

Particle Depletion Does Not Remediate Acute Effects of Traffic-Related Air Pollution and Allergen

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At a Glance Commentary (200 characters max)

Scientific Knowledge on the Subject

Health benefits of particulate-reducing technologies have been largely untested.

What This Study Adds to the Field

Particle-depletion of diesel exhaust increased NO₂ and exacerbated allergen-induced loss of lung function, particularly in those genetically at risk.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org.

Abstract

Rationale: Diesel exhaust (DE), an established model of traffic-related air pollution, contributes significantly to the global burden of asthma and may augment the effects of allergen inhalation. Newer diesel particulate-filtering technologies may increase NO₂ emissions, raising questions regarding their effectiveness in reducing harm from associated engine output.

Objectives: To assess the effects of diesel exhaust and allergen co-exposure on lung function, airway responsiveness, and circulating leukocytes, and determine whether diesel exhaust particle-depletion remediates these effects.

Methods: In this randomized double-blinded crossover study, 14 allergen-sensitized participants (9 with airway hyper-responsiveness) underwent inhaled allergen challenge after 2h exposures to DE, particle-depleted DE (PDDE) or filtered air. The control condition was inhaled saline after filtered air. Blood sampling and spirometry were performed before and up to 48h after exposures. Airway responsiveness was evaluated at 24h.

Measurements and Main Results: PDDE-allergen co-exposure impaired lung function more than DE-allergen, particularly in those genetically at risk. DE-allergen and PDDE-allergen each increased airway-responsiveness in normally-responsive participants. DE-allergen increased blood neutrophils, and was associated with persistent eosinophilia at 48h. DE and PDDE each increased total peripheral leukocyte counts in a manner affected by participant genotypes. Changes in peripheral leukocytes correlated with lung function decline.

Conclusions: Co-exposure to diesel exhaust and allergen impaired lung function, which was worse after particle-depletion (which increased NO₂). Thus, particulates are not necessarily the sole or main culprit responsible for all harmful effects of diesel exhaust. Policies and technologies aimed at protecting public health should be scrutinized in that regard.

(246/250 words)

Keywords: Diesel exhaust, asthma, allergy, filter, genetic susceptibility

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Introduction

Air pollution was responsible for an estimated 6.5 million global deaths in 2015 (1); in some countries, half of all mortality relating to air pollution can be attributed to motorized traffic (2). Traffic-related air pollution (TRAP) exposure has been strongly linked to airways disease, including asthma incidence, hospitalization, and related symptoms, particularly in children (2-4). However, the mechanism(s) through which TRAP may lead to the development or exacerbation of asthma are not well understood.

Previous acute human exposure studies have demonstrated that short-term exposure to TRAP can impair lung function, induce systemic inflammation, and in asthmatics, increase airway hyperresponsiveness (AHR) (5-9). Recent work suggests that the effects of co-exposure to diesel exhaust (DE) and allergen on nasal/pulmonary inflammatory markers may be greater than the added effects of either one alone (10, 11), a synergy which is important to understanding the true health effects of this nearly ubiquitous pollutant. Our group previously revealed that co-exposure to inhaled DE, along with allergen instilled during lung segmental allergen challenge, augmented allergic inflammation in the lower airways relative to allergen alone (10). Furthermore, such effects were more pronounced in genetically 'at-risk' individuals, who were glutathione-S-transferase T1 (GSTT1) null (10). Similar augmented co-exposure effects have been demonstrated in a nasal challenge model (11). The novelty of this paper reflects our motivation, in this context, to understand the following unknowns: the effects of particle depletion on DE's augmentation of allergenic effects, the potential role of genetic susceptibility in modifying this response, and role of a more realistic inhaled (as opposed to instilled) allergen challenge model therein. We

hypothesized that particle-depletion would protect against the harmful effects of DE and allergen co-exposure, and that the effects of co-exposures would be augmented in genetically at-risk individuals.

It is worth noting therefore that a number of diesel engine technologies have emerged in an effort to curtail the growing public-health impact of TRAP (12). Amongst them, diesel particulate filters (DPFs) generally remove ~90% of the particulate fraction of DE emissions, but often increase NO₂ emissions (13). By 2011, an estimated 250,000 on-road heavy-duty vehicles were retrofitted with DPFs worldwide, and since 2000, millions of new diesel passenger cars in Europe have been produced with DPFs (14). In Canada and the U.S., all heavy-duty diesel highway engines produced since 2007 were equipped with DPFs in an effort to meet increasingly stringent emissions standards set by Environment Canada and the Environmental Protection Agency (14). Although the particulate emissions from DPF-fitted engines are drastically reduced, their effectiveness in protecting health in the context of TRAP and allergen co-exposure is not well-understood. Previous controlled human exposure studies achieving ~50% particle depletion have demonstrated that symptoms, lung function deficits, and pulmonary inflammation induced by DE were not attenuated by particle-depletion (8, 9). However, the effectiveness of particle depletion has not been tested in the context of DE and allergen co-exposures, using technology that more effectively reduces particulate matter (at the cost of higher gaseous output). Accordingly, we aimed to address two particular concerns: 1) to directly assess the effects of DE and allergen co-exposure on lung function, airway responsiveness, and circulating leukocyte counts, by simulating real-world conditions with inhaled DE and inhaled allergen challenge models; 2) to determine the efficacy of

particle depletion in protecting against these harmful effects. Some of the results of these studies have been previously reported in the form of an abstract (15).

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METHODS

Study Design

14 allergen-sensitized individuals participated in this randomized, double-blinded, controlled human exposure crossover study taking place between April, 2013 and April, 2017 (Clinical Trials ID: NCT02017431). All participants gave written informed consent to the study protocol which was approved by the University of British Columbia Research Ethics Board (H11-01831), Vancouver. The sample size was based on previous studies detecting a change in blood eosinophil counts after allergen challenge with 12 subjects (16), and blood neutrophil counts after DE exposure with 15 subjects (5). Exposures were carried out at the Air Pollution Exposure Laboratory at Vancouver General Hospital. Each individual was exposed to all of the four co-exposure conditions in random order, each separated by a 4-week washout period: filtered air + 0.9% saline (FA-S, the negative control); FA + allergen (FA-A); DE diluted to 300 $\mu\text{g}/\text{m}^3$ of particulate matter sized 2.5 microns in diameter or less (PM_{2.5}) + allergen (DE-A); and particle-depleted DE + allergen (PDDE-A) (**Figure 1**). Participants stopped bronchodilator use at least 48h prior to study visits, and exposures were not performed on days when participants reported any change in bronchodilator use in the days prior to a visit or exhibited cold or flu symptoms (Common Cold Questionnaire; see detailed methods in the supplemental materials).

