



Ozone exposure leads to changes in airway permeability, microbiota and metabolome: a randomised, double-blind, crossover trial

To the Editor:

The associations between atmospheric ozone pollution and increased risks of respiratory diseases have been well established [1, 2], but the underlying biological mechanisms are not fully ascertained. Lung epithelial cells may be injured by inhaled ozone, but results have not been fully consistent [3, 4]. Furthermore, respiratory microbiota and metabolic homeostasis have been deemed to be key factors in maintaining human respiratory health, and any disturbances in this balance have the potential to increase susceptibility to respiratory infectious diseases [5]. However, few human studies have investigated the potential effects of ozone inhalation on respiratory microbiota and metabolome.

To address these gaps, we conducted a randomised, double-blind, crossover, controlled exposure trial in 30 healthy young adults to explore the respiratory effects of short-term ozone exposure. The sample size was calculated using an equivalence test, and was comparable to that in previous controlled exposure studies [4, 6–8]. Each subject was exposed sequentially to both filtered air and 200 ppb ozone continuously for 2 h in a random order. A washout period of at least 2 weeks was determined according to previous controlled exposure trials [8, 9]. Lung function was measured according to the recommended methods [10]. As a well-established biomarker of lung epithelial injury, serum club cell protein (CC16) was measured using enzyme-linked immunosorbent assays. Respiratory microbiota was analysed with the nasal secretion using 16S rRNA amplicon sequencing. Airway metabolome was analysed with the exhaled breath condensate (EBC) using gas chromatography-time of flight-mass spectrometry (GC-TOF-MS).

We applied linear mixed-effect (LME) models to estimate the acute effects of ozone exposure on lung function and serum CC16. For microbiota analysis, we first evaluated alpha-diversity by calculating abundance-based coverage estimator (ACE), and Simpson and Shannon indices; then, we assessed beta-diversity by calculating weighted Unifrac distance and visualising it *via* principal coordinate analysis; lastly, we identified taxa characterising the differences between ozone and filtered air groups using a linear discriminant effect size analysis, and a taxon with a linear discriminant analysis score >4 was considered significantly different between two groups. For metabolomics analyses, we first conducted an orthogonal partial least squares discriminant analysis and used the variance importance in the projection (VIP) scores to define contributions of each metabolite to the overall between-group difference; then, for metabolites with a VIP score >1, we used LME models to evaluate the differences between groups. All tests were two-sided and a p-value <0.05 was considered statistically significant. The study protocol was registered at ClinicalTrials.gov (NCT03697174), and all participants provided written informed consent at enrolment.

Ozone concentrations in the chamber were very close to the target values, with an average of 201.0±1.6 ppb in the ozone group and 8.0±2.6 ppb in the filtered air group. The levels of fine particulate matter, nitrogen dioxide, temperature and relative humidity were quite similar between the two groups. Relative to the filtered air group, exposure to ozone resulted in significant declines in lung function at lag 2 h, and the decrements became more prominent at lag 15 h in the next morning. At lag 15 h, there were decrements of 3.70% (95% CI 0.58–6.82%) in forced vital capacity (FVC) and 3.14% (95% CI 0.02–6.30%) in forced

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Ozone inhalation could lead to depleted diversity of respiratory bacterial community, imbalanced proportion between commensal and pathogenic bacteria, and elevated levels of glucose and its metabolites in respiratory tract <https://bit.ly/2JS9OJQ>

