ENVIRONMENTAL HEALTH PERSPECTIVES
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http://dx.doi.org/10.1289/ehp.1307240
Received: 18 June 2013
Accepted: 15 April 2014
Advance Publication: 18 April 2014

NIH National Institute of Environmental Health Sciences

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Acknowledgments: We would like to thank the following for their contributions: Isabelle Poirier, Hervé Casterman, Stéphanie Billot-Bonef, Monique Bonnet-Belfais, Elisabeth Pigeon, Sabine Maestri, Bastien Vandercasteele, Loubna Alavoine, Laurent Nardelli, Alexia Boulmeau, Ludovic Dejoie, Yann Retory, Solène de Ferrières, and David Ormandy.

Independent Statistical Analysis: All statistical analyses were performed by Serge Koscielny, PhD, Service de biostatistiques et épidémiologie, Gustave Roussy, Villejuif, France.

Declaration of all sources of funding: The study was supported by the Center for Research & Innovation in Gas and New Energy Sources, CRIGEN, GDF SUEZ, the Medical Studies Department of EDF, and the Clinical Center of Investigation at Bichat Hospital, Paris, France.

Patrick Mure, Luc Ropert, and Sandra Tokarek are employed by GDF-SUEZ, Center for Research & Innovation in Gas and New Energy Sources CRIGEN, St-Denis, France.

Véronique Ezratty, Gaëlle Guillossou, Pierre-André Cabanes, and Jacques Lambrozo are employed by EDF, Service des Etudes Médicales (SEM), Levallois-Perret, France.

The rest of the authors declare no competing interests.

The authors' freedom to design, conduct, interpret and submit for publication the research described in this work was not compromised by any of the funding bodies.

Abstract

Background: Nitrogen dioxide (NO_2), a ubiquitous atmospheric pollutant, has been reported to enhance the asthmatic response to allergen through eosinophilic activation in the airways. The effect of NO_2 on inflammation without exposure to allergen is poorly studied.

Objectives: We investigated whether repeated peaks of NO_2, at various realistic concentrations, induce changes in airway inflammation in asthmatics.

Methods: 19 nonsmoker asthmatics were exposed at rest in a double-blind, crossover study, in randomized order, to 200 ppb NO_2, or 600 ppb NO_2, or clean air for 1x30 min day 1, and 2x30 min day 2. The three series of exposures were separated by 2 weeks. Inflammatory response in sputum was measured 6 hours (day 1), 32 hours (day 2), and 48 hours (day 3) after first exposure and compared to baseline measured twice 10 to 30 days before.

Results: Compared to baseline, the percentage of eosinophils in sputum increased by 57% after 600 ppb NO_2 (P=0.003) but did not change significantly after 200 ppb. The slope of the association between the percentage of eosinophils and NO_2 exposure level was significant (p=0.04). Eosinophil cationic protein (ECP) in sputum was highly correlated with eosinophil count and increased significantly after exposure to 600 ppb NO_2 (p=0.001). Lung function assessed daily was not affected by NO_2.

Conclusions: To our knowledge, this is the first study on repeated NO_2 peaks performed without allergen exposure that demonstrates a dose-related effect on airway eosinophilic inflammation in asthmatics.

Introduction

Nitrogen dioxide (NO_2), an ubiquitous atmospheric pollutant, is a respiratory irritant that is still a matter of concern (WHO European Centre for Environment and Health and WHO Regional Office for Europe 2013). Indoor concentrations often exceed those found outdoors, especially when unvented combustion appliances are used. Inside homes, peaks of NO_2, associated with the use of gas and solid fuel appliances for cooking and heating, have been measured in the range of 80-1100 ppb (150-2090 $\mu g/m^3$) (Basu and Samet 1999; Dennekamp et al. 2001; Kotzias et al. 2005; Pilotto et al. 1997). Outdoors, hourly NO_2 concentrations in cities rarely exceed 200 ppb (380 $\mu g/m^3$) (US EPA (United States Environmental Protection Agency) 2008), although urban levels can reach levels up to 500 ppb (950 $\mu g/m^3$) (WHO (World Health Organization) 2006), especially for short period in streets with heavy traffic and in road tunnel (Larsson et al. 2010).

Epidemiological and controlled human exposure studies suggest that people with asthma are more susceptible to the effects of NO_2 compared with healthy individuals (Bauer et al. 1986; Belanger et al. 2006; Bylin et al. 1988; Hasselblad et al. 1992; Jorres and Magnussen 1990; Strand et al. 1996).

However, despite the extensive literature on NO_2 induced health effects, some inconsistencies in the results of studies have been pointed out (Jarvis et al. 2010). In asthmatics, NO_2 exposure without challenge did not result in lung functional changes in most studies (Avol et al. 1988; Kleinman et al. 1983; Linn et al. 1986; Mohsenin 1987) and inconsistent results were found in airway responsiveness after non specific bronchoconstrictor challenges (Bylin et al. 1988; Hazucha et al. 1983; Jorres and Magnussen 1991; Kleinman et al. 1983; Roger et al. 1990; Strand et al. 1996). After allergen challenge, exposure to NO_2 in asthmatics increased airway

hyperresponsiveness (Jenkins et al. 1999; Strand et al. 1997; Strand et al. 1998; Tunnicliffe et al. 1994) and eosinophilic inflammation (Barck et al. 2005; Barck et al. 2002) .

A few studies have investigated inflammatory response to a single exposure of NO_2 without allergen challenge in asthmatics but the findings have been inconsistent (Jorres et al. 1995; Solomon et al. 2004; Vagaggini et al. 1996).

We investigated whether repeated brief exposures to 200 ppb (380 $\mu g/m^3$) and 600 ppb (1130 $\mu g/m^3$) NO_2 that mimic NO_2 peaks indoors, enhance airway inflammation in asthmatics. This clinical study involved 19 adults with intermittent asthma and used a randomized double-blind protocol with assessment of inflammatory response in induced sputum.