Participants

We recruited non-smoking adults, sensitized to at least one of birch, grass, or house dust mite. AHR was assessed at the first screening visit by a methacholine challenge using the 2-min tidal breathing technique (17). The provocative concentration of methacholine eliciting a 20% drop (PC₂₀) in forced expiratory volume in 1 second (FEV₁) during screening was used to define

participants as either hyper-responsive ($PC_{20} \leq 8\text{mg/mL}$) or normally responsive ($PC_{20} > 8\text{mg/mL}$). Six hyper-responsive and two normally responsive participants reported that they had previously received an asthma diagnosis from a physician. Due to this discordance, groups are classified herein based on our measured airway responsiveness, rather than asthma diagnosis. Participant characteristics are detailed in **Table 1**.

Exposures

Exposures (FA, DE, PDDE) were 2h in duration and contained trivial levels of endotoxin (18) (**Table 2**). For PDDE, particulates were removed by HEPA filtration and electrostatic precipitation, generating a representation of newer DPF technology that creates PM-reduced, but NO_2 -enriched, exhaust (**Figure E1**) (19).

Inhaled allergen challenge

One hour after exposure, a 2-min inhaled allergen challenge was performed using an allergen PC_{20} dose that was determined at screening based on methacholine PC_{20} and skin prick wheal size (20).

Lung function, AHR, and blood leucocytes

Spirometry was performed in accordance with the American Thoracic Society's guidelines (21) before and through 48h after exposure, and airway responsiveness was re-assessed by methacholine challenge 24h after each exposure (22). Circulating leukocyte counts were measured before and after exposures. 13 participants performed all three days of each of the four exposure conditions, and one participant voluntarily withdrew from the study after the third visit due to scheduling conflicts.

Genotyping and genetic risk score

Sixteen null alleles, micro insertion/deletion sites or single nucleotide polymorphisms (SNPs) were selected as targets to construct a genetic risk score for each participant who completed the study (**Table E1**) (23-26). These allelic variants were previously suggested to modulate the response to air pollution (24-26). Each individual was assigned an unweighted genetic risk score, which is defined as the unweighted sum of the number of SNP risk alleles (or null for GSTT1 or GSTM1). Details on the genotyping procedure are provided in **Table E2 and E3** and the supplemental methods.

Statistical analyses

Outcomes presented herein for this clinical trial are not the primary or secondary outcomes registered, which will be presented elsewhere. Effects of exposures were assessed using linear mixed effects models (nlme package version 3.1-131) in R (version 3.4.3). Initially, conditions (FA-S, FA-A, DE-A or PDDE-A) were used as the fixed effect, and participant ID as the random effect. Previous work showed that asthmatic individuals have increased AHR after exposure to DE or NO₂ (7, 27, 28). Thus, it was hypothesized that hyper-responsive individuals would have a greater reduction in methacholine PC₂₀ after co-exposures. Therefore, a second model was employed where condition-by-group (normally responsive vs. hyper-responsive) interaction was the fixed effect. A third model, with condition-by-genetic risk score interaction as the fixed effect, was used to assess the potential role of genetic susceptibility in modulating responses. P-values less than 0.05 were considered statistically significant. The potential for order effects were not statistically assessed, as there were 13 unique orders in which the exposures were delivered in our

study. Therefore, the likelihood of a carryover effect producing a false positive result is extremely low. Nonetheless, 18 days was previously shown to be sufficient to avoid carryover in immunological endpoints from a DE particle challenge (29). The area under the FEV₁ curve (AUC) was calculated across time in minutes, from -2min to 4h (representative of sensitive post-exposure period), and 48h (representative of full follow up period) time points shown in figure 1. Blood cell counts that were significantly modified by exposure were tested for their association with 30 min FEV₁, as well as the 48h AUC, by repeated measures correlation using the rmcrr package (version 0.3.0).

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RESULTS

Effective particle depletion decreased total volatile organic compounds, but increased NO₂ levels

PM_{2.5} was effectively depleted on average 93% in the PDDE (20.0 µg/m³) condition relative to DE (290.5 µg/m³) (**Figure 2**). In the gaseous fraction, particle depletion led to a decrease in total volatile organic compounds (TVOC) and an increase in NO₂. The geometric mean aerodynamic diameter of particles in the mixture was significantly greater in DE (84 nm, 95% CI = 77 nm to 91 nm, p<0.0001) and PDDE (75 nm, 95% CI = 66 nm to 83 nm, p<0.05) than FA (63 nm, 95%CI = 58 nm to 68 nm), with no difference between DE and PDDE. As noted in the following mean differences, other measured components of the exhaust were not statistically different in DE relative to PDDE: CO (0.8; 95%CI = -1.7 to 3.2), CO₂ (-58; 95%CI = -208 to 93), NO (215; 95%CI = -149 to 579) and NO_x (117; -242 to 476). Consistent with our previously published exposure characteristics (18), endotoxin levels in the current study were below the threshold limit of detection (0.5 EU/m³).

Particle depletion exacerbated acute airflow impairment induced by diesel exhaust and allergen co-exposure, particularly in those genetically at-risk

There was no effect of DE or PDDE on lung function prior to delivering the allergen challenge (**Figure 3**). As expected, lung function was significantly reduced in all three conditions that included allergen (FA-A, DE-A and PDDE-A), relative to FA-S. At 30 min after exposure, the typical peak of response to allergen-induced lung function decline (30), PDDE-A elicited a 7.5% greater impairment in FEV₁ than DE-A (95% CI = -0.07% to -14.8%, p=0.047), suggesting that particle depletion exerted a deleterious, rather than protective, effect on lung function (**Figure E2**).

After Bonferroni correction for multiple comparisons, which is conservative in this context of non-independent measures and modest risks of type II error (31), there was no difference between DE-A and PDDE-A. However, a post-hoc analysis to estimate the isolated effect of PDDE vs DE showed that, after normalization by subtracting FA-A, the 30m change in FEV₁ associated with PDDE-A was greater than DE-A (mean difference of PDDE-A minus FA-A *versus* DE-A minus FA-A = 8.3%; 95%CI = -16.3 to -0.25, p = 0.05). Furthermore, the effect of condition on FEV₁ was significantly modified by genetic risk score only in the PDDE-A condition; individuals with higher genetic risk score had a greater decrease in FEV₁ at 30 min particularly with PDDE-A (p=0.008 for condition-by-genetic risk score interaction). FEV₁ resolved to the point of non-significance relative to baseline by 48h across all conditions. The 4h AUC was significantly increased by FA-A (2.0x10³ L.min, 95% CI = 0.8 to 3.2, p = 0.001), DE-A (+1.6x10³ L.min, 95% CI = 0.4 to 2.7, p=0.011) and PDDE-A (+2.1 x 10³ L.min, 95% CI = 1.0 to 3.3, p = 0.001), and the 48h AUC was increased by FA-A (+2.4x10⁴ L.min, 95% CI = 1.1 to 3.7, p=0.001), DE-A (+1.9x10⁴ L.min, 95% CI = 0.6 to 3.2, p=0.007) and PDDE-A (+2.2x10⁴ L.min, 95% CI = 0.9 to 3.5, p=0.002) relative to FA-S, with no significant differences between these three conditions. A significant effect of genetic risk score on AUC is detailed in the supplemental materials.

