marker of eosinophilic airway inflammation, and is increasingly used in the diagnosis and management of asthma, but so far there have been few studies of how FeNO is influenced by microbial exposures, especially of indoor origin, that have been shown to be associated with allergic airway diseases. The manuscript is well-written and well organized, and the methodology appears to be appropriate. As discussed by the authors, the main limitation of the study is the heterogeneity of the three cohorts included.

SPECIFIC COMMENTS

Minor essential revisions:

1. Although the authors refer to the ATS guidelines in the methods section, a brief description of the FeNO measurements should be included. Were the FeNO values based on a single measurement, or the mean of several? Were children prescribed asthma medications excluded from the study?

Thank you for your comment. We have included the requested information in the “Fractional exhaled Nitric Oxide (FeNO)” section of the Methods section (pages 7 and 8). Now it reads:

“FeNO was measured in LISA at 10 years of age, in PIAMA at 8 years of age [43] and in INMA at 10 to 13 years of age, according to the American Thoracic Society guidelines [44] using the NIOX MINO® in LISA and INMA and the NIOX analyser (Aerocrine, Solna, Sweden; http://www.aerocrine.com) in PIAMA. In LISA and INMA children were refrained from eating or drinking one hour before the measurement. They were asked to inhale to near-total lung capacity through the NIOX MINO®, and to exhale immediately at a constant flow rate of 50 mL/sec until a NO plateau of at least 3 seconds could be identified during an exhalation of at least 6 seconds. Measurements in both cohorts were performed until a correct FENO measurement was displayed. If necessary, the FENO test was repeated to obtain one acceptable measurement.

In PIAMA, children were instructed to inhale to near-total lung capacity through a NO scrubber (Dräger combination filter, Dräger, Lübeck, Germany), integrated in the NIOX analyzer, and to exhale immediately at a constant flow rate of 50 mL/second, until a NO plateau of at least 2 seconds could be identified during an exhalation of at least 4 seconds. A maximum of 6 attempts were performed to obtain three acceptable FENO measurements. FENO in PIAMA is expressed as the average of the 3 measurements.

In the LISA cohort children who had taken any anti-asthmatic or anti-inflammatory medication did not perform the test. In PIAMA and INMA the use of anti-asthmatic medication (in the past 24 hours in INMA, in the past 48 hours in
PIAMA) or anti-inflammatory medication (in the past 24 hours for INMA and PIAMA) was recorded. Four children in PIAMA and 49 in INMA had taken anti-asthmatic or anti-inflammatory medication. In the three birth cohorts, all values were above the LOD. Children did not have any active upper or lower respiratory tract viral infection on the day of the measurement.”

In addition, we have added a paragraph to the statistical analyses section referring to the treatment of individuals who took anti-asthmatic medication before the test (page 12):

“For additional sensitivity analyses, we excluded individuals who had taken anti-asthmatic or anti-inflammatory medication during the 48 or 24 hours previous to the FeNO measurement.”

Finally, we have included the results of the mentioned sensitivity analyses in the results section of the manuscript (page 15):

“Finally, the exclusion of individuals who had taken anti-asthmatic or anti-inflammatory medication in the 48 or 24 hours previous to the FeNO measurement did not modify our results.”

2. Were any of the measurements below the LOD for the NIOX MINO device? If so, how were those measurements treated statistically?

We did not have any children with measurements below the LOD. We have included a sentence in the Methods section (see the response to the previous comment).

3. The combined random-effects adjusted coefficient for endotoxin and its 95% CI given in table 3 do not agree with the text in the abstract and in the results section (p. 13).

We apologize for this. We have changed it. Now all values are consistent across the abstract, text and tables.

Discretionary revisions:

4. On p. 15 the authors state that FeNO was higher in asthmatic and allergic children, but the data are not shown. Since this is a key finding that validates the measurements in any study of eNO levels, I suggest adding these data to the results section, e.g. in Table 2.

We followed the reviewer’s suggestion and included these data in Table 2.