Materials and Methods

Participants

Nineteen patients (fourteen men and five women; median age, 29 years; range, 20 to 69 years; median BMI 26 kg/m^2, range 20 to 39 kg/m^2) were included in the study (Table 1). All had intermittent asthma as defined by the Global Initiative for Asthma (GINA) guidelines (GINA (Global Initiative for Asthma) 2011) and were non-smokers (18 had never smoked and 1 had stopped smoking some 10 years ago). Only those who had a diagnosis of asthma confirmed by a positive methacholine challenge performed twice at baseline 10 to 30 days before the first exposure were included. A positive methacholine test was defined as a methacholine provocative dose causing a 20% decrease in forced expiratory volume in one second (FEV_1) from control FEV_1 (PD_{20} methacholine) <4 mg. All participants had allergy to house dust mite (HDM) and/or pollen confirmed by a positive skin prick test done at least 4 weeks before the inclusion in the study. The study was performed outside the pollen season for those who had been diagnosed

with allergy to pollen. Six out of the 19 participants had a personal history of atopic dermatitis and/or an atopic familial history. None of them used inhaled or oral corticosteroids or other anti-inflammatory therapy and the only permitted medication was inhaled beta 2-agonist as needed during the study period (from baseline, i.e., 30 days before the first NO_2 exposure until the end of the study 2 weeks after the last exposure). Participants who had a gas stove and/or unvented combustion appliances at home were told not to use them at baseline and on the days of exposures and during the two days before and after. All participants had to be free of airway infection for at least 6 weeks prior to baseline.

The study was registered by the French Ministry of Health (DGS 2006/0016) and approved by the Ethics Committee of Hotel-Dieu Hospital, Paris, France (project 0611398, registered on 28 February 2007). Prior to enrolment in the study, all participants signed an informed consent.

Study design

The study had a double-blind, crossover design, each participant acting as his or her own control. For each participant, the study involved a three series of three exposures at rest: one series to clean air, one series to 200 ppb (380 $\mu g/m^3$) of NO_2, and one series to 600 ppb (1130 $\mu g/m^3$) of NO_2. The order of the three series of exposures was randomized (Figure 1). The design for each series of exposures with the timing of pulmonary function testing, and sputum inductions from day 1 to day 3 is described in Figure 2. For each series, participants were exposed to the same level of NO_2 or to clean air 1x30 min on day 1, and 2x30 min on day 2, at the same time and on the same days of the week. The two exposures performed on day 2 were separated by one hour. There was no exposure on day 3. There was an interval of two weeks between each series of exposures. Only the engineer in charge of injection into the chamber knew whether the participant was being exposed to NO_2 or clean air.

Sputum was induced twice at baseline (10 to 30 days before first exposure with at least one week and at most 3 weeks between the sputum inductions) and for each series of exposures, 6 hours (on day 1), 32 hours (on day 2), and 48 hours (day 3) after the end of first exposure. Spirometry with flow-volume curves was carried out at baseline (immediately before first exposure) and daily from day 1 to day 3, before and immediately after exposures, and immediately before sputum inductions. Allergen challenge was not performed, either before or after the exposures.

Nitrogen dioxide/clean air exposure

The exposures were performed in an 8.8 m^3 exposure chamber installed in the investigation clinical center at the Hospital Bichat in Paris as previously described (Ezratty et al. 2007). The chamber was supplied with fresh, particle-free air at mean temperature of 25°C and a mean relative humidity of 32%. The air supply passed through a high-efficiency particulate absolute (HEPA) and activated carbon filters. We used NO_2 concentrated at 950 ppm compressed in a 20 liters gas bottle under a 150 bar pressure for the 600 ppb exposures, and a gas bottle of NO_2 concentrated at 520 ppm under a 150 bar pressure for the 200 ppb exposures (Air Liquide SA, Paris, France). A mass flow meter secured the stability of the injected flow at the expected concentration (200 ppb or 600 ppb).

During exposures, NO_2 concentration inside the exposure chamber was continuously monitored (chemiluminescence NOx analyser, Model AC 32 M, Environnement S.A, 78300 Poissy, France). The mean concentration was 581 ppb +/-3.2% for 600 ppb NO_2 exposures and 203 ppb +/-1.5% for 200 ppb NO_2 exposures. During exposures to clean air, NO_2 concentration was $\leq$10 ppb.

Pulmonary function and methacholine-challenge testing

Flow-volume curves were obtained using a Biomedin spirometer (Biomedin srl, Padova, Italy) according to the European Community Respiratory Health Survey specifications to determine forced expiratory volume in one second (FEV_1) and peak expiratory flow (PEF) (Quanjer 1983).

Methacholine challenge tests were done twice at baseline with an automatic inhalation-synchronized Mefar MB3 dosimeter jet nebulizer (Mefar spa, Bovezzo, Italy) as described elsewhere (Aubier et al. 1992; Ezratty et al. 2007). Methacholine challenge tests at baseline were conducted 10 to 30 days before the start of the exposures to avoid any putative interference of methacholine challenge with the effect of NO_2 (Jorres et al. 1995).

Sputum induction and measurements of inflammatory markers

Sputum induction was performed with an aerosol of hypertonic saline using the method of Pin et al. (Pin et al. 1992). The sputum was analyzed within one hour according to Pizzichini et al. (Pizzichini et al. 1996), as described elsewhere (Ezratty et al. 2007). Total non-squamous inflammatory cell counts were expressed as 10^3 cells per mg of induced sputum. Differential cell counts were performed by counting 400 cells on May Grünwald Giemsa stained slides by two expert observers blinded to the participant's exposure. Results were both expressed as percentage and as number of inflammatory cells per mg of induced sputum. Only samples with cell viability >70% and squamous cell contamination <20% were considered adequate.

Sputum supernatant concentrations of eosinophil cationic protein levels (ECP) were measured by a commercially available enzyme assay (CAP-FEIA, Pharmacia, St Quentin-en-Yvelines, France), with a 2 ng/ml lower detection limit.

Follow-up during the study period

After 0, 15, and 30 min of exposure to NO_2 or clean air in the chamber, participants were asked questions relating to respiratory symptoms and perception of discomfort.

FEV_1 and PEF were monitored twice during exposure at 15 min intervals and hourly for 6 hours after leaving the chamber, with a portable combined spirometer (One Flow Tester, Mediflux, Croissy Beaubourg, France).

During the 10 to 30 days between baseline and first exposure and during the 2-week interval following each series of exposures, subjective symptoms and medications were recorded every day. Each participant measured FEV_1 and PEF twice daily with a portable combined spirometer.