Co-exposure to allergen and diesel exhaust, with and without particulates, significantly increased airway responsiveness in normally responsive participants

Both DE-A and PDDE-A significantly augmented airway responsiveness only in the normally responsive group (mean decrease in methacholine PC₂₀ -13.55 mg/mL after DE-A, p=0.035; -13.2 mg/mL after PDDE-A, p=0.027 [*i.e.*, 1.8 and 2.5 doubling doses, respectively]) (**Figure 4**). There was no significant difference between DE-A and PDDE-A, indicating that particle-depletion did

not protect against the effects of co-exposure on AHR. Alternatively, there was no main effect of exposure on airway responsiveness (across all participants) and, contrary to our hypothesis, no effect in the hyper-responsive participants.

Diesel exhaust and allergen co-exposure led to blood neutrophilia and prolonged eosinophilia

Effects of exposure on systemic WBC counts are summarized in **Table 3**. Only DE-A and PDDE-A increased total WBC counts at 24 hours (**Table 3, Figure 5**). Only DE-A increased neutrophil counts at 24h, and was associated with persistent blood eosinophilia at 48h. Monocyte counts were increased at all time points, and eosinophils at 24h, after FA-A, DE-A and PDDE-A exposure. Changes in cell counts were, almost universally, negatively correlated with changes in FEV₁ at 30 mins and in the FEV₁ AUC (**Table 4**). Significant effect modification by genetic risk score is detailed in the supplemental materials.

Discussion

Particulate-filtering technologies are attractive for their desired potential to mitigate the harmful effects of particulate air pollution, and are already promoted by a number of environmental regulatory agencies (13, 32). However, the current study demonstrates for the first time that exposure to particle-depleted DE, in the presence of allergen, may actually exacerbate some adverse effects of DE on biological and clinical endpoints compared to typical DE. Our data suggest that this deleterious effect of particle depletion may be attributable to differences in the gaseous fraction of PDDE. Of the measured gaseous components of DE (TVOCs, CO, CO₂, NO, and NO₂), the only differences between DE and PDDE in our study were that PDDE contained lower TVOCs and higher NO₂ levels in the emissions, pointing to NO₂ as a potentially important

player in these responses. This is particularly important as recent studies show diesel truck engines trending towards higher NO_x when assessed in real-world conditions (33).

Perhaps most noteworthy in our results is that PDDE-A impaired FEV₁ to a larger extent than DE-A. Although the cause of this worsening is unclear, the increased NO₂ in PDDE seems a plausible contributor. Previous work has demonstrated that acute NO₂ mono-exposure can increase airway resistance (27), and NO₂ can also augment airway responsiveness to an allergen challenge (34). Furthermore, indoor NO₂ exposures are associated with airflow obstruction in atopic children (35). It has been previously determined that a change of 0.23L in FEV₁ could be considered clinically important in the context of asthma (36). In the current study, PDDE-A reduced FEV₁ 7.5% more than DE-A on average, or 0.39L in absolute terms. Therefore, the magnitude of the worsening effect of PDDE-A is, although transient, clinically relevant (37). These findings are in contrast with a previous study, which demonstrated lower levels of thrombus formation and improved response to vasodilators following exposure to particle-depleted exhaust relative to regular diesel exhaust in healthy male volunteers (38). Differences between this and the current study, including subject population, endpoints, and the use of co-exposures, highlights the importance of delineating the effects of particle traps across a range of exposure conditions and biological endpoints.

The method of particle-depletion in the current study, electrostatic precipitation, oxidizes NO, which increases levels of NO₂ in the exhaust. Although in one sense it is a limitation that we were not able to assess the isolated effects of particle depletion (with all gaseous components remaining equal), our exposure characteristics may in fact be more reflective of real-world DPFs and diesel

oxidation catalysts, which tend to suffer from the same trade off (reduced PM and increased NO₂) (39). It should be noted that other after-treatment methods exist, such as selective catalytic reduction technologies, which are designed to reduce NO_x emissions. However, these only recently began to be fitted on new vehicles, still face durability challenges, have suffered from efforts that bypass their effectiveness (40), and have highly variable performance that is dependent on operating conditions (41). DPF technologies have already contributed to a measurable change in roadside pollutants: between 2002-2004, the roadside ratio of NO₂/NO_x in London approximately doubled, coinciding with the London bus fleet being fitted with DPFs (13). Future work would benefit from assessing the longer-term impact of repeated exposures under similar co-exposure conditions to our current study.

This study is the first to directly observe an increase in airway responsiveness, in individuals normally responsive at baseline, in the context of acute air pollution exposure (although an increase in airway resistance has been shown previously in response to diesel exhaust (42), that observation may or may not have been due to changes in airway responsiveness, which was unassessed in that study, and was also not in the context of allergen co-exposure). Specifically, following DE-A or PDDE-A exposure, methacholine PC₂₀ was significantly reduced in those who had normal PC₂₀ values at baseline, despite allergen alone having no effect. While these phenomena are presumed transient, and thus not truly representative of *de novo* disease, co-exposures should be duly considered (43) in future observational efforts to understand new-onset AHR and/or asthma development. Previously, both DE and NO₂ were shown to increase airway responsiveness in some asthmatics (7, 27). The effect in our study was not mitigated by particle-depletion, suggesting an important role for the gaseous fraction of DE. It is unclear why individuals from the hyper-

responsive group did not see an increase in airway responsiveness as has been suggested previously (7, 27). While speculative, we suggest that increased airway responsiveness is more likely in the context of allergen co-exposure than with diesel exhaust alone, as our previous work also demonstrated a trend in this direction (44) (with only a segmental allergen challenge, expected to be less potent in driving this effect than would be inhaled whole-lung allergen, as in our current study).

As noted previously, we earlier tested the effects of DE and allergen co-exposure using a segmental allergen challenge (10), which precluded us from delineating systemic effects. Accordingly, the current study is the first to examine the effects of DE-A and PDDE-A co-exposures on circulating leukocytes. In the current study, blood eosinophilia persisted up to at least 48h only after DE-A exposure. This may be a consequence of particle deposition prolonging the effective exposure seen by the lung (45). This is consistent with previous work demonstrating that ultrafine particles, including the average particle diameter of 100 nm in the current study, have high levels of deposition, may evade phagocytosis, and may penetrate into interstitial sites and even the circulation (45).