5. For the sake of clarity, the authors may want to rephrase the last sentence on p.14 (Similarly, no statistically significant associations…….). E.g. “In this study, no statistically significant associations were found…..”. As the authors mention in the introduction, some recent studies have found associations between early exposure to mold and later asthma development. What could be the reason for
the conflicting results of the various studies including this one? The authors might want to add some discussion about that.

We agree with the reviewer’s comment and modified the mentioned paragraph as follows:

“Regarding the exposure to fungal agents such as EPS or glucans, our study did not find statistically significant associations with FeNO. These results are in line with those obtained in previous studies on the association of EPS and glucan levels with respiratory symptoms [6, 21]. However, previous studies have reported increased risks of asthma during childhood in association with mould exposure [15–17]. In our study, we did not find an association between early life reports of home dampness and FeNO at school age. The lack of associations in our study could be explained by the fact that our exposure was parental-reported dampness and not objective observation. However, results on previous studies regarding early life exposure to dogs are in line with our findings. Dog ownership was suggested to be inversely associated with allergic sensitization and respiratory symptoms [18, 19, 50].”

6. The authors state on p. 15 that there were no differences in the associations between endotoxin and dog exposure and FeNO according to the report of asthma or allergy. In the manuscript, the results for all study subjects are compared to the results for the non-asthmatic subjects as shown in Table E3. However, since only a small number of the study subjects were asthmatic, it is to be expected that the results for the all study subjects and the results for non-asthmatics would be very similar. Were coefficients obtained for the group of asthmatic children alone? If not, was this due to limited power?

Thank you for your comment. As you suggest, the power was extremely limited to calculate coefficients for asthmatic children per cohort as, for example, in LISA, only 10 children (6%) were classified as asthmatic. The birth cohort with the highest number of asthmatic children was INMA (n=33; 10%) (see table 1).

We calculated the coefficients for asthmatics in INMA. They were not statistically significant and the direction of the effect estimate was the same as shown for non-asthmatic children. For example, the adjusted coefficient for endotoxin concentrations was -0.14 (95%CI: -0.4; 0.08).

We also calculated the coefficients for asthmatics in pooled analyses additionally adjusted for cohort. The number of included children was 70. The adjusted coefficient for endotoxin concentration was -0.16 (95%CI: -0.3; 0.01).

Nevertheless, we would rather not show these results in the manuscript because they may induce the reader to confusion. The power was very limited to draw conclusions from the results per cohort and the differences between cohorts were big enough to choose a meta-analyses technique instead of pooled analyses.
Reviewer 2

Overall comments

In this study the authors have investigated early life exposures (mould and pets) on exhaled nitric oxide (FeNO) in childhood. The paper is well written and the design, in the main, is sound. The analyses seem appropriate and the authors have mostly interpreted the results adequately. However, I have some concerns over the use of FeNO as a single outcome measure for the purpose of the study as well as the lack of inclusion in the analyses of some important factors that can affect FeNO, or at least the lack of acknowledgement of the potential importance of these factors.

Many studies have investigated the associations between environmental exposures and FeNO but none, that I am aware of, have investigated this so distant from the exposure (8 – 10 years). This makes the idea novel but given the number of factors that can influence FeNO in both the short and long term it is harder to justify the observed associations. This is particularly so as I would suggest the understanding of what FeNO represents, particularly in the general (ie those not necessarily with resp disease) population is still uncertain (see below). The time factor does not make the concept wrong and the authors have tried to account for some of the factors that could also affect FeNO, including asthma and allergy. However, some important factors have not been assessed or acknowledged: at least it is not clear if they have or not. These include, but are not restricted to, atopic sensitization (independent of allergic disease), current exposures, season of testing and current medications. Some of these data may be available.