Sample size

The primary end point was the change in the percentage of eosinophils in sputum, expressed as the ratio between the percentage after exposure and the baseline percentage. When the study was designed, literature reports were insufficient to determine the variance of the ratio which was mandatory to estimate the sample size. Variance was estimated after inclusion of the first eight participants without unblinding. Based on the variance found of 0.10, a sample size of 18 participants was considered sufficient to demonstrate a doubling of the percentage of eosinophils in sputum with a power of 80% and a significance level of 0.05 (see Supplemental Material, Table S1). Additionally, a doubling has been found consistent with clinically relevant changes in clinical status of asthmatics (Green et al. 2002).

Statistical analysis

The parameters studied in sputum were the percentage of eosinophils, the number of eosinophils per mg, ECP, the number of neutrophils per mg, and the number of macrophages per mg. All

parameters were log-transformed to normalize the distributions. We used a generalized linear model (GLM procedure, SAS, Cary, NC, USA) for the analyses. We included in the statistical model the effect of the participant, the time, the dose, and the interaction between the time and the dose. The interpretation of the results was based on the type III sum of squares. In case of a significant interaction between day and dose, a global analysis was performed, followed by per-day analyses. In the global analysis, all the data concerning the parameter were summarized by their geometric mean (in case of sputum inductions, this relates to three sputum inductions over 3 days, one per day) and we tested the relation between NO_2 concentration, used as a quantitative value (0, 200 and 600 ppb) and the parameter studied. The global analysis provided the *p*-Value (trend). To display the results in a meaningful way we also analyzed the data using classes of exposure (without any preconceived idea on the form of the relationship); for each class of exposure we estimated the least-square mean (LSMEAN) and its 95% confidence limits. The LSMEANS and the confidence limits were exponentiated to obtain the changes relative to baseline and their confidence intervals. The per-day analyses were reported only when the trend in the global analysis was significant.

p-Values<0.05 were considered significant.

Results

Among the nineteen participants, eighteen completed the three series of exposures and were included in the analysis. Among those, two did not produce an adequate sample of sputum at all time-points and were not included in analyses of sputum (Table 1).

Respiratory function, FEV_1 and PEF, measured by spirometry, did not significantly change after NO_2 exposure compared to clean air (Table 2). No major clinical adverse reactions, such as

coughing, wheezing, or chest tightness suggesting asthma attacks, were observed during exposures or follow-up. During the two weeks after each exposure, subjective symptoms and peak flow measurements were not significantly different whatever the participants had been exposed to.

The primary analysis tested the exposure level (dose), the time (since the study design included assessment at 3 time points after exposure, one each day) and the interaction between time and exposure level. The dose was significantly related to the change in the percentage of eosinophils, the time was not. There was a significant interaction between time and exposure level, meaning that the association between dose and effect on eosinophils was different according to the day. As planned in the case of a significant interaction, we performed both a global analysis, averaging the measurements over the 3 days, and per day analyses.

In the global analysis, the slope of the association between the percentage of eosinophils and NO_2 exposure level was significant (p=0.04) (Table 3).

Compared to baseline, the percentage of eosinophils in sputum increased by 57% (95% CI: 18, 109%) after 600 ppb NO_2 (p=0.003) but did not change significantly after clean air and after 200 ppb NO_2.

Similar results were found for the association between the number of eosinophils per mg of sputum and NO_2 exposure level. In the global analysis, the slope of the association was significant (p=0.02) (Table 3). The number of eosinophils per mg of sputum increased by 120% (95% CI: 60, 202%) after 600 ppb NO_2 (p<0.001) but not after clean air and after 200 ppb NO_2 (Table 3).

Per day analysis showed that the slope of the association between the percentage of eosinophils and NO_2 exposure level was not significant at day 1 (p=0.81) but was significant at day 2 (p=0.01) and day 3 (p=0.03). A similar pattern was found for the slope of the association between the number of eosinophils per mg of sputum and the level of exposure to NO_2 which was not significant at day 1 (p=0.10), close to significance at day 2 (p=0.06), and significant at day 3 (p=0.03) (Table 3 and Supplemental Material, Figure S1).

Compared to baseline, there was no significant change of the percentage of eosinophils in sputum whatever the level of exposure to NO_2 at day 1. At day 2, the percentage and the number of eosinophils increased significantly at 600 ppb, but not at 200 ppb NO_2. At day 3, the number of eosinophils increased significantly at 200 ppb and at 600 ppb NO_2, and the increase of the percentage of eosinophils was close to significance at 600 ppb NO_2 but not significant at 200 ppb (Table 3 and Supplemental Material, Figure S1).

Absolute values not baseline adjusted (geometric means and 95% confidence intervals) of the percentages of eosinophils are reported in Supplemental Material (see Supplemental Material, Table S2). Individual plots of the percentage of eosinophils are displayed in Supplemental Material (see Supplemental Material, Figure S2,). In order to see if results were driven by a participant in particular, we did a sensitivity analysis: a series of analyses with one different participant removed for each analysis. The trend test that measures the relationship between the dose of NO_2 and the increase of the percentage of eosinophils in sputum from baseline was significant (p<0.05) in 10 of the tests and was close to significance (p<0.10) for the other participants. Moreover, the increase of the percentage of eosinophils in sputum compared to baseline was always significant at 600 ppb, whatever participant was removed from the analysis (see Supplemental Material, Table S3).

There was a significant correlation between the number of eosinophils per mg of sputum and ECP (ng/ml) ($p < 0.001$) (See Supplemental Material, Figure S3).

Compared to baseline, ECP in sputum increased significantly after exposure to 600 ppb NO_2 (43%; 95% CI: 17, 75%; p=0.001) but not after clean air or 200 ppb of NO_2. However the slope of the association between ECP and the level of exposure was not significant (Table 3).

Exposure to NO_2 did not affect the other cell types (macrophages, neutrophils) measured in sputum (Table 3).

There was no correlation between methacholine challenge tests and eosinophil responses (data not shown).

Discussion

This study demonstrates that repeated brief exposures to NO_2 without allergen exposure increase eosinophilic airway inflammation in subjects with intermittent asthma without inducing any changes in lung function. As only mild asthmatics were tested, the results cannot be extrapolated to healthy individuals.

