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Effect of controlled human exposure to diesel exhaust and allergen on airway surfactant protein D, myeloperoxidase, and club (Clara) cell secretory protein 16

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Running Head: Diesel and allergen effects lung biomarkers

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Abstract

Background

Air pollution is a major cause of global morbidity and mortality. Air pollution and aeroallergens aggravate respiratory illness, but the variable effects of air pollutants and allergens in the lung are poorly understood.

Objective

To determine the effects of diesel exhaust (DE) and bronchial allergen challenge as single and dual exposures on aspects of innate immunity in the airway as reflected by surfactant protein D (SPD), myeloperoxidase (MPO) and club (Clara) cell secretory protein 16 (CC16) in 18 atopic individuals.

Methods

In this double-blind, randomized crossover study, atopic individuals were exposed to DE or filtered air, followed by endobronchial allergen or saline 1 hour after inhalational exposure.

Bronchoalveolar lavage (BAL), bronchial washings (BW), nasal lavage and blood samples were obtained 48-hours after exposures and assayed for CC16, MPO and SPD by ELISA.

Results

In bronchial samples the concentration of SPD increased from 53.3 ng/mL to 91.8 ng/mL after endobronchial allergen, with no additional contribution from DE. MPO also increased significantly in response to allergen (6.8 ng/mL to 14.7 ng/mL) and there was a small additional contribution from exposure to DE. The concentration of CC16 decreased from 340.7 to 151.0 ng/mL in response to DE, with minor contribution from allergen. These changes were not reflected in nasal lavage fluid or plasma samples.

Conclusions & Clinical Relevance

These findings suggest that allergen and DE variably influence different aspects of the innate immune response of the lung. SPD and MPO, known markers of allergic inflammation in the lung, are strongly increased by allergen while DE has a minor effect therein. DE induces a loss of CC16, a protective protein, while allergen has a minor effect therein. Results support site- and exposure-specific responses in the human lung upon multiple exposures.

Trial Registry

ClinicalTrials.gov; No.: NCT01792232, URL: www.clinicaltrials.gov

Introduction

Air pollution has been increasingly recognized for its detrimental effects on cardiovascular and respiratory outcomes.(1,2) Recently, ambient air pollution has been cited the 9th greatest contributor to the global burden of disease overall, and the 3rd largest contributor global contributor to disability-adjusted life years due to respiratory diseases.(3) Patients with chronic obstructive pulmonary disease (COPD) and asthma are more likely to be hospitalized due to exacerbations of these diseases when exposed to higher levels of ambient air pollution.(4-7) Notably, there is an well-known association between air pollution and sensitization to allergens and incident asthma,(8,9) but the underlying mechanisms are unclear.

Diesel exhaust (DE) is a common air pollutant, and the major contributor to ultrafine particulate matter pollution in cities.(10) Exposure to diesel exhaust has been shown to induce local changes in inflammatory cytokines and induce T-helper type 2 (Th2) responses that predispose individuals to asthmatic and allergic responses.(8,10) Co-exposure to diesel exhaust and allergen has been shown in animal models and in humans to have synergistic effects on the immunological response to allergen,(11-14) but the exact mechanisms are poorly understood.

In this study, we determined the effects of diesel exhaust and allergen co-exposure on three important airway proteins involved in host response to aeroallergens and irritants: club (Clara) cell secretory protein 16 (CC16), surfactant protein D (SPD) and myeloperoxidase (MPO). CC16 and SPD are produced predominantly in lung tissue and have been shown to be dysregulated in a number of respiratory illnesses, including asthma and COPD.(15,16) CC16 is an important anti-inflammatory molecule that modulates immune responses to inhaled irritants.(17) SPD, a collectin, binds to aeroallergens and pathogens and induces their clearance from the airways.(18) Myeloperoxidase (MPO) is produced by granulocytes and plays a central role in neutralizing aeropathogens and effecting their clearance.(19) The concentration of MPO in sputum and serum is positively associated

with airways diseases.(19-21) Our aim was to characterize the effect of allergen and DE on these airway biomarkers.