Additionally, only DE-A significantly increased neutrophil counts, while there was a strong trend for PDDE-A ($p=0.07$), and only these co-exposures, but not FA-A, significantly increased total WBC count. Previous controlled human exposure studies to DE alone found effects on differential blood cell counts to be modest (i.e. neutrophils alone being affected at 6h) or absent (5, 46). However, DE + ozone was previously shown to induce peripheral monocyte, lymphocyte, and neutrophils effects, the latter two of which persisted up to 22h, highlighting the importance of co-

exposures in eliciting potentially greater responses (47). The negative correlation between blood cell counts and FEV₁ suggests that circulating leukocytes may play a meaningful role in eliciting lung function decrements in the context of these exposures. Elevated peripheral WBC counts, which were observed after DE-A and PDDE-A, are also an independent predictor of all-cause mortality at the epidemiological level (48). Nonetheless, there was no difference between DE-A and PDDE-A exposure on blood leucocyte counts, indicating that contrary to the hypothesis, particle depletion did not demonstrate a protective effect therein, suggesting that DE-A was no worse than PDDE-A in this regard, but we were unable to perform an appropriate non-inferiority analysis due to the lack of established minimally important clinical difference for these endpoints. Furthermore, our study may be underpowered for such an analysis.

Finally, it is worth noting that long-term exposure to PM_{2.5} increases risk of cardiovascular disease and mortality steeply at low concentrations, but subsequently responses tend to plateau (49). Dose-response relationships with regards to short-term settings are less well understood, but it is notable that in our acute exposure PM_{2.5} was reduced from 290±25 µg/m³ in DE to 20±9 µg/m³ in PDDE. If these remaining low levels are near the plateau of the dose-response curve in our acute setting, it could explain why overall DE-A and PDDE-A were more similar than different. However, this is speculation until dose-response relationships are better defined in the context of acute exposures.

In conclusion, we showed that diesel exhaust and allergen co-exposures decreased FEV₁ and increased peripheral WBC counts. These adverse effects persisted even after particle depletion, which suggests that some diesel particulate-filtering technologies may not protect against the harmful effects of DE, particularly in the context of allergen co-exposure. Future work should aim

to delineate the effects of longer-term exposures to TRAP and allergen and the potential role of peripheral inflammation in development of asthma as it relates to these co-exposures. Furthermore, using genetic risk scores we identified susceptible subgroups defined by their number of oxidative stress-associated risk alleles. Future work should continue to assess who is most at risk, which components of DE are most harmful, and what technologies, policies, and practices could be employed to reduce TRAP emissions and hence the substantial harm to public health attributable to air pollution exposure.

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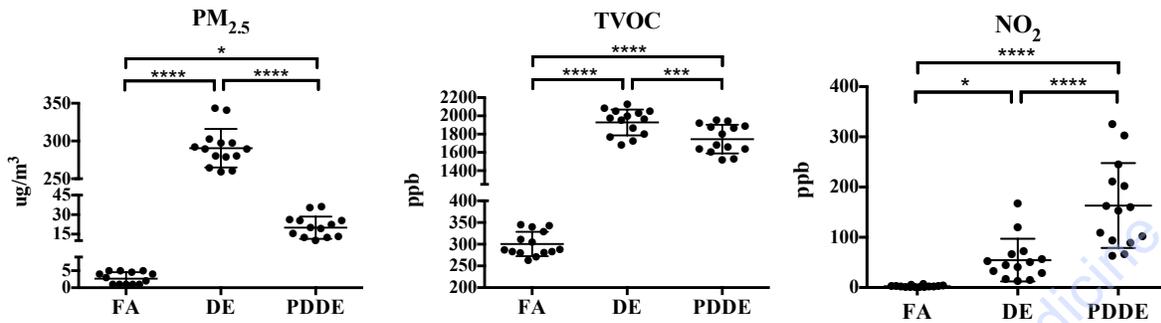


Figure 2. Comparison of filtered air (FA), diesel exhaust (DE) and particle-depleted diesel exhaust (PDDE). $PM_{2.5}$ was depleted on average 94% in PDDE, while TVOC decreased 9.5% and NO_2 increased on average 350%. Each point represents one exposure, lines represent mean \pm SD and differences were quantified using one-way ANOVA with Tukey's post-hoc test, * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

FA = filtered air; DE = diesel exhaust; PDDE = particle-depleted diesel exhaust.

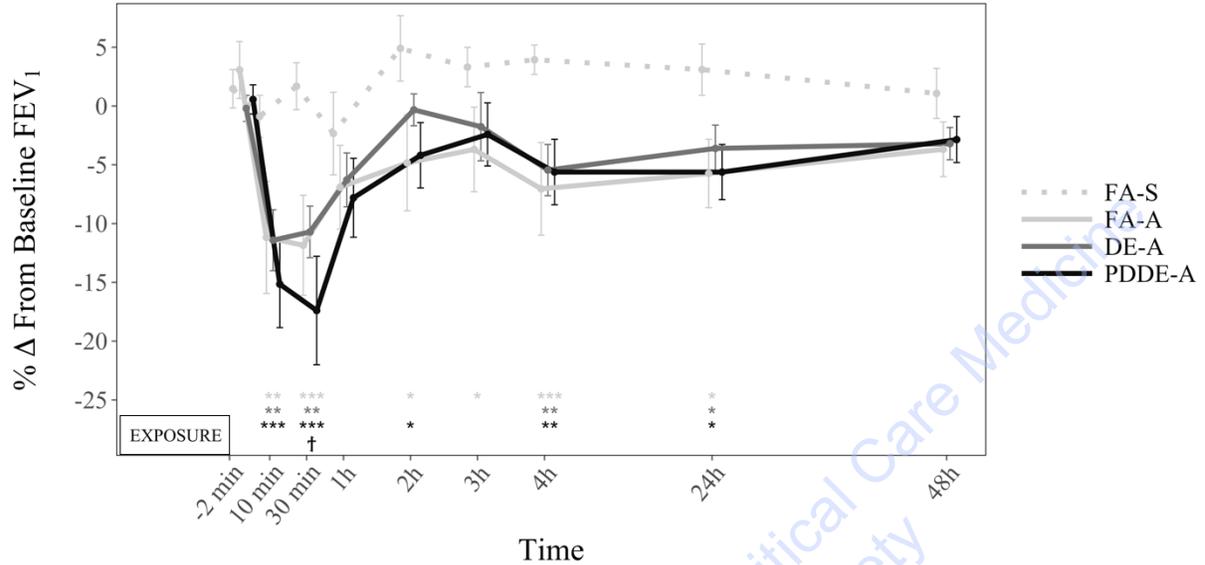


Figure 3. Effect of exposures on forced expiratory volume in 1 second (FEV₁). Values are expressed as % change from same-condition baseline measurements. Lines represent mean of all participants (N=14), error bars represent standard error (SE). Significance relative to FA-S: *p<0.05, **p<0.01, ***p<0.001. Significance relative to DE-A: †p<0.05.

