Responses to reviewer 1

Comments and Suggestions for Authors

The authors reported their experience on the relationship between airborne pollutants concentration and DNA damage in the buccal mucosa cells of pre-school children.
They retrospectively evaluated 152 children who lived in Brescia, Northern Italy.
They couldn't find any significant association between air pollution and DNA damage.
Overall, the paper is interesting.

Some concerns should be addressed by the authors.

The analyses were carried out in the saliva of children aged 3-6. Since the authors are interested in air pollution, I was wondering why they did not evaluate the volatile organic compounds or the exhaled breath condensate in this patients' cohort. In my opinion it would have been more appropriate than the saliva examination. Could the authors comment on that?

With this regard, these two papers might be considered and possibly cited by the authors. They might be also considered these techniques in the introduction and discussion of the manuscript.

- *Bianchi F, Riboni N, Carbognani P, Gnetti L, Dalcanale E, Ampollini L, Careri M. J Pharm Biomed Anal. 2017 Nov 30;146:329-333. Solid-phase microextraction coupled to gas chromatography-mass spectrometry followed by multivariate data analysis for the identification of volatile organic compounds as possible biomarkers in lung cancer tissues.*
- *Corradi M, Poli D, Banda I, Bonini S, Mozzoni P, Pinelli S, Alinovi R, Andreoli R, Ampollini L, Casalini A, Carbognani P, Goldoni M, Mutti A. Exhaled breath analysis in suspected cases of non-small-cell lung cancer: a cross-sectional study.J Breath Res. 2015 Jan 29;9(2):027101. doi: 10.1088/1752-7155/9/2/027101.*

The aim of this study was to investigate the genotoxic effects of air pollutants using comet assay to detect primary DNA damage in salivary leukocytes of pre-school children living in a town with heavy air pollution. It is known that among air pollutants there are substances with mutagenic activity. We chose to focus on a biomarker of DNA damage in children because some data suggest that genetic damage occurring early in life may influence the risk of carcinogenesis and other chronic diseases in adulthood more than later damage does. In this study we investigated biological responses rather than symptoms or diseases. The use of a direct measure of biological effect, i.e. DNA breakages, which can be detected a long time before clinical disease develops and has already been shown to be predictive of cancer development later in life, can give us useful information about the substances to which children are exposed. Salivary leukocytes and mucosa buccal cells are an excellent target tissue for detecting early genotoxic effects induced by mutagenic airborne compounds introduced into the body by inhalation. The study of these biomarkers suggests an exposure. Moreover, the collection of these cells is a simple and non-invasive method, easily acceptable even by young children. Finally, it must be remembered that the study subjects are not patients but healthy children. In fact, the parents of the participating children did not report any respiratory diseases in the answers to the questionnaire.

The study of exhaled is certainly a very interesting approach to study childhood asthma and exhaled VOC profiles but it detects something very different from what we wanted to investigate.

We accepted the reviewer's suggestion and cited this type of studies in the Discussion section and included some related articles in the list of references.

In the text the following sentences have been added:

“This study focused on a biomarker of DNA damage in children because some data suggest that genetic damage occurring early in life may influence the risk of carcinogenesis and other chronic diseases in adulthood more than later damage does. The use of a direct measure of biological effect, i.e. DNA breakages, which can be detected a long time before clinical disease develops and can give us useful information about the substances to which children are exposed.” (lines 157-161)

“New and interesting approaches that use exhaled, a non-invasive technique now used above all for diagnostic purposes, could also have applications in vitro tests and be combined with other biomarkers to evaluate the risks in children of future respiratory diseases [56-59]. The non-invasiveness is an important aspect in preventive studies involving children. Leukocytes from saliva as well as epithelial cells of the oral mucosa, representative of epithelial respiratory tract cells, were easy to collect and especially in the pediatric population a collection more easily acceptable by children and their parents, which favored participation in the study.” (lines 233-241)

In the list of references the following articles have been added:

Corradi, M.; Poli, D.; Banda, I.; Bonini, S.; Mozzoni, P.; Pinelli, S.; Alinovi, R.; Andreoli, R.; Ampollini, L.; Casalini, A.; Carbognani, P.; Goldoni, M.; Mutti, A. Exhaled breath analysis in suspected cases of non-small-cell lung cancer: a cross-sectional study. J Breath Res 2015, 9(2):027101. doi: 10.1088/1752-7155/9/2/027101.

Bianchi, F.; Riboni, N.; Carbognani, P.; Gnetti, L.; Dalcanale, E.; Ampollini, L.; Careri, M. Solid-phase microextraction coupled to gas chromatography-mass spectrometry followed by multivariate data analysis for the identification of volatile organic compounds as possible biomarkers in lung cancer tissues. J Pharm Biomed Anal 2017, 146, 329-333.

Ferraro, V.; Carraro, S.; Bozzetto, S.; Zanconato, S.; Baraldi, E. Exhaled biomarkers in childhood asthma: old and new approaches. Asthma Research and Practice 2018, 4, 9. doi: 10.1186/s40733-018-0045-6.