Although we cannot exclude that repeated challenges with hypertonic saline could have potentialised the effect of NO_2, the repetition of sputum inductions is not the cause of the effect as it is expected to be the same for each of the 3 series of exposures (Pavord 1998). Eosinophils in sputum increased according to NO_2 exposure level and this significant trend supports a dose-related relationship. The effect on eosinophils and on ECP in sputum was significant at 600 ppb NO_2. A strong correlation between ECP and eosinophils was found suggesting that eosinophils were activated.

In subjects with asthma, several studies have found that NO_2 exposure increased eosinophilic inflammation in response to inhaled allergen, in the distal lower airways assessed by bronchial wash and bronchoalveolar lavage (BAL) (Barck et al. 2002), and in sputum (Barck et al. 2005). The only three previous studies that have investigated effects of NO_2 without allergen challenge in asthmatic subjects did not find changes in inflammatory cell distributions in BAL (Jorres et al. 1995) and in sputum (Solomon et al. 2004; Vagaggini et al. 1996). However, these studies involved small numbers of participants, and no repetition of exposure, and, in the Jörres et al. study (Jorres et al. 1995), the evaluation of inflammation could have been performed too soon after exposure.

In the present study, asthmatic subjects were exposed to two realistic NO_2 concentrations: 200 ppb and 600 ppb of NO_2, close to NO_2 peaks likely to be found indoors during the use of combustion appliances for cooking and heating (Basu and Samet 1999; Dennekamp et al. 2001; Kotzias et al. 2005; Pilotto et al. 1997) and outdoors for short periods in streets with heavy traffic and in road tunnels (Larsson et al. 2010). Exposures lasted 30 minutes, close to the average time spent cooking dinner in France (38 min during the week and 46 min the week-end in a 2003 survey) (Hébel 2012). Furthermore, exposure to intermittent peaks of NO_2 may have greater effects than long-term low-level exposure (Gardner et al. 1979).

Exposures in this study were repeated over two days to mimic how exposures take place in real life and in order to assess a possible cumulative effect. At day 1, after one exposure, there was no significant change in eosinophilic airway inflammation contrary to day 2 and day 3, after three exposures. These findings suggest that inflammatory response to NO_2 exposure may be delayed or that a single exposure may be insufficient to enhance eosinophilic airway inflammation, suggesting a cumulative effect of NO_2. These results are consistent with those of Barck et al.

who found that 2 to 3 brief exposures at 260 ppb NO_2 were needed to promote an increase in the airway inflammatory response to inhaled allergens (Barck et al. 2005). In addition to the study of Barck et al., our study shows that NO_2, without an exposure to a high concentration of allergens, as with an allergen challenge, is sufficient to enhance inflammation in the airways. This finding could be of significance as exposure to peaks of NO_2 is common, particularly indoors, while exposure to both high concentrations of NO_2 and the specific stimulus for a susceptible individual is less likely (Hesterberg et al. 2009).

Many cities in Europe show an increase in concentrations of NO_2 measured close to traffic due to the increasing number of vehicles, in particular diesel vehicles. Exhaust emissions from such vehicles are lower for Carbon Monoxide (CO), Non Methanic Volatile Organic Compounds (NMVOC) and Particulate matter (PM) but may be higher for NO_2 (Guerreiro et al. 2012). While epidemiological studies on NO_2 have several limitations in particular because of the potential for exposure misclassification and co-pollutant effects, our results provide important evidence suggesting that NO_2 alone has a direct effect on airway inflammation in asthmatics.

Conclusions

To our knowledge, this is the first study to demonstrate that repeated peaks of NO_2 at realistic concentrations without exposure to allergen increase eosinophilic inflammation in the airways of asthmatics and to support a dose-response relationship. Although it is difficult to evaluate the clinical implications of these findings in the present study, an increased eosinophilic inflammation may lead to exacerbation or loss of control of asthma (Green et al. 2002; Jacobsen et al. 2007). Therefore, we cannot rule out the effects of repeated exposures to NO_2 over a longer period of time or effects in subgroups.

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Table 1. Characteristics of participants.

Participant	Age (years)	History of atopy[b]	Smoking Status[c]	Gender	Height (cm)	Weight (kg)	BMI[d] (kg/m^2)	Asthma duration (years)	FEV_1 at inclusion (% pred)	PD20 methacholine[e] at baseline (µg)	% eosinophils in sputum[f] at baseline
1[a]	26	Yes	N	F	164	57	21	8	3.23 (110%)	1600	NA
2	29	No	E	M	185	90	27	11	4.13 (89%)	1490	14.73
3	29	No	N	M	182	87	27	10	4.43 (99%)	690	11.88
4	31	No	N	M	161	84	33	8	2.87 (81%)	3200	6.79
5	28	No	N	M	174	74	25	4	3.69 (88%)	1550	1.68
6	27	No	N	M	180	88	28	21	3.57 (80%)	1220	0.93
7	24	No	N	M	178	70	22	8	3.88 (87 %)	1070	4.25
8	30	No	N	F	159	56	22	20	3.03 (103%)	500	2.68
9	29	Yes	N	M	168	72	26	22	3.89 (100%)	3200	1.88
10[a]	28	Yes	N	M	186	92	27	2	4.49 (96%)	800	ANR
11	20	Yes	N	F	158	49	20	9	2.29 (76%)	930	0.26
12	69	No	N	M	178	90	29	5	2.49 (78%)	3200	17.72
13	30	No	N	F	163	55	21	20	2.68 (87%)	1950	32.08
14	32	Yes	N	M	179	82	26	24	4.12 (92%)	340	2.51
15	24	No	N	F	171	60	21	14	3.41 (97%)	310	5.12
16	28	No	N	M	174	62	21	16	3.82 (91%)	2100	20.58
17	32	No	N	M	176	115	38	21	4.16 (100%)	2170	2.99
18[a]	30	Yes	N	M	169	89	32	21	3.75 (95%)	190	NA
19	30	No	N	M	174	115	39	23	3.16 (76%)	210	1.55

[a]Participants excluded from the analysis: participants 1 and 18 did not produce adequate sputum specimens for cell analysis at baseline (squamous cells>20%); participant 10 did not complete the 3 series of exposure. [b]History of atopy: personal history of atopic dermatitis and/or atopic familial history. [c]Smoking status: N: neversmoker; E: ex-smoker. [d]BMI: Body Mass Index = Weight/Size^2. [e]PD_{20} methacholine: provocative dose of methacholine causing a 20% decrease of $FEV_{1.}$ [f]% eosinophils at baseline (10 to 30 days before first exposure): NA: non available; ANR: available but not relevant as participant 10 didn't complete the 3 series of exposure.