Methods

Participants

18 participants were recruited to the Air Pollution Exposure Laboratory in Vancouver, British Columbia, Canada. Written consent was obtained from all subjects, and the Clinical Research Ethics Board at the University of British Columbia approved the protocol (approval number H11-01831). Participants were adults aged 19-50 years, and had a history of atopic illness. All enrolled subjects had a positive skin prick test (>3 mm induration), to Timothy/Pacific Grasses, Birch, or House Dust Mite (*D. pterinyssinus*). Smoking status was determined for all subjects, with non-smokers having quit for greater than one year. All subjects were asked to hold all long-acting anti-histamines for 7 days and short-acting anti-histamines for 3 days prior to skin testing. Subjects were instructed to hold short acting β -agonists for 6 hours, and long acting β -agonists for 48 hours prior to exposures. Patients requiring frequent use of β -agonists (greater than three times per week) were excluded from the study. Patients on inhaled corticosteroids or regular antihistamines were excluded. Active exacerbation of respiratory disease or inflammatory conditions, and active infection were ruled out in all subjects by careful clinical review by the study physician and multiple questionnaires. If a participant was felt by the study physician to have an active illness, participation was delayed until a period of clinical stability.

Experimental design and procedures

This study (ClinicalTrials.gov; No.: NCT01792232, URL: www.clinicaltrials.gov) followed a double-blind, crossover design where participants were exposed to either filtered air (FA) or diesel exhaust (DE) using a system previously reported,(22) altered only in that we utilized a 2.5kW constant load for the present study. Blinding to inhalational exposure was previously validated.(23)

Briefly, a portion of raw exhaust was drawn into a dilution system at standard temperature (20°C) and relative humidity (40%) levels, diluted and then aged 4 min before entering the 4x6x7 ft. exposure booth. The average concentration of DE was through the study was $302.0 \pm 30.5 \mu\text{g PM}_{2.5}/\text{m}^3$, for a total exposure time of 2 hours as previously reported.(24) During exposures, participants alternated between rest (45 min/hr) and light exercise (15 min/hr) on a stationary bicycle. The load of the exercise was set to achieve a minute ventilation of approximately 15 L/min/m² body surface area. A washout period of 4 weeks was observed between exposures. The sequence of inhalational exposure was randomized and counterbalanced, and exposures took place within a given subject in a fashion that fully avoided the season of the allergen used for endobronchial challenge of that subject.

All subjects underwent spirometry and methacholine challenge (using the 2 minute tidal breathing method) as per American Thoracic Society (ATS) guidelines prior to exposures.(25,26) Bronchial hyperresponsiveness was defined as $\text{PC}_{20} \leq 8 \text{ mg/mL}$ of methacholine. Blood samples were collected immediately prior to exposure, as well as 4-, 24-, and 48-hours after exposure. Nasal lavage with a total of 4 mL of sterile saline was performed prior to inhalational exposure according to a protocol used previously(27,28), and repeated at 6- and 24-hours after exposure.

1 hour after the initial inhalational exposure, subjects underwent bronchoscopy, where one of the 3 study allergens (5 mL solution of allergen extract in a concentration 10-fold lower than that minimal dose producing a positive skin wheal) was deposited in the right middle lobe or the lingula, and sterile saline was deposited in the equivalent segment of the contralateral lung (5 mL diluent control), in a randomized and blinded fashion. Subjects then underwent a second bronchoscopy at 48-hours post-exposure. Bronchial washing (BW) and bronchoalveolar lavage (BAL) samples were obtained, with a wedged bronchoscope, from the sites where allergen and control solutions has been instilled. After the second exposure, allergen was deposited in the segment where saline was previously deposited and vice-versa.

Blood, nasal lavage and lung samples were processed within 4 hours of collection and were stored at -80°C until used for assays.

Assays

Concentration endpoints in all samples were determined using commercially available ELISA assay kits (MPO and SPD: R&D Systems Inc., Minneapolis, MN, USA, catalog numbers DMYE00B and DSFPD0, respectively; CC16: BioVendor, Karasek, Czech Republic, catalog number RD191022200). The lower limits of detection were 1.57 ng/mL, 1.56 ng/mL and 0.63 ng/mL for CC16, MPO and SPD, respectively.

Statistical Analysis

All statistical models were created using R (<https://www.r-project.org/>). Demographic data are presented for each subject and presented in the text as means and standard errors of the mean. Experimental data were transformed on a natural log scale prior to fitting with a mixed effects model and calculating means and confidence intervals. The means were assumed to depend on the exposure (DE versus FA; saline versus allergen), and repeated measures analyses included terms to estimate error between and within subject data. Ordinal models used orthogonal polygonal contrast to estimate trends in the data. Means and 95% confidence intervals of the means were back-transformed by the delta method for presentation in figures and in the text. P-values of <0.05 were considered significant.