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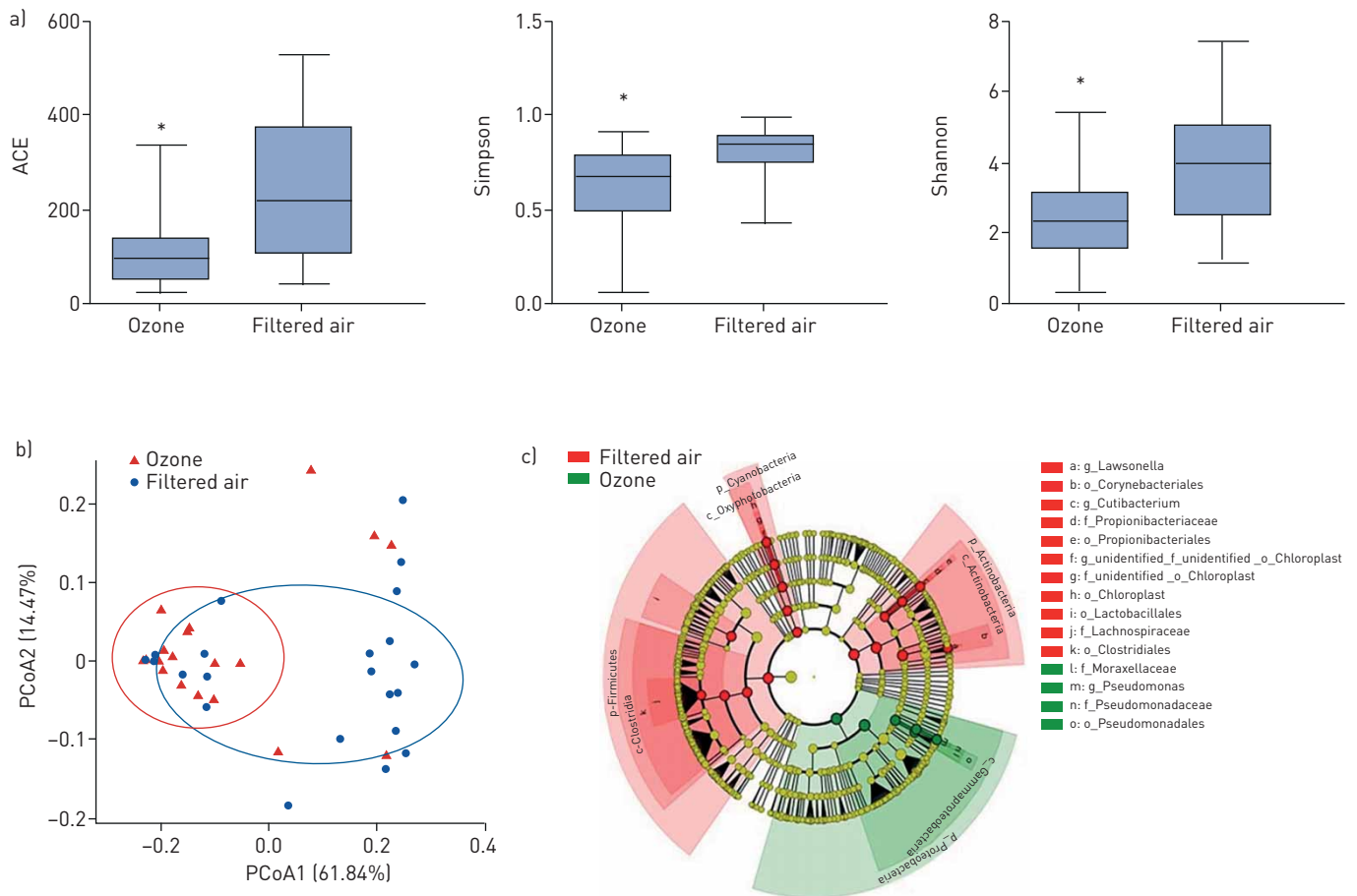


FIGURE 1 Changes in the diversity and composition of nasal bacterial community comparing the ozone group to the filtered air group. a) Alpha-diversity metrics of nasal bacterial community in the two groups, including abundance-based coverage estimator (ACE), Simpson, and Shannon. *: $p < 0.05$ versus the filtered air group. b) Beta-diversity of nasal bacterial community in the two groups. The circles refer to the 95% confidence ellipses. c) The cladogram of nasal bacterial community from a linear discriminant effect size analysis. The red area represents significantly enriched taxa in the filtered air group; the green area represents significantly enriched taxa in the ozone group; and the yellow area represents no differences in taxa between the two groups. The central point represents the root of the tree (bacteria), and each ring represents the next lower taxonomic level (from phylum to genus). The diameter of each circle represents the relative abundance of the taxon. PCoA: principal coordinate analysis.

expiratory volume in 1 s (FEV_1). The findings were consistent with previous chamber studies that revealed delayed impairment of lung function after acute ozone exposure [6, 7].