Bannier, M.A.G.E.; Rosias, P.P.R.; Jöbsis, Q.; Dompeling, E. Exhaled Breath Condensate in Childhood Asthma: A Review and Current Perspective. Front Pediatr 2019, 7:150. doi: 10.3389/fped.2019.00150.

The authors did not use "pure saliva" but they took samples of mouthwashes with mineral water. The authors should comment on that and explain the reasons of such a choice. Did the authors know the effect (if any) of mineral water on fine particulate matter? What about distilled water?

The children rinsed their mouth with mineral water before spitting to eliminate epithelial cells and any food residues that could annoy the Comet test. We have used mineral water for rinsing before spitting because numerous laboratory tests have shown that mineral water does not damage the cells and does not give artifacts. Although the use of physiological solution is usually recommended, we preferred to use mineral water because it is more tolerated than physiological solution by young children (3-5 years), and therefore allowed a better collaboration by participants in the sample

collection. The collected spit was then added to the physiological solution. However, the time of contact of cells with the mineral water was minimal because the samples were processed in a very short time after sampling. Distilled water, on the other hand, can not used with this sampling method because it breaks the cells.

The authors should clearly stated the number of samples per patient they collected during the study period. The authors reported that "the children rinsed their mouths twice with mineral water and the mouthwashes were collected...". Did they perform this procedure only one single time per patient? If so, I think it should have been repeated several times. Could the authors comment on that?

The RESPIRA study design involved 200 pre-school children, aged 3-6 years, to evaluate markers of early biological effect, such as primary DNA damage evaluated with the comet assay in salivary leukocytes and induction of micronuclei (MN) in exfoliated buccal cells to investigate the genotoxic effects of air pollutants (for the MN test see Ceretti et al., 2014). As comet test detects DNA damage caused by recent exposure, the associations between DNA damage and air pollutants were analysed using air mean levels of polutants at 1, 2 and 7 days before sampling. The biological sampling was conducted only once because of practical difficulties in collecting splitting samples by very young children, in line with other studies investigating the association between air pollution exposure and biological damage in the general population. However, we agree with the reviewer that repeating the sampling in different times could provide useful information on variability of the relationship between cellular damage and air pollution. Indeed, we investigated the relationship between air pollution exposure and early biological effects in different seasons and years in another study (see Feretti et al., Monitoring air pollution effects on children for supporting public health policy: the protocol of the prospective cohort MAPEC study. BMJ Open 2014;4:e006096. doi:10.1136/bmjopen-2014-006096), the results of which have been published or submitted for publication.
A comment on this point has been added to the text (lines 225-229): “Another limitation of the study is that biological sampling was carried out only once for each child, in line with other studies investigating the association between air pollution exposure and biological damage in the general population [25,26,44,46,54]. The sampling repetition in different times could provide useful information on variability of the relationship between cellular damage and air pollution.”

Why did the authors choose january-march and not november or december? Why didn't they take samples once a month?

The RESPIRA study started in spring 2011. The ethics committee approved the study protocol in August 2011, we identified the urban pre-schools in which to conduct the study and we contacted teachers and other school personnel and organized meetings with parents to describe the study from September to December. All the samplings had to be carried out in winter, characterized by high concentrations of air pollutants in our town. However, we had also to take account of weather conditions, as all samplings had to be done after some consecutive days with absence of rain, snow and wind. Therefore, we completed the collection of all environmental and biological samples in January, February and early March.

I was wondering why the authors decided to publish these data after so many years. Could the authors comment on that?

Other parts of this study have already been published (e.g. the data on the MN in the mucosa buccal cells of children and the results of environmental genotoxicity (see Ceretti et al., 2014 and Feretti et al., 2019). At the end of this project, another more demanding project has started funded by the European Program Life plus. This new project has completely taken our time and our attention and the data regarding the comet test in children of the Respira study have been pending so far. Anyway, we still think that these data may of interest for the scientific community, since few data on biomarkers of early biological damage by air pollution in very young children are available so far.

How many of these patients developed an allergic asthma? Did the authors look at that? In my opinion that might be a very interesting point for people studying air pollution.

The children's parents were interviewed using an *ad hoc* questionnaire designed to gather information on exposure to air pollutants from both indoor and outdoor sources and children's respiratory diseases and drug consumption. At the time of the study, no children were suffering from asthma. In this regard the following sentence has been added in the text (lines 125-126): "None of the participants had asthma or other respiratory disorders investigated with the questionnaire."
The RESPIRA study was a cross-sectional study and therefore no investigation was planned in the subsequent years for these children. However, it is of note that asthma has no relationship with the genetic damage we investigated in our study according to current knowledge.

Did the authors follow-up those patients? They will be 10-13 years old at the moment. So it might be very attractive to know their state of health.

The reviewer's suggestion is appealing. Although the RESPIRA study was a cross-sectional study, we are really considering the hypothesis of going on with the study and contacting again the participant children to evaluate their health status, given that we have the financial resources.

The authors should report the approval of the study by the local Ethical Committee.

The project was approved by the Ethics Committee of the Brescia Local Health Authority/Lombardy Region on 10th August 2011 (with number of general protocol 0112487). Approval of the ethics committee was mentioned in the submitted manuscript. Now also the indication of the date and the protocol number has been added (line 87).