FA-S = filtered air + saline; FA-A = filtered air + allergen; DE-A = diesel exhaust + allergen; PDDE-A = particle-depleted diesel exhaust + allergen.

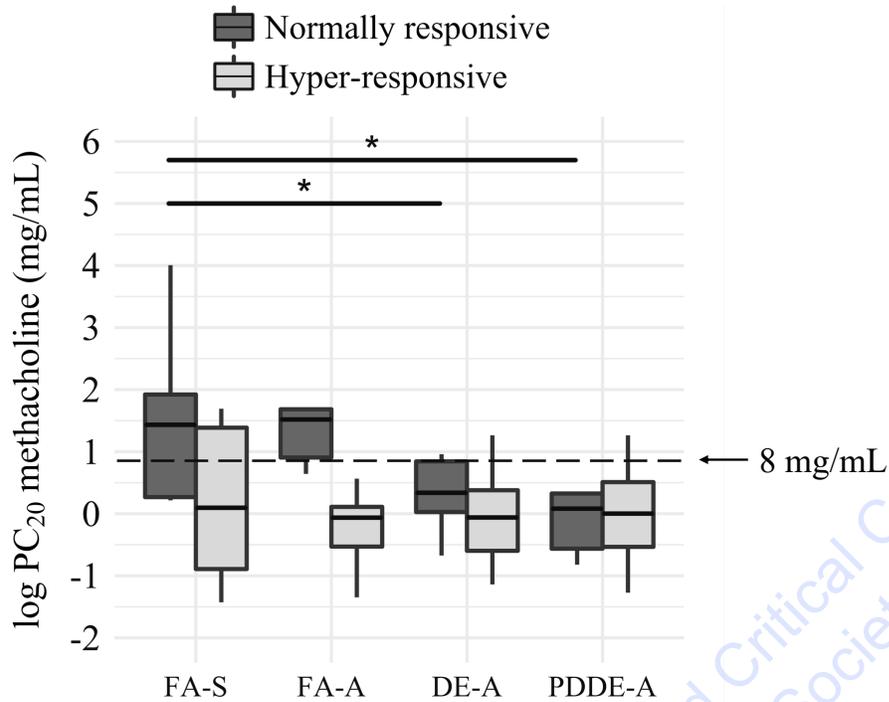


Figure 4. Effect of exposure on airway hyperresponsiveness (AHR). Methacholine PC₂₀ (provocative concentration eliciting a 20% drop in FEV₁) was measured 24h after exposure. Significance bars represent differences induced by exposure, which existed only in the normally responsive group, *p<0.05. Boxes show mean with 25th and 75th percentiles, whiskers extend to largest and smallest value at most 1.5*IQR from box edge. Hashed line represents 8 mg/mL methacholine (for reference as a ‘threshold’ for hyperresponsiveness).

FA-S = filtered air + saline; FA-A = filtered air + allergen; DE-A = diesel exhaust + allergen; PDDE-A = particle-depleted diesel exhaust + allergen.

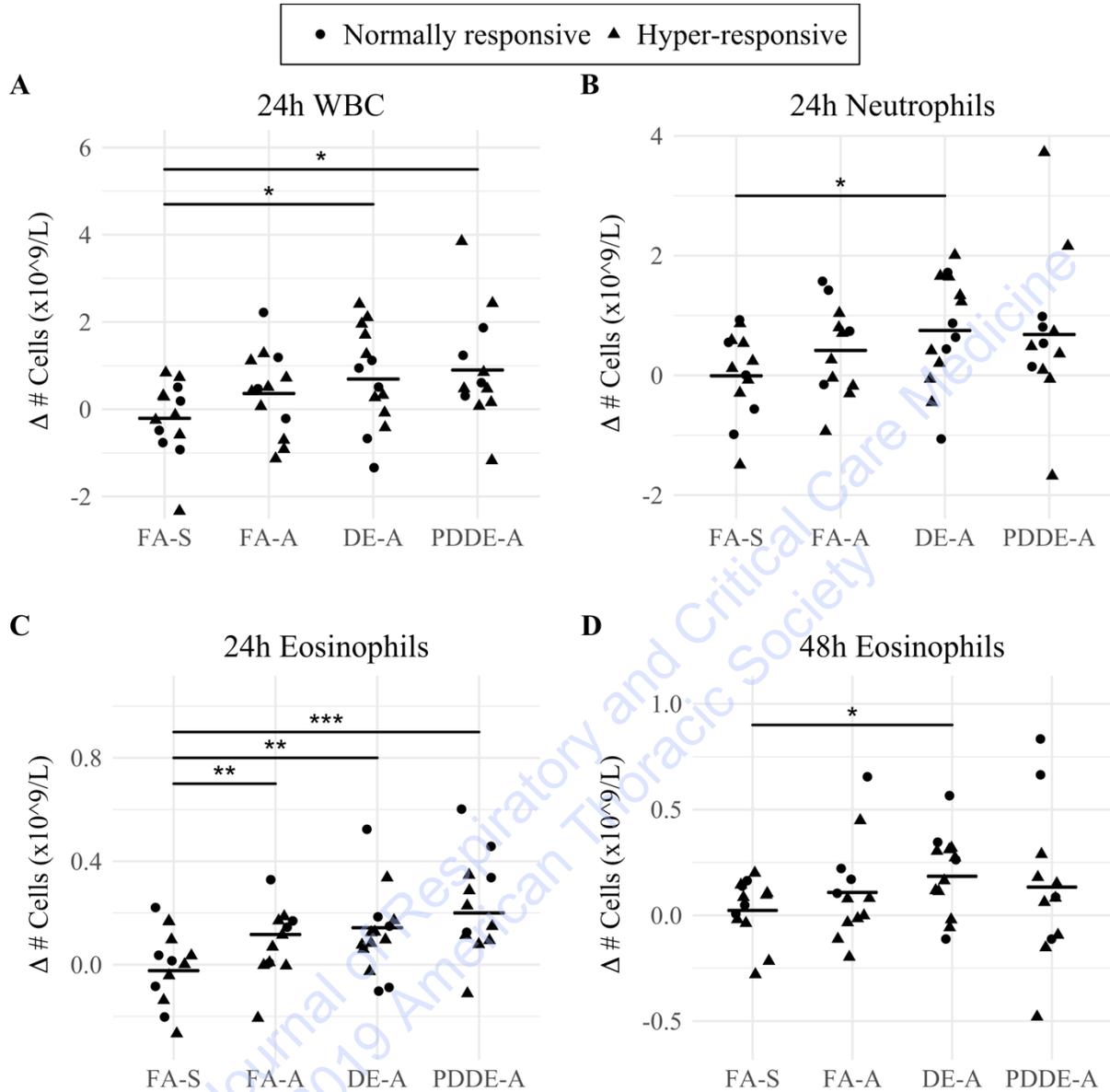


Figure 5. Effect of exposure on blood inflammatory cell counts. Each point represents the change in blood cell counts from baseline to post-exposure for one participant. Significance denoted by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FA-S = filtered air + saline; FA-A = filtered air + allergen; DE-A = diesel exhaust + allergen; PDDE-A = particle-depleted diesel exhaust + allergen. WBC = white blood cells.