Impaired lung function was always accompanied by increased epithelial permeability, which can be indicated by elevated serum levels of CC16 [4]. Similarly, we found serum CC16 increased by 41.93% (95% CI 31.96–51.89%) 2 h after exposure to ozone compared with filtered air, and then the effect attenuated considerably but remained significant at lag 15 h. Elevated serum CC16 levels at lag 2 h was significantly correlated with decrements in both FVC and FEV_1 at lag 15 h, suggesting a potential temporal pattern: from ozone exposure, increased epithelial permeability to impaired lung function.

Notably, we presented, to our knowledge for the first time, the human-based evidence that short-term ozone exposure could significantly decrease the diversity of nasal bacterial community. As shown in figure 1a, ACE, Simpson and Shannon were significantly lower in the ozone group than in the filtered air group, suggesting distinct reductions in both nasal bacterial community richness and evenness after ozone inhalation. Meanwhile, we observed a clear separation between the ozone group and the filtered air group in the figure 1b ($p = 0.007$), indicating an evident difference in nasal bacterial community between groups. Some *in vitro* studies also demonstrated the anti-microbial potential of ozone [11]. Diminished diversity of bacterial community in upper respiratory tract has been associated with several respiratory infectious diseases in clinical studies [12, 13]. In addition, we found the composition of nasal bacterial community was altered by ozone inhalation. Specifically, we observed significant decrements in the relative abundance of two phyla, *Actinobacteria* and *Firmicutes* (figure 1c), also known as harmless commensal bacteria in nasal capacity. The

decreases in the two commensal bacteria may result in diminished resistance against the colonisation of foreign pathogens, and thus increase risks of bacterial or viral infection in the respiratory system [5]. In contrast, at the family level, we observed relative enrichment of *Moraxellaceae* and *Pseudomonadaceae* following ozone exposure (figure 1c), which were often involved in the development of pneumonia and other infectious diseases [12]. Therefore, it is reasonable to assume that the imbalance between commensal and pathogenic bacteria in the upper respiratory tract induced by ozone exposure may lead to individual vulnerability to respiratory infection.

The colonisation and proliferation of bacteria in the respiratory tract may also be influenced by some substances in airway surface liquid. In this metabolomic analysis with EBC, we found that glucose was significantly elevated after an inhalation of ozone relative to filtered air with a fold change (FC) of 1.59, accompanied by significantly increased D-glyceric acid (FC 1.27) and lactic acid (FC 1.15). The alteration of airway glucose metabolism by ozone was in accordance with a previous study [8]. Increased glucose in EBC possibly resulted from an enhanced leakage of glucose from the blood into the airway through impaired lung epithelial tight junctions, which could be supported by an elevation of serum CC16 in this study. Higher level of airway glucose may potentially enhance the proliferation of some specific bacteria that take glucose as carbon source by providing nutrient [14, 15]. This kind of imbalanced overgrowth may lead to perturbation in bacterial community composition, and ultimately increase the risk of respiratory infection.

This randomised, double-blind, crossover trial has the advantage of allowing for causal inference on the associations between ozone and adverse respiratory outcomes. However, this study has several limitations. First, the sample size is relatively small, adding statistical uncertainty to our results. Second, we only enrolled healthy college students as the subjects to better control behavioural risk factors, but this strategy might restrict the generalisability of our findings to vulnerable populations. Third, some differential metabolites in EBC might have been missed because we only performed GC-TOF-MS analyses due to the limited amount of EBC. Fourth, the microbiota and metabolome were analysed with samples collected immediately after exposure, which did not allow for an exploration of temporal relationships.

To conclude, this randomised, double-blind, crossover, controlled exposure trial revealed that an acute inhalation of ozone could impair lung function and increase airway permeability. Our results further provided a novel mechanistic insight that ozone inhalation may increase susceptibility to respiratory infection through disturbing microbiota and glucose homeostasis in the respiratory tract.

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The study protocol was registered at ClinicalTrials.gov (NCT03697174). Individual participant data that underlie the results reported in this article will be shared, after de-identification, beginning 6 months and ending 24 months following article publication, for those investigators whose proposed use of the data has been approved by an independent review committee and in order to perform individual participant data meta-analysis. Proposals should be directed to the corresponding author.

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