Results

Clinical Characteristics

Demographic and clinical data for the cohort are presented in Table 1. In general, subjects were young, with an average age of 28 ± 1.82 years, and most (61.1%) were of Caucasian descent and female (61.1%). Most had normal lung function, with average FEV₁ (percent predicted) of $103.6 \pm 4.5\%$. Approximately one-third, (33.3%) of participants were hyperresponsive to methacholine. Three subjects had baseline FEV₁ below 80% of predicted; of these, two were hyperresponsive to methacholine and all were never smokers.

All subjects had a history of atopy, and were skin test positive to a common allergen, most frequently to dust mite (*D. pterinysinus*) allergen (41.1%, Table 1). A complete description of the subject's demographics and lung function was previously reported.(24)

Concentration of SPD, MPO and CC16 in Endobronchial Samples After Exposures

Figure 1 shows the concentration of SPD, MPO and CC16 in BAL samples 48 hours after exposure, for each of the four possible conditions; filtered air plus endobronchial saline (FAS), filtered air plus endobronchial allergen (FAA), diesel exhaust plus endobronchial saline (DES) and diesel exhaust plus endobronchial allergen (DEA).

The concentration of SPD in BAL samples after exposure to FAS was 53.3 (95% CI: 32.3-86.9) ng/mL; after DES, 47.5 (95% CI: 29.1-77.6) ng/mL; after FAA, 91.8 (95% CI: 57.0-147.8) ng/mL; and after DEA, 89.4 (95% CI: 54.1-147.8) ng/mL (Figure 1A); a significant increase in the concentration of SPD in BAL samples after exposure to allergen compared to saline ($p=0.01$), but not for exposure to DE vs. FA ($p=0.75$). A similar result for bronchial washings was found (data not shown).

The concentrations of MPO in BAL samples were 6.8 (95% CI: 4.0-11.3) ng/mL for FAS; DES: 8.2 (95% CI: 4.9 to 13.7); FAA: 14.7 (95% CI: 8.9-24.3); DEA: 15.9 (95% CI: 9.3-27.0) ng/mL. A significant effect of allergen vs. saline ($p=0.008$), but not DE vs. FA ($p=0.61$), on the

concentration of MPO was again detected in BAL samples (Figure 1B). Similar results were noted for BW (data not shown).

Based on the stepwise increase in MPO to each in exposure (Figure 1B) we hypothesized increasing strength of response for the combined exposures, in spite of no significant effect of DE vs. FA. As such, we explored additive effects of inhalational exposures on the concentration of MPO in BAL using ordinal modeling, with the hypothesis that FAS would have the weakest effect on concentration of MPO, with increasing effect of DES, FAA, and DEA. For MPO, the linear model alone was significant ($p=0.01$; $p=0.8$ for quadratic and $p=0.4$ for cubic models), suggesting a linear increase in the concentration of MPO in BAL for the experimental conditions in the hypothesized order (FAS<DES<FAA<DEA). A similar hypothesis was considered for SPD but rejected given that a stepwise pattern was not present on inspection (Fig 1B).

The pattern of CC16 in response to exposures was strikingly different than that found for MPO and SPD (Figure 1C). In BAL samples after FAS, the concentration of CC16 was 340.7 (95% CI: 158.3-733.5) ng/mL and after DES it was 151.9 (95% CI; 70.5-327.0) ng/mL. After FAA the concentration of CC16 was 247.2 (95% CI; 117-521.0) ng/mL and after DEA it was 122.7 (95% CI; 55.7-270.1) ng/mL; representing a significant decrease in the concentration of CC16 in BAL samples for DE vs. FA ($p=0.04$). No significant effect of allergen vs. saline was found ($p=0.46$). In BW, no significant changes in the concentration of CC16 were detected (data not shown). As expected from the pattern of response in Figure 1C, none of the best-fit ordinal models tested were significant.

CC16 and MPO in Nasal Lavage Samples After Exposures

There was no significant difference in the concentration of MPO in NLF after exposure to FA (78.4; 95% CI: 52.1-118.0 ng/mL) vs. DE (65.6; 95% CI; 43.5-98.9 ng/mL, $p=0.45$). There was no significant difference in the concentration of CC16 after FA (3.6; 95% CI: 1.7-7.4 ng/mL) vs. DE (3.3; 95% CI: 1.6-6.9 ng/mL, $p=0.84$). We were unable to reliably measure the concentration of SPD in NLF.