Table 1. Participant characteristics.

Participant	Age	FEV ₁ % Predicted	FEV ₁ /FVC	Genetic Risk Score	Methacholine PC ₂₀ (mg/mL)	Sex	Allergen (Concentration)
1	30	108	0.89	15	>128	M	HDM (1/32)
2	28	105	0.87	12	>128	F	HDM (1/32)
3	46	97	0.78	13	>128	F	Grass (1/256)
4	50	122	0.84	N/A	>128	F	Birch (1/64)
5	23	105	0.87	9	>128	M	Grass (1/1024)
6	23	102	0.69	6	0.5	M	Grass (1/66538)
7	32	111	0.76	5	5.9	F	HDM (1/128)
8	28	104	0.78	7	2.6	M	HDM (1/512)
9	33	86	0.73	9	0.8	M	Grass (1/4096)
10	24	100	0.74	7	0.9	F	HDM (1/16384)
11	25	107	0.84	12	6.8	M	HDM (1/512)
12	23	84	0.79	12	1.6	F	Grass (1/256)
13	44	114	0.82	6	6.9	F	HDM (1/4)
14	28	123	0.87	10	3.5	M	Birch (1/8)
Mean ± SD							
	31 ± 9	105 ± 11	0.81 ± 0.06	8 ± 3	5 normally responsive 9 hyper-responsive	7 M 7 F	7 HDM 5 Grass 2 Birch

Definition of abbreviations: FEV₁ = forced expiratory volume in one second. PC₂₀ = provocative concentration eliciting a 20% drop in FEV₁, HDM = house dust mite, SD = standard deviation. Genetic risk score is the unweighted sum of the number of SNP minor alleles (or null for GSTT1 or GSTM1) of fourteen selected SNPs.

Table 2. Exposure characteristics.

	CO (ppm)	CO₂ (ppm)	NO₂ (ppb)	NO (ppb)	NO_x (ppb)	PM_{2.5} ($\mu\text{g}/\text{m}^3$)	TVOC (ppb)
FA	3 \pm 2.1	696.8 \pm 56.2	2.8 \pm 1.7	22 \pm 14.3	24.8 \pm 14.8	2.6 \pm 1.8	300.7 \pm 29.2
DE	14 \pm 3.4	2044.1 \pm 202.4	52.5 \pm 40.1	2770.1 \pm 583	2822.5 \pm 569.6	292.2 \pm 24.2	1932.3 \pm 145.4
PDDE	15.2 \pm 8.3	2101.6 \pm 220.2	150.3 \pm 86.2	2555.2 \pm 449.4	2705.6 \pm 447.6	18.9 \pm 8.5	1750.9 \pm 150.6

Definition of abbreviations: FA = filtered air. DE = diesel exhaust. PDDE = particle-depleted diesel exhaust. CO = carbon monoxide. CO₂ = carbon dioxide. NO₂ = nitrogen dioxide. NO = nitric oxide. NO_x = nitric oxides. PM_{2.5} = particulate matter 2.5 microns in diameter or less. TVOC = total volatile organic compounds.

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Table 3. Effect of exposures on blood cell counts and effect modification by genetic risk score.

	Time	FA-S Mean	FA-A Effect (95%CI)	FA-A p-value	DE-A Effect (95%CI)	DE-A p-value	PDDE-A Effect (95%CI)	PDDE-A p-value
WBC	24h	-0.21	0.57 (-0.24 to 1.38)	0.18	0.90 (0.11 to 1.69)	0.03	1.10 (0.28 to 1.93)	0.01
	48h	-0.45	0.30 (-0.42 to 1.03)	0.42	0.64 (-0.07 to 1.36)	0.09	0.64 (-0.09 to 1.37)	0.10
Neutrophils	24h	0.03	0.38 (-0.32 to 1.08)	0.29	0.72 (0.05 to 1.40)	0.04	0.67 (-0.03 to 1.38)	0.07
	48h	-0.36	0.29 (-0.33 to 0.91)	0.37	0.21 (-0.40 to 0.83)	0.50	0.56 (-0.07 to 1.19)	0.09
Lymphocytes	24h	-0.07	0.08 (-0.21 to 0.38)	0.59	-0.06 (-0.34 to 0.22)	0.67	0.07 (-0.23 to 0.37)	0.64
	48h	0.01	-0.13 (-0.37 to 0.12)	0.32	-0.19 (-0.42 to 0.05)	0.14	-0.18 (-0.42 to 0.07)	0.16
Monocytes	24h	-1.18	1.28 (0.67 to 2.30)	0.001 †	1.19 (0.41 to 1.97)	0.005 †	1.22 (0.41 to 2.04)	0.006 †
	48h	-1.22	1.18 (0.47 to 1.91)	0.003 †	1.23 (0.53 to 1.94)	0.002 †	1.24 (0.51 to 1.96)	0.002 †
Eosinophils	24h	-0.03	0.14 (0.04 to 0.24)	0.01	0.17 (0.07 to 0.27)	0.002	0.23 (0.13 to 0.33)	0.0001
	48h	0.01	0.08 (-0.07 to 0.23)	0.31	0.17 (0.02 to 0.32)	0.03	0.12 (-0.03 to 0.27)	0.13
Basophils	24h	-0.006	-0.002 (-0.035 to 0.031)	0.896	0.006 (-0.025 to 0.038)	0.71	0.014 (-0.019 to 0.047)	0.41
	48h	-0.008	-0.001 (-0.043 to 0.042)	0.98	0.008 (-0.034 to 0.049)	0.72	0.024 (-0.018 to 0.067)	0.27

Cell count values are expressed as unit change (cells x 10⁹/L). Filtered air plus saline (FA-S) mean represents the mean change in counts after FA-S relative to pre-exposure. Effect columns represent mean change across the exposure relative to FA-S mean. Significant exposure effects (p<0.05) are bolded. †Denotes exposure effects that were significantly modified by genetic risk score, such that a higher genetic risk score was associated with a greater increase in cell counts following exposure (p<0.05).