For this study FeNO is being measured as a non-invasive biomarker of eosinophilic inflammation (implied in last paragraph of Introduction and stated explicitly in the 1st paragraph of Discussion). It is very much an indirect marker and a number of uncertainties about FeNO, NO biology and eosinophilic airway inflammation remain. Although there is reasonably, but not totally, consistent evidence of a moderate correlation between FeNO and eosinophils, what FeNO truly reflects is still not known (this is nicely summed up by Teague JACI 2010; 125: 1234). Indeed some of the responses of FeNO to environmental exposures are not always consistent with the eosinophilic inflammation, with which FeNO is most closely related. The authors do focus on inflammation throughout the text but correctly imply that both the biological and clinical implications of their findings, if any, are not known

Major Compulsory Revisions

1. Important data that can affect FeNO are either missing or not included in the analyses. If they are available they should be assessed and if not they should be acknowledged. I am aware that there are potentially many things that may have some effect on FeNO and I am not suggesting that everything that has been published needs to be considered. The authors state that ‘Potential confounders were a-priori identified from the literature and selected based on their relationship with FeNO and the exposure variables in the present study.’

We appreciate your comment. We have included a list with the potential confounders that have been tested in this study in the supplementary material and referenced it in the methods and results sections. Also, we have included a table in the Online Repository with the effect estimates for the associations between FeNO and endotoxin and early life dog ownership after the additional association for each of the potential confounders that are not included in the model (see table E2 in the Online Repository).

We have included additional information to the “potential confounders and effect modifiers” section in the methods sections (pages 10 and 11). Now it reads:

“Atopy was assessed in 74% of the study population. Specific immunoglobulin E (sIgE) was measured in blood in the three cohorts at different time points: 10 years of age in LISA, 8 years of age in PIAMA and 4 years of age in INMA. In LISA, atopy was defined as sIgE of at least 0.35 kU/l for ‘sx1 inhalant mixture’ (timothy, rye, mugwort, house dust mite (*Dermatophagoides pteronyssinus*, Der p), cat, dog and mould mixture). In PIAMA, atopy was defined as sIgE of at least 0.35 kU/l for house dust mite (Der p), cat, dog, birch, *Alternaria alternata* and *Dactylis glomerata*. Finally, atopy in INMA was defined as sIgE ≥0.35U/mL of IgE for Der p, cat or grass/pollen. A list of all the potential confounders evaluated in this study is provided in the Online Repository.”

In addition we have included a short report of the results after additional adjustments at the 4rth paragraph of the results section (page 14). Now it reads:

“These associations were adjusted for sex, age at FeNO measurement, asthma, parental indoor smoking at the time of the FeNO measurement, and parental education. Models including microbial agents measurements were additionally adjusted for season of dust sampling. Additional adjustment for other potential confounders such as season of FeNO measurement, asthma or anti-inflammatory medication prior to the FeNO measurement, outdoor NO2 at school age or ever moving to another home did not modify the effect estimates. Additional adjustment for atopy resulted in an attenuation of the effects. Also, the effect estimates for endotoxin and early life pet ownership became statistically non-significant. The effect estimates for endotoxin and early life dog ownership after these additional adjustments are shown in Table E2 in the Online Repository.”

Finally, the list in the Online Repository reads as follows:

- “Potential confounders evaluated in our study:
  - Sex.
  - Age at FeNO measurement.
  - Reported allergies (hay fever, rhinitis and eczema).
  - Parental smoking at the time of the FeNO measurement.
  - Parental education.
• Season of dust collection (winter, spring, summer or fall).
• Ever moving to another home between the dust sampling (early life) and the FeNO measurement.
• Atopy: specific IgE (see methods section).
• Having cat(s) or dog(s) in the home at the age when measurement of FeNO took place
• Modelled annual average levels of NO\textsubscript{2} at the home address at the age when FeNO measurement took place. These levels were obtained from land-use regression models described in detail elsewhere [2].
• Season at the time of the FeNO measurement (winter, spring, summer or fall).
• Use of asthma or anti-inflammatory medication in the 24hr or 48hr prior to the FeNO measurement.

Important factors that I was unclear about and feel should have been included in the analyses include;

• An objective measure of atopic sensitization: The authors have taken into account reported allergy but atopy per se may also be important. It is possible that many of the non-allergic children are SPT positive (atopic). Indeed it could be up to 25%.