Plasma samples from prior to, and 4-, 24-, 48-hours after inhalational exposures were assayed for SPD, MPO and CC16. No significant changes in the concentration of SPD, MPO or CC16 were found in response to inhalational exposure to DE vs. FA (data not shown). In all cases, a significant independent effect of time was found, indicating diurnal variation of all proteins assayed.

Discussion

This study demonstrates that in atopic individuals exposed to DE and allergen the airway concentrations of SPD and MPO increase while airway CC16 decreases. The observed increases in the levels of SPD and MPO were primarily driven by allergen exposure, whereas the decrease in CC16 was driven by exposure to diesel exhaust. While the different exposures appear to drive the primary response, diesel exhaust adds a modest additional contribution to MPO.

We also found the levels of MPO and CC16 do not change in NLF after inhalational challenge. Plasma levels of SPD, MPO and CC16 either did not change in response to DE, or such change was masked by a large systemic inflammatory effect from the segmental allergen challenge. We show that plasma levels of SPD, and CC16 have diurnal variation that has been reported previously.(16,29,30) This is the first report of circadian changes in MPO in healthy patients; diurnal variation in MPO has only been previously observed in patients with myocardial infarction.(31)

SPD is primarily produced by Type II pneumocytes in the distal airways, and has both direct and indirect effects on the innate immunity of the lung, including the regulation of eosinophils.(15) Bronchial SPD is reduced in healthy smokers compared to controls,(32,33) and increased in asthmatic patients when compared to healthy controls.(34) Increasing bronchial SPD also correlates with the severity of asthma.(35) Previous reports show an increase in SPD in response to segmental allergen challenge in asthmatics, but not healthy controls.(36) This is the first study to evaluate bronchial

concentrations of SPD in humans after exposure to DE; although a previous mouse study showed decreases SPD in BAL after 5 days of exposure to DE(37) we did not see this in our human subjects.

Granulocyte-mediated inflammation is important in the response of the lung to inhaled air pollutants,(9,38) and allergen.(39) Inflammatory changes in the lung in response to diesel exhaust are well documented.(40-42) MPO, produced by neutrophils, serves as a surrogate marker for this inflammation.(19) Previous reports have shown that DE can increase neutrophil counts and MPO concentration in bronchial samples.(41,43) The concentration of MPO is also responsive to allergen; MPO increases in bronchial washings from asthmatics after segmental allergen,(44) and in NLF after nasal challenge in patients with allergic rhinitis.(45) We found that the major driver of increases in MPO, in the context of our model, was the exposure to allergen but the result of our ordinal analysis suggests that the increase in MPO in response to allergen is modified by exposure to DE.

CC16 decreased in response to DE, with relatively little contribution from allergen. Several previous studies have evaluated serum levels of CC16 in response to air pollutants,(46-49) but there are no prior reports of the effect of DE specifically on BAL concentrations of CC16. Our finding that BAL levels of CC16 are suppressed after exposure to DE is consistent with reports of CC16 repression in airway tissues and bronchoalveolar lavage fluid (BALF) of smokers;(50-52) but contrasts with the report of Broeckaert et al.,(53) showing no change in BALF levels of CC16 in response to ozone, another common air pollutant. We know of only one other human study on BAL levels of CC16 after allergen challenge,(54) which showed variable increases in individual patients with asthma after allergen exposure. The mechanism by which DE reduces airway CC16 is unknown. However, it is well established that CC16 plays an important role in protecting airway epithelium against oxidative stress.(55,56) We postulate that DE may have similar effects as cigarette smoking in depleting CC16, which over time, may have deleterious effect in airway repair and remodeling that may help explain the epidemiologic association of air pollution with COPD. Further studies will be required to validate this hypothesis.

The strengths of our study include its crossover double-blind design, which allows us to internally control for variability within individual subjects and allows us to investigate the effects of allergen and DE at the level of the airways and systemically. However, our research protocol does not allow us to look for very early changes in response to allergen or DE in bronchial samples, which may occur prior to our 48-hour post-exposure bronchoscopy.