Table 2. Geometric mean of values relative to baseline[a] ([95% confidence interval]) of forced expiratory volume in one sec (FEV_1) and peak expiratory flow (PEF) evaluated in the 18 participants who completed the study.

Variable	**0 ppb NO_2 (clean air)**	**200 ppb NO_2**	**600 ppb NO_2**	***p*-Value**
FEV_1/baseline	1.02 (1.00, 1.05)	1.00 (0.97, 1.03)	1.00 (0.98, 1.03)	0.41
PEF/baseline	1.05 (0.99, 1.11)	1.04 (0.98, 1.10)	1.01 (0.96, 1.07)	0.36

[a]Baseline values were measured immediately before first exposure (H0 on day 1) for each series of exposure.

Each value corresponds to the geometric mean change from baseline of the 6 values (H0', H6, H24, H26, H32, H48; see Figure 2) obtained by spirometry with flow-volume curves.

The flow volume curves were measured with a spirometer according to the European Community Respiratory Health Survey specifications (Quanjer 1983).

The effects of exposure, whatever the dose, on FEV_1 and PEF are not significant.

Table 3. Changes relative to baseline measurements (performed 10 to 30 days before the first exposure) for parameters measured in sputum [geometric mean percentage (95% confidence interval), n=16].

Variable	0 ppb NO_2 (clean air)	200 ppb NO_2	600 ppb NO_2	*p*-Value (trend)
Percentage of eosinophils[a]	-12% (-34, 16)	-5% (-28, 26)	57% (18, 109)**	0.04
Day 1	16% (-28, 86)	-34% (-59, 6)	12% (-31, 79)	0.81
Day 2	-5% (-37, 44)	-3% (-36, 46)	102% (32, 211)**	0.01
Day 3	-39% (-67, 13)	36% (-26, 149)	79% (-3, 230)	0.03
Number of eosinophils/mg[a]	-5% (-31, 29)	23% (-10, 68)	120% (60, 202)[#]	0.02
Day 1	-5% (-44, 60)	-24% (-55, 29)	64% (-3, 177)	0.10
Day 2	9% (-39, 95)	23% (-31, 119)	142% (32, 344)**	0.06
Day 3	-18% (-57, 56)	99% (5, 278)*	163% (38, 398)**	0.03
Eosinophil cationic protein[a]	21% (-1, 47)	0% (-18, 22)	43% (17, 75)**	0.23
Number of neutrophils/mg[a]	12% (-17, 51)	-9% (-32, 22)	10% (-19, 49)	0.97
Number of macrophages/mg[a]	-18% (-33, 0)	-2% (-20, 21)	-6% (-24, 15)	0.46

[a]Geometric mean of all changes of the parameter for the 3 days (one sputum induction per day).

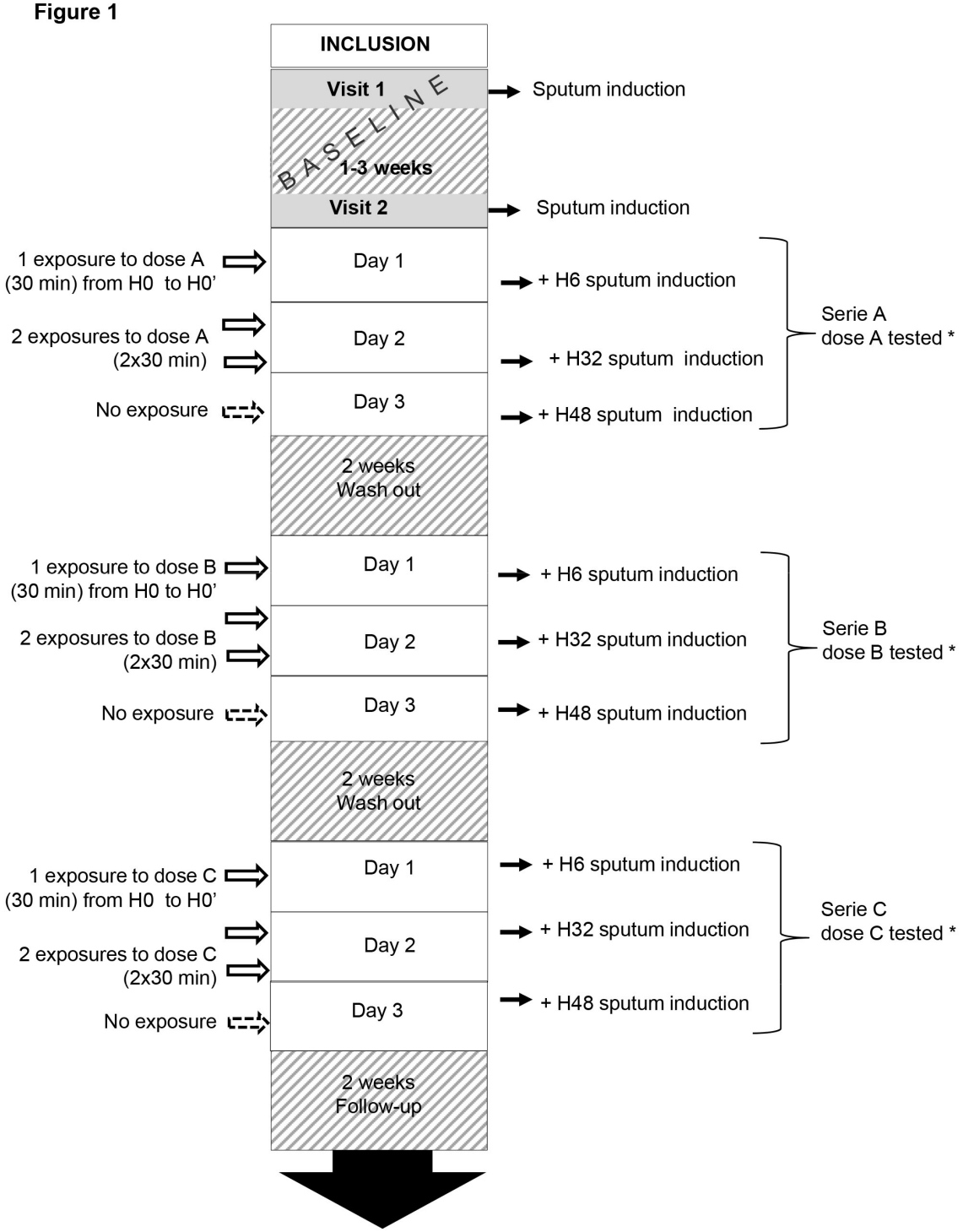
P-values for differences compared to baseline: *$p < 0.05$; **$p < 0.01$; [#]$p < 0.001$.