Unfortunately, SPT was not available in all three cohorts. However, we had information on specific IgE levels in 74% of our study population at the age of 10 years in LISA, 8 years in PIAMA and 4 years in INMA. We dichotomized these variables into two categories (<0.35U/mL vs ≥0.35U/mL). A description of the methods and results is included in the revised manuscript. The results of these additional analyses are also included in Table E2 in the online repository (see the response to the previous comment)

• Current exposures: There are many studies of current environmental exposures and FeNO, some which were referenced in the Introduction. These include, among other things, air pollution and current allergen exposure. Different air pollutants have been associated with increased FeNO while current allergen exposure has been shown to increase FeNO in sensitised children (these are referenced in the manuscript - ref 34, 35, 37). The only ‘current’ exposures mentioned in the manuscript were location of home and parental smoking (it is not clear if it is current smoking or ever smoking). Even pets are classified as none, ever in 1st 2 years, ever after 1st 2 years but there is no measure of pet ownership at time of FeNO test.

Air pollution: we have included outdoor NO\textsubscript{2} measurements obtained from land-use regression models in our models. The levels of NO\textsubscript{2} were significantly associated with FeNO in PIAMA and INMA but not in LISA. Nevertheless, the effect estimates of the exposures assessed in our study did not change (see the response to the first comment and Table E2 in the Online Repository). Because this variable does not change our results, we will not show our results including this adjustment in the main tables.
Current allergen exposure: unfortunately, we do not have information on current allergen exposure other than the report of cat and dog ownership. We have included this information in the models, none of these reports were significantly associated with the outcome and their inclusion in the models did not modify the effect estimates.

Parental smoking: the model is adjusted for current parental smoking. We agree that this is not clear in the manuscript and we have added this information to the foot notes of Table 3 and Figure 1 in the manuscript and Tables E2 and E3 in the supplementary material.

• Season: This may be particularly important for those who had seasonal rhinitis for the reasons stated above (relationship between sensitization and exposure)

As we did with the previous suggested variables, we have included the season of FeNO measurement in the models and we have not found a significant change of our effect estimates (see the response to the first comment). The results are now shown in Table E2 in the Online Repository

• Medications: Data on asthma medication was available and used to define asthmatics but it is not clear if they were also included in analyses. What about treatment for rhinitics (nasal steroids)?

Thank you for your comment. Asthma medication in the 24 or 48 hours before the FeNO measurement was recorded in INMA and PIAMA respectively. The use of nasal steroids in the last 24 hours was recorded in PIAMA, children of these birth cohort that are included in our study did not take nasal steroids previous to the FeNO measurement.

In LISA, children that had taken asthma medication in the hours previous to the measurement were rescheduled to perform the measurements on another day.

This information and how these data were treated are explained in detail in the response to the first comment of the previous reviewer and in the methods and results sections of the manuscript.

2. The differences in FeNO for the different levels of exposure seemed to be small and were mostly of borderline significance. It is possible that these were purely statistical results with no biological or clinical relevance. This possibility is not raised

We agree with your comment. We have included a paragraph at the end of the discussion section, before the conclusions. It reads as follows:

“Finally, because the differences in FeNO across the different levels of exposure were small we must consider the possibility that our findings are due to a statistical association without a biological basis. Nevertheless, the consistency between our findings with dog ownership only in the first two years of life and endotoxin exposure supports the biological basis of our findings.”
Discretionary Revisions

1. I suggest more caution is used when discussing FeNO as a biomarker of eosinophilic inflammation and some of the uncertainty around this be acknowledged.

Thank you for your comment. We have included a few sentences in the discussion section (page 17) to acknowledge the limitations of the use of FeNO:

“The association between the biochemical events involved in the release of NO in the airways and the inflammatory mechanisms needs to be further studied [56], but, at present, FeNO is, the most extensively studied biomarker of airway inflammation and its non-invasive nature makes it suitable for epidemiological studies [27, 44, 55, 57].”

2. In the 3rd paragraph of the Discussion the authors state ‘FeNO is one of the few tests that has diagnostic value in asthma.’. I don’t agree and even the ATS guideline that is referenced has acknowledged that their recommendation that ‘FeNO may be used to support the diagnosis of asthma’ is a weak recommendation based only on moderate quality of evidence.

We have changed that. Now it reads:

“FeNO is a test that may be used to support the diagnosis of asthma.”