DE and allergen exposure have differential effects on SPD, MPO, and CC16. That SPD and MPO levels in BALF are responsive to allergen, whereas CC16 is more responsive to DE exposure, highlights a complex interaction between the lung and environmental exposures. Further study is needed to elucidate the mechanisms driving these changes and compare the effect of DE and allergen with patients who have pulmonary disease such as COPD, a group likely more vulnerable to the health effects of air pollution.

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Contributions: CC conceived of and recruited subjects for the study. ST and YRC carried out all experimental assays, with input from DDS. BJB conducted data analyses. BJB and CC wrote the manuscript. CC had full access to the all study data, and takes responsibility for the integrity of the data and the accuracy of the data analyses. All authors read and approved the final manuscript.

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Figure Legends

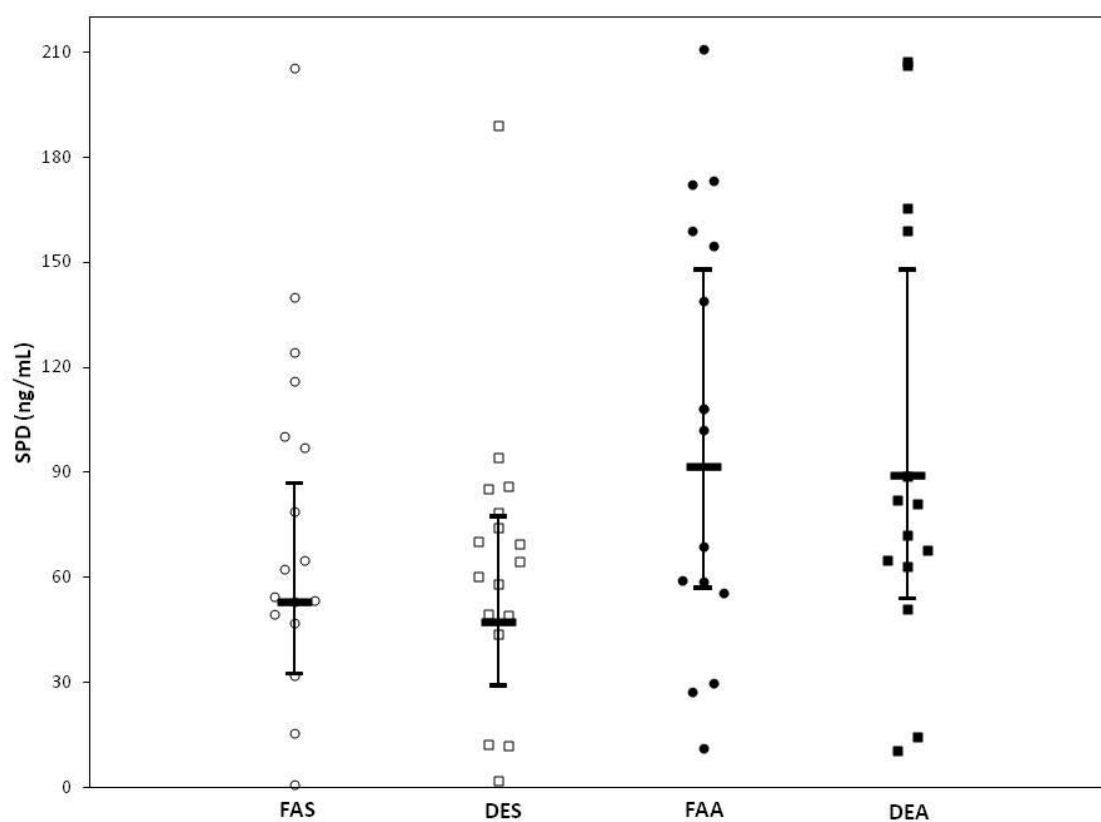
Figure 1: Concentration of SPD (A.), MPO (B.) and CC16 (C.) after exposure to inhaled diesel exhaust or filtered air (squares and circles, respectively) and endobronchially-instilled saline or allergen (open and solid shapes, respectively). Raw individual patient data is shown. Open circles represent filtered air and endobronchial saline (FAS), and open squares represent diesel exhaust and endobronchial saline (DES). Solid circles represent filtered air and endobronchial allergen (FAA) and solid squares represent diesel exhaust and endobronchial allergen (DEA). Black bars represent means and 95% confidence intervals.