Figure legends

Figure 1. Flow diagram of the study period for each participant

Figure 2. Study design for each of the three series of exposures separated by 2 weeks. Participants were exposed at rest in a double-blinded, randomized, crossover design, to 200 ppb NO_2, or 600 ppb NO_2, or clean air for 1 x 30 min on day 1, and for 2 x 30 min on day 2. There was no exposure on day 3.

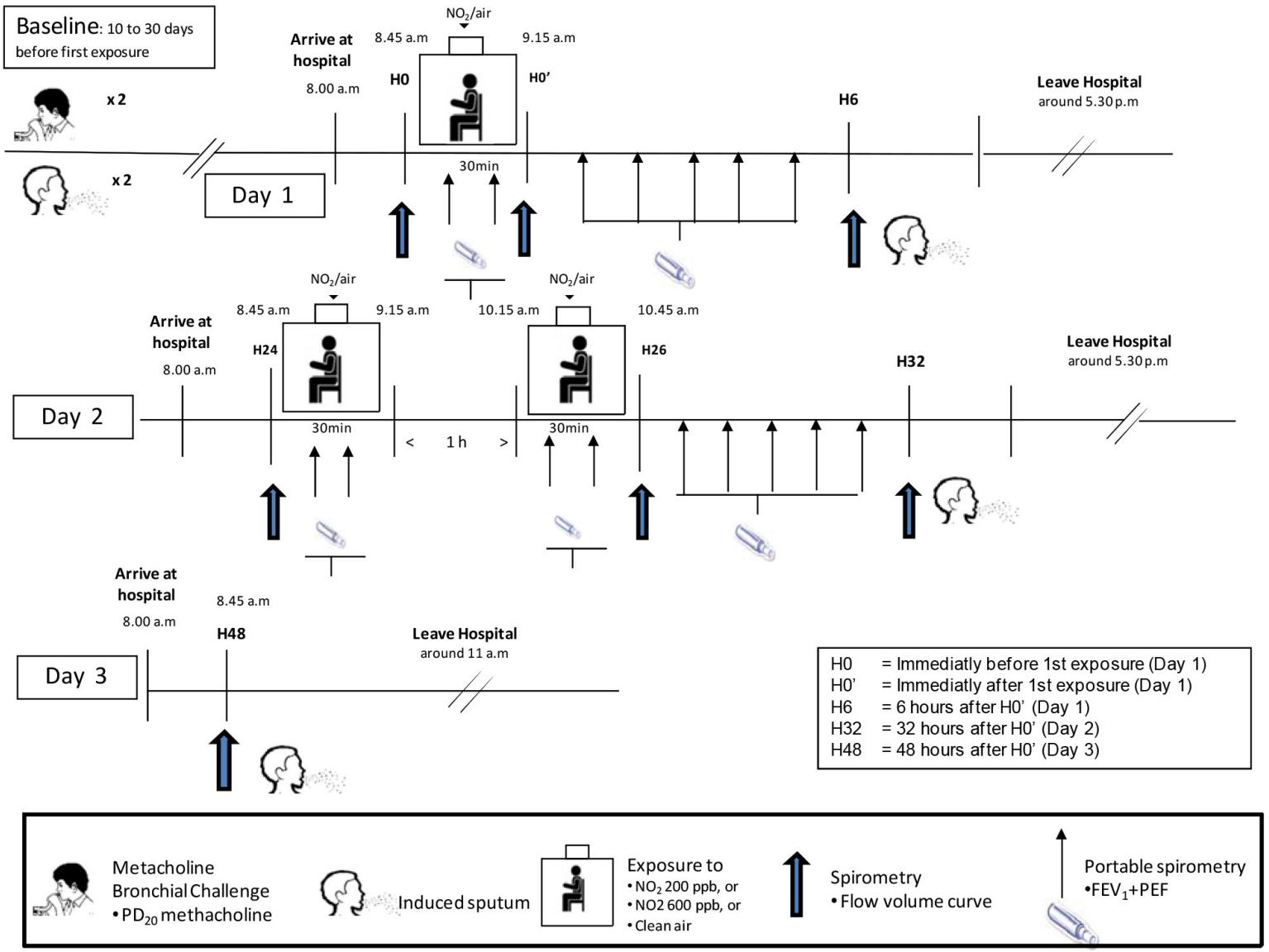
Figure 1



H0 = Immediatly before 1st exposure (Day 1)
H0' = Immediatly after 1st exposure (Day 1)
H6 = 6 hours after H0' (Day 1)
H32 = 32 hours after H0' (Day 2)
H48 = 48 hours after H0' (Day 3)

* Note: the dose order is attributed randomly. so, A,B,C, corresponds to clean air or 200 ppb or 600 ppb

Figure 2


Baseline: 10 to 30 days before first exposure
x 2
x 2
Day 1
Arrive at hospital
8.00 a.m
8.45 a.m
NO2/air
9.15 a.m
H0
H0'
30min
H6
Leave Hospital
around 5.30 p.m
Day 2
Arrive at hospital
8.00 a.m
8.45 a.m
NO2/air
9.15 a.m
H24
30min
< 1 h >
10.15 a.m
NO2/air
10.45 a.m
30min
H26
H32
Leave Hospital
around 5.30 p.m
Day 3
Arrive at hospital
8.00 a.m
8.45 a.m
H48
Leave Hospital
around 11 a.m
H0 = Immediatly before 1st exposure (Day 1)
H0' = Immediatly after 1st exposure (Day 1)
H6 = 6 hours after H0' (Day 1)
H32 = 32 hours after H0' (Day 2)
H48 = 48 hours after H0' (Day 3)
Metacholine Bronchial Challenge
• PD20 methacholine
Induced sputum
Exposure to
• NO2 200 ppb, or
• NO2 600 ppb, or
• Clean air
Spirometry
• Flow volume curve
Portable spirometry
•FEV1+PEF

Environmental Health Perspectives doi:10.1289/ehp.1307240

Supplemental Material

Repeated Nitrogen Dioxide Exposures and Eosinophilic Airway Inflammation in Asthmatics: A Randomized Crossover Study

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Table S3. Changes from baseline [geometric mean percentage (95% confidence interval)], for the percentage of eosinophils in sputum with one participant excluded each time. **4**

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Figure S3. Correlation between eosinophil cationic protein (ECP) concentration in sputum supernatant (ng/ml) and the number of eosinophils per mg of sputum (n=16). **7**

Table S1. Sample sizes required to estimate statistically significant differences (alpha = 0.05) in percentage of eosinophils in sputum between two exposure groups with a variance of 0.10 and 80% power.