Definition of abbreviations: WBC = white blood cells, CI = confidence interval, FA-A = filtered air + allergen, DE-A = diesel exhaust (300µg/m³ PM_{2.5}) + allergen, PDDE-A = particle-depleted diesel exhaust + allergen.

Table 4. Repeated measures correlation between change in blood cell counts and change in forced expiratory volume in one second (FEV₁) across exposures.

Cell	Time	30 min FEV ₁ % Change			AUC FEV ₁ % Change		
		r	95%CI	p-value	r	95%CI	p-value
Monocytes	24h	-0.454	-0.12 to -0.70	0.008	-0.42	-0.66 to -0.11	0.009
	48h	-0.53	-0.21 to -0.75	0.002	-0.39	-0.64 to -0.06	0.019
Eosinophils	24h	-0.573	-0.27 to -0.77	0.0005	-0.57	-0.76 to -0.29	0.0002
	48h	-0.394	-0.03 to -0.66	0.026	-0.47	-0.70 to -0.16	0.003
WBC	24h	-0.432	-0.10 to -0.68	0.011	-0.29	-0.57 to 0.04	0.076
Neutrophils	24h	-0.396	-0.05 to -0.66	0.022	-0.24	-0.53 to 0.10	0.154

Blood cell counts that were significantly altered by exposure (Table 3) were assessed for their correlation with changes in airflow. Columns labeled “r” reflect correlation coefficient between change in blood cell counts relative to pre-exposure, and (in first instance) % change in FEV₁ at 30 minutes relative to pre-exposure and (in second instance) area under the FEV₁ % change curve (AUC), across the entire 48h follow-up period (see Figure 3).

Definition of abbreviations: WBC = white blood cells. AUC = area under the FEV₁ % change curve across the 48h follow-up period.

Particle Depletion Does Not Remediate Acute Effects of Traffic-Related Air Pollution and Allergen

Denise J. Wooding, Min Hyung Ryu, Anke Hüls, Andrew D. Lee, David T. S. Lin, Christopher F. Rider, Agnes C. Y. Yuen, Chris Carlsten

Online Data Supplement

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Detailed Methods

Study Design

14 allergen-sensitized individuals participated in this randomized, double-blinded, controlled human exposure crossover study taking place between April, 2013 and April, 2017 (Clinical Trials ID: NCT02017431). All participants gave written informed consent to the study protocol which was approved by the University of British Columbia Research Ethics Board (H11-01831), Vancouver. The sample size was based on previous studies detecting a change in blood eosinophil counts after allergen challenge with 12 subjects (E1), and blood neutrophil counts after DE exposure with 15 subjects (E2). Exposures were carried out at the Air Pollution Exposure Laboratory at Vancouver General Hospital. Diesel exhaust was generated with a Yanmar L100 EE 4-stroke diesel generator and particle filtering performed by high efficiency particulate air (HEPA) filtration followed by electrostatic precipitation (E3). Each individual was exposed to all of the four co-exposure conditions in random order, each separated by a 4-week washout period: filtered air + saline (FA-S, the negative control); FA + allergen (FA-A); DE diluted to $300\mu\text{g}/\text{m}^3$ of particulate matter sized 2.5 microns in diameter or less ($\text{PM}_{2.5}$) + allergen (DE-A); and particle-depleted DE + allergen (PDDE-A) (**Figure 1**). Operation of the exposure system and preparation of allergen or saline dilutions was performed by individuals not involved in other study procedures or analyses, in order to maintain blinding.

Participants

We recruited via online and paper flyers, and assessed study eligibility during a telephone screening. Inclusion criteria was as follows: age 19-50, non-smoker, and sensitized to at least one of birch, grass, or house dust mite (assessed at first screening visit, where a wheal $\geq 3\text{mm}$ was

considered a positive skin prick). If birch or grass were selected, visits were conducted outside of the regular allergy season. Participants were excluded using the following criteria: pregnant/breastfeeding, use of inhaled corticosteroids, use of bronchodilator medication more than three times per week, unstable asthma symptoms, presence of co-morbidities assessed by the primary investigator, or regular use of antihistamines, non-steroidal anti-inflammatories, anticoagulants, acetylsalicylic acid, or decongestants. AHR was assessed at the first screening visit by a methacholine challenge using the 2-min tidal breathing technique (E4). Participants were defined as either normally responsive or hyper-responsive, based on the provocative concentration of methacholine eliciting a 20% drop (PC_{20}) in forced expiratory volume in 1 second (FEV_1) during screening (hyper-responsive if $PC_{20} \leq 8\text{mg/mL}$). Participant characteristics are detailed in **Table 1**.

Exposures

Exposures (FA, DE, PDDE) were randomized to order, 2h in duration, contained trivial levels of endotoxin (E3), and followed our previously described system with the exception that a constant 2.5kW engine load was used in the present study (**Table 2**). Importantly, we have previously shown under similar conditions that such an approach leads to effective blinding (E5). For PDDE, particulates were removed by HEPA filtration and electrostatic precipitation (Trion HE 1400), generating an approximation of newer DPF technology that creates PM-reduced, but NO_2 -enriched, exhaust (E6).

Many details of our exposure system are described previously (E3). Here, we include added detail surrounding the particle depletion protocol (**Figure E1**). For PDDE exposures, particulate

matter was depleted by the electrostatic precipitator (10 in diagram) immediately prior to where the exhaust enters the exposure booth (11 in diagram). The flow drop from before to after the precipitator is 0.3L/min (1.5L/min pre and 1.2 L/min post). The filters were cleaned on a schedule per manufacturers recommendations, which varied according to how heavily the system was used. It would be impossible to exclude some temporal variability in the day-to-day system operation due to a range of factors, in spite of being minimized by our quality control and maintenance, but any such variability would bias towards the null given the crossover design and order randomization built into the protocol.

Inhaled allergen challenge

Concentrated allergen extract (Omega Laboratories, Montreal QC, Hollister Stier, Birch Cat# LH1169ED, Grass Cat# LH0831TS, *D. pteronyssinus* Cat# LH6692UP) was diluted with 0.9% normal saline. One hour after exposure, a 2-min inhaled allergen challenge was performed using an allergen PC₂₀ dose that was determined at screening. Briefly, the dose was first estimated by a previously described algorithm, based on methacholine PC₂₀ and skin prick wheal size (E7). At a second screening visit, participants were given increasing allergen concentrations surrounding the estimated allergen PC₂₀ until a 20% drop in FEV₁ was achieved. This concentration was used for test days and is reported in Table 1.