ID	Age	Gender	Smoker	Allergen	Asthmatic	Hyperresponsive to Methacholine (<8 mg/mL)	FEV ₁ (% Predicted)
1	25	F	No	Dust Mite	No	No	117.3
2	27	M	No	Dust Mite	No	No	105.1
3	34	F	No	Timothy/Pacific Grasses	Yes	Yes	78.6
4	32	M	Yes	Timothy/Pacific Grasses	No	No	123.4
5	24	F	No	Dust Mite	Yes	No	116.2
6	31	F	No	Dust Mite	No	No	112.9
7	20	F	No	Dust Mite	Yes	Yes	104.0
8	22	F	Yes	Timothy/Pacific Grasses	Yes	Yes	107.3
9	22	F	No	Birch	Yes	No	143.6
10	23	M	Former	Dust Mite	No	No	112.3
11	23	F	No	Dust Mite	No	Yes	100.8
12	23	M	Former	Timothy/Pacific Grasses	Yes	Yes	104.2
13	28	M	No	Timothy/Pacific Grasses	Yes	No	100.5

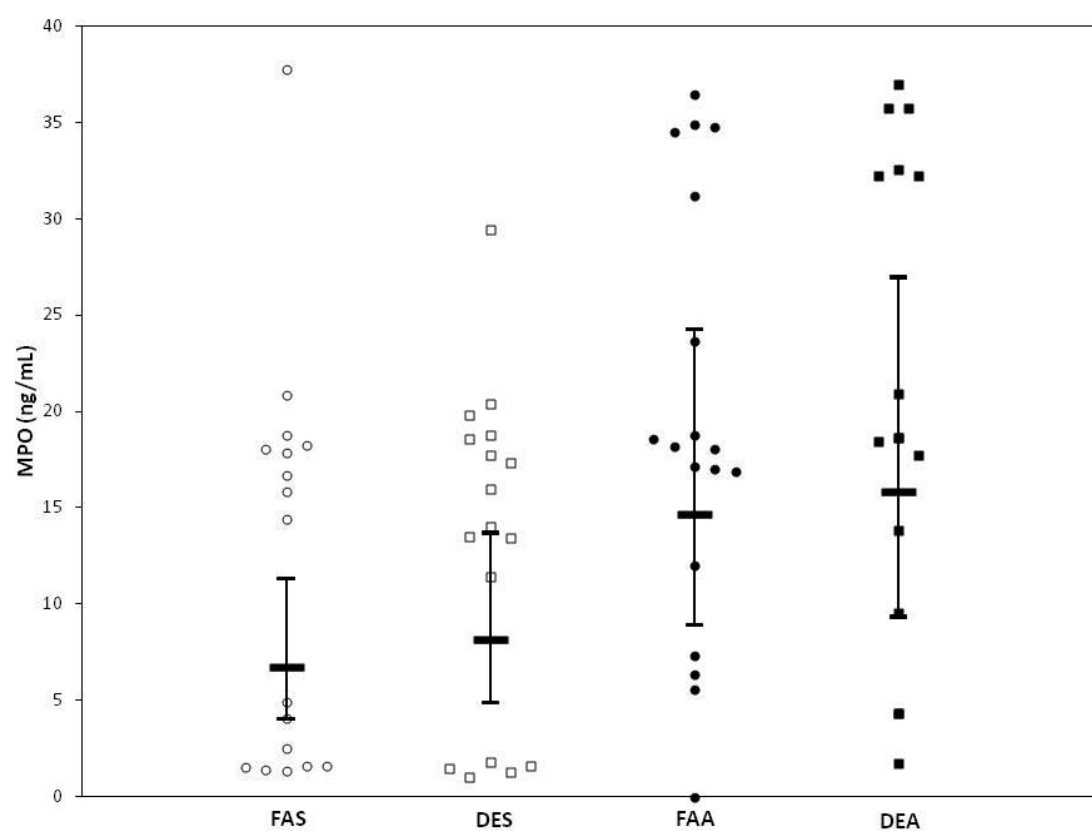
14	31	F	Former	Birch	No	Yes	103.5
15	20	F	No	Timothy/Pacific Grasses	No	Yes	98.4
16	46	F	No	Dust Mite	Yes	No	63.3
17	27	M	Former	Timothy/Pacific Grasses	No	No	107.4
18	46	M	No	Birch	No	Yes	65.8

Table 1: Demographic and clinical data for the 18 subjects.

A.



B.



c.