Percentage increase in eosinophils	**Sample size (n)**
50%	51
100% (doubling)	18
150%	10

Table S2. Absolute values not baseline adjusted (geometric means and 95% confidence intervals) for percentages of eosinophils measured in sputum (n = 16).

Variable	**0 ppb NO_2 (clean air)**	**200 ppb NO_2**	**600 ppb NO_2**	***p*-trend**
Percentage of eosinophils in sputum	3.92 (1.92, 8.01)	4.05 (1.99, 8.28)	6.44 (3.15, 13.2)	0.30

Table S3. Changes from baseline (geometric mean percentage (95% confidence interval)), for the percentage of eosinophils in sputum with one participant excluded each time.

Variable	**0 ppb (clean air)**	**200 ppb**	**600 ppb**	***p* trend**
Participant 2 excluded	1% (-23, 32)	-5% (-27, 25)	77% (35, 132)	0.03*
Participant 3 excluded	-11% (-33, 18)	-4% (-28, 28)	63% (22, 117)	0.04*
Participant 4 excluded	-4% (-28, 27)	-1% (-26, 32)	71% (28, 129)	0.05*
Participant 5 excluded	-7% (-31, 24)	1% (-24, 35)	70% (27, 127)	0.05*
Participant 6 excluded	-13% (-35, 17)	-5% (-29, 27)	53% (14, 105)	0.06
Participant 7 excluded	-14% (-35, 14)	-5% (-28, 25)	48% (12, 97)	0.07
Participant 8 excluded	-20% (-40, 7)	-10% (-33, 2)	46% (8, 96)	0.05*
Participant 9 excluded	-15% (-35, 12)	-1% (-25, 3)	38% (4, 83)	0.09
Participant 11 excluded	-17% (-38, 11)	-3% (-27, 3)	52% (13, 104)	0.05*
Participant 12 excluded	-20% (-40, 7)	-13% (-35, 17)	53% (14, 106)	0.03*
Participant 13 excluded	-7% (-30, 24)	6% (-20, 41)	81% (35, 141)	0.03*
Participant 14 excluded	-22% (-42, 4)	-14% (-34, 15)	38% (3, 86)	0.06
Participant 15 excluded	-26% (-45, -1)	-19% (-39, 09)	40% (4, 89)	0.03*
Participant 16 excluded	4% (-21, 35)	25% (-5, 63)	62% (24, 113)	0.08
Participant 17 excluded	-13% (-45, 16)	-10% (-33, 21)	51% (13, 103)	0.06
Participant 19 excluded	-6% (-28, 24)	-12% (-33, 15)	71% (29, 125)	0.02*

Participants 1, 10 and 18 did not contribute and so were not included in the analysis.

*p-Value<0.05

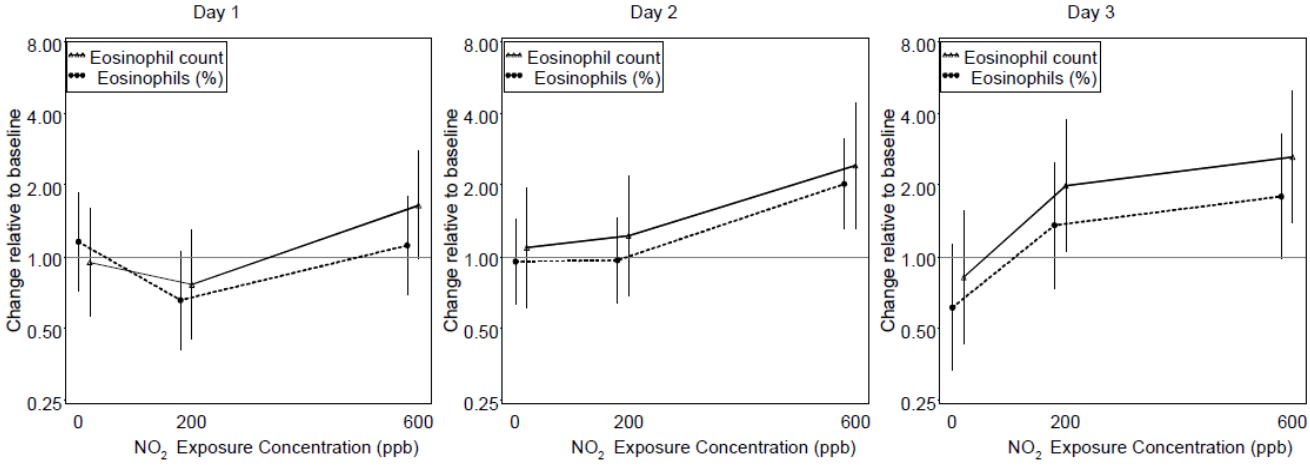

Figure S1. Changes relative to baseline of eosinophil count and percentage of eosinophils for days 1, 2, and 3 (n=16).

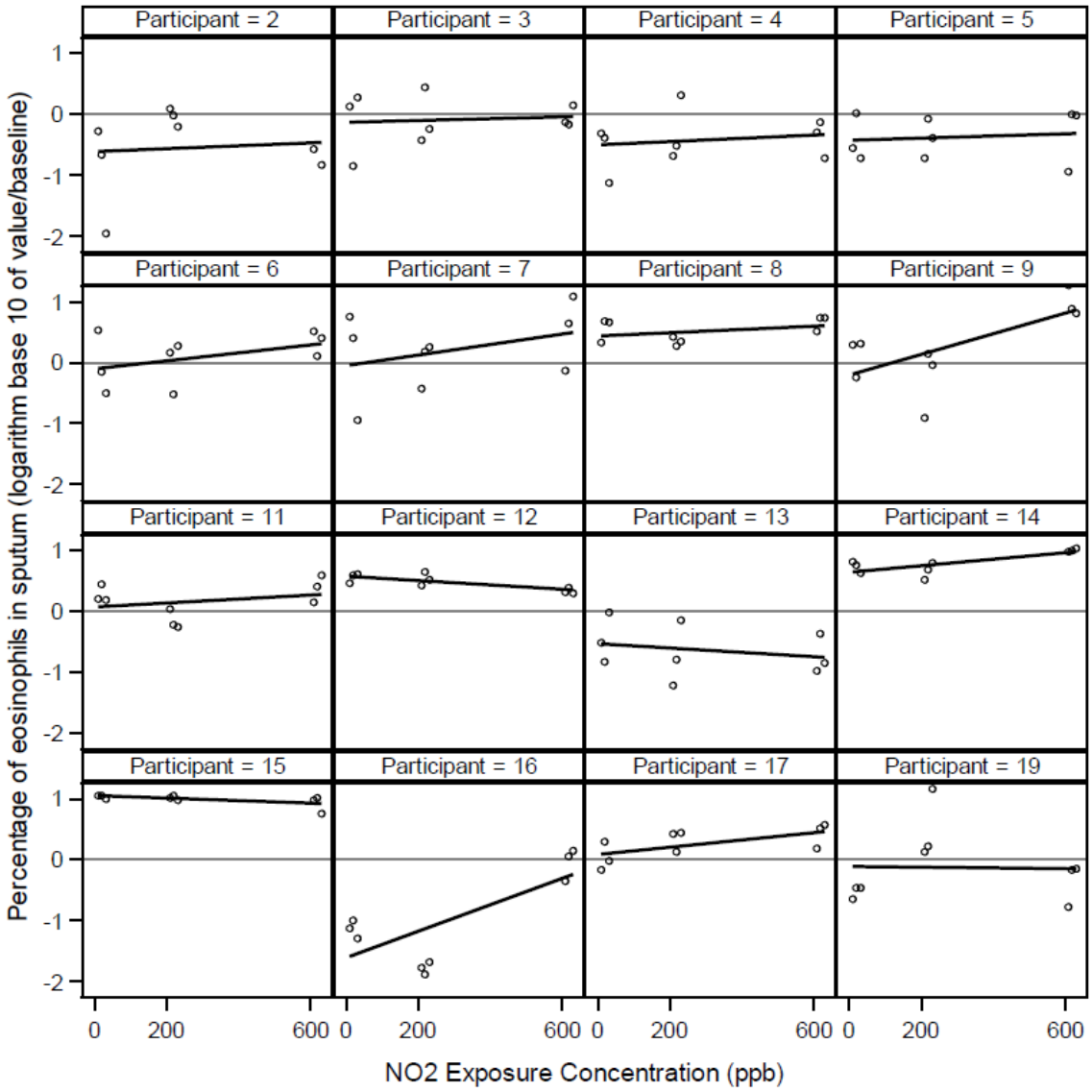

Figure S2. Change from baseline in the logarithm (base 10) of the percentage of eosinophils, for each participant (n=16). Individual plots are organized according to the subject number from subject 2 to subject 19. A change of 1 corresponds to a ten-fold increase from baseline. Each subject is identified by his or her percentage of eosinophils at baseline (measured 10 to 30 days before the first exposure).

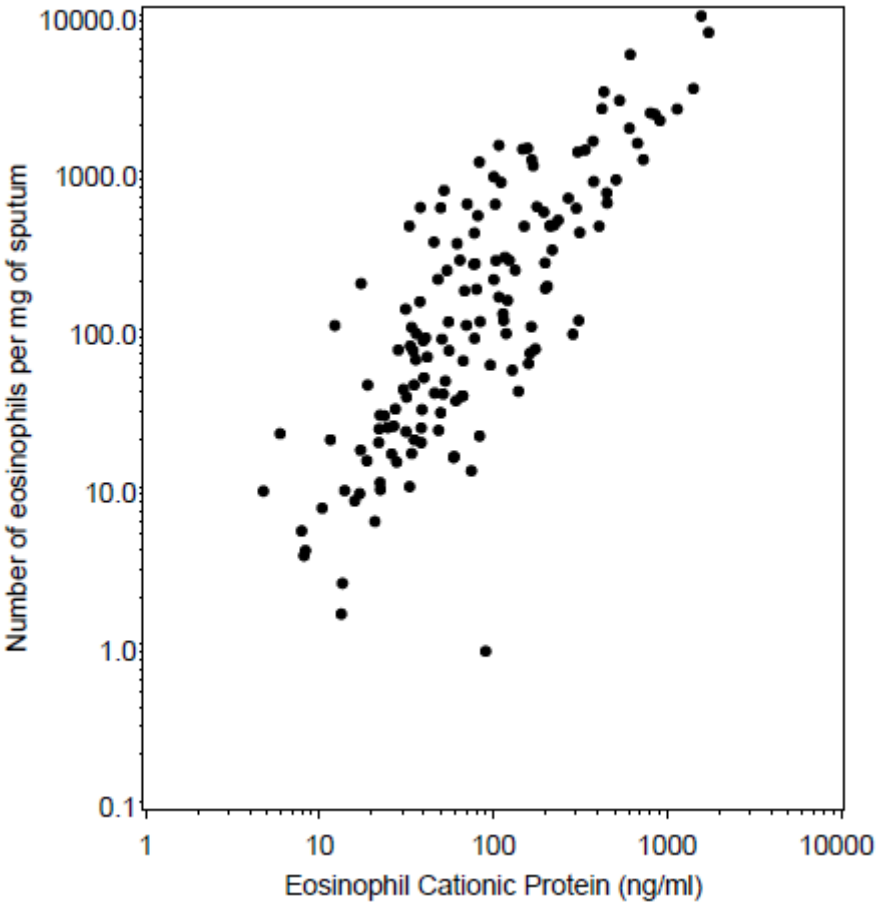

Figure S3. Correlation between eosinophil cationic protein (ECP) concentration in sputum supernatant (ng/ml) and the number of eosinophils per mg of sputum (n=16).