Lung function, AHR, and blood leucocytes

Spirometry was performed in accordance with the American Thoracic Society's guidelines (E8) before and through 48h after exposure, and airway responsiveness was re-assessed by methacholine challenge 24h after each exposure (E9). Circulating leukocyte counts were measured

before and after exposures, at a centralized hospital laboratory located at the Vancouver General Hospital in Vancouver, BC. The effects of exposures on total white blood cell (WBC) counts, lymphocytes, monocytes, neutrophils, and eosinophils are presented herein. 13 participants performed all three days of each of the four exposure conditions, and one participant voluntarily withdrew from the study after the third visit due to scheduling conflicts.

Genotyping and genetic risk score

Peripheral blood mononuclear cells (PBMCs) were isolated using BD Vacutainer CPT collection tubes and stored at -80°C until study completion. Fourteen null alleles, micro insertion/deletion sites or single nucleotide polymorphisms (SNPs) were selected as targets to construct a genetic risk score for each participant who completed the study (**Table E1**) (E10-E13). These allelic variants were previously suggested to modulate the response to air pollution and have null or minor allele frequencies such that at least one genetic variant at each allele was anticipated to be present among our 14 participants (E11-E13). PBMC DNA was extracted using Qiagen AllPrep DNA/RNA/miRNA universal kits, according to the manufacturer's recommendations. GSTM1 and GSTT1 genotypes were determined by real-time PCR using a Rotor-Gene 6000 (Qiagen; Toronto, ON) in 15µl PerfeCTa SYBR Green FastMix (Quanta Biosciences; Gaithersburg, MD) reactions, using primers (IDT; Skokie, IL, USA) (**Table E3**). Cycling conditions were: 10min at 95°C, then 40 cycles of 15s at 95°C and 1min at 60°C, followed by melt curve analysis. Samples that did not start amplifying until cycle 32 were classified as 'null' and this was confirmed by analysis of melt curves.

Pyrosequencing assays for SNPs were designed using the PyroMark Assay Design 2.0 software (Qiagen). Regions containing each SNP to be genotyped were amplified using PCR (HotstarTaq DNA polymerase kit, Qiagen) with 15min at 95°C, followed by 45 cycles of 95°C for 30s, 58°C for 30s, and 72°C for 30s, and a final 5min extension step at 72°C, using biotinylated primers (**Table E2**). Streptavidin-coated beads were used to isolate DNA, before strand separation (Pyromark Vacuum Prep Workstations, Qiagen) and sequencing by synthesis on a Qiagen Pyromark Q96 MD Pyrosequencer (**Table E3**) (E14). The genotype of each participant was determined using Pyromark MD software (Qiagen). Each individual was assigned an unweighted genetic risk score, which is defined as the unweighted sum of the number of SNP risk alleles (or null for GSTT1 or GSTM1). This was because the selected SNPs were derived from a number of separate studies with different endpoints, no adequate external weights of the relative contribution of each variant were available for the construction of weighted risk scores (E10). Due to the small sample size of our study, it was further not possible to estimate valid internal weights (E10). Therefore, we used unweighted risk scores, which are more conservative than weighted risk scores, but still much more powerful than single SNP approaches, with a well-controlled type I error (E15).

Statistical analyses

Effects of exposures were assessed using linear mixed effects models (nlme package version 3.1-131) in R (version 3.4.3). Initially, conditions (FA-S, FA-A, DE-A or PDDE-A) were used as the fixed effect, and participant ID as the random effect. Previous work showed that asthmatic individuals have increased AHR after exposure to DE or NO₂ (E16-E18). Thus, it was hypothesized that pre-existing AHR would modify the response to DE, such that hyper-responsive

individuals would have a greater reduction in methacholine PC₂₀ after co-exposures. Therefore, a second model was employed where condition-by-group (normally responsive vs. hyper-responsive) interaction was the fixed effect. A third model, with condition-by-genetic risk score interaction as the fixed effect, was used to assess the potential role of genetic susceptibility in modulating responses. P-values less than 0.05 were considered statistically significant. The potential for order effects could not be reasonably assessed, as there were 13 unique orders in which the exposures were delivered in our study (in this circumstance, the likelihood of such a carryover effect producing a false positive result is extremely low; nonetheless, 18 days was previously shown to be sufficient to avoid carryover in immunological endpoints from a DE particle challenge) (E19). The area under the FEV₁ curve (AUC) was calculated across time in minutes, from -2min to 48h time points shown in Figure 1, using the AUC function from the DescTools package (version 0.99.24). Where there was missing spirometry data, it was replaced with the sample mean to produce a complete curve for each participant in the AUC calculation. Blood cell counts that were significantly modified by exposure were tested for their association with 30 min FEV₁, as well as the AUC, by repeated measures correlation using the rmcrr package (version 0.3.0).

Results

Lung Function

While the average FEV₁ response over time is shown in Figure 3 of the main manuscript, a comparison of the individual FEV₁ responses to DE-A and PDDE-A at the 30-minute time point is shown here (**Figure E2**).

Genetic Risk Score

For some outcomes, higher individual genetic risk scores were associated with greater physiological responses to exposures. The effect of exposure on 4h FEV₁ area under the curve (AUC) was significantly modified by genetic risk score after FA-A (p=0.02) and DE-A (p=0.02), such that the genetically at-risk individuals saw a greater increase in FEV₁ AUC during this acute post-exposure period. Conversely, genetic risk score did not significantly modify the effect of exposures on airway responsiveness. Higher genetic risk scores were associated with a greater increase in monocyte counts after FA-A, DE-A and PDDE-A exposure (data not shown).

Although there is a strong association between air pollution and a number of health effects at the epidemiological level, the degree of this response varies significantly between individuals, which may at least in part be explained by genetics (E20). Our group previously identified the null allele of the GSTT1 gene as one modulator of the response to acute diesel exhaust and allergen co-exposure (E21). A large pooled population-level study also identified GSTP1 variants (rs1138272 and rs1695) as associated with asthma susceptibility in children in the context of air pollution (E12). Given that it is unlikely that a single gene allele is ultimately responsible for individual susceptibility to air pollution, we utilized a composite genetic risk score to assess whether susceptibility to acute co-exposures could be related to a selection of target genetic variants. The genetic risk score is a statistically powerful tool for detecting gene-environment interactions, and facilitates the detection of the cumulative effects of what may be relatively minor individual contributions from each genetic polymorphism (E10). Notably, we found that individuals with higher genetic risk scores had significantly increased peripheral blood cell counts following allergen, and more prominently, co-exposure to DE-A and PDDE-A, as well as greater FEV₁

suppression across the first 4h post-exposure to FA-A and DE-A, and at 30-min in PDDE-A. However, although our genetic risk score indeed supports the hypothesis that at-risk individuals experience greater deleterious effects, these results should be interpreted cautiously due to the small sample size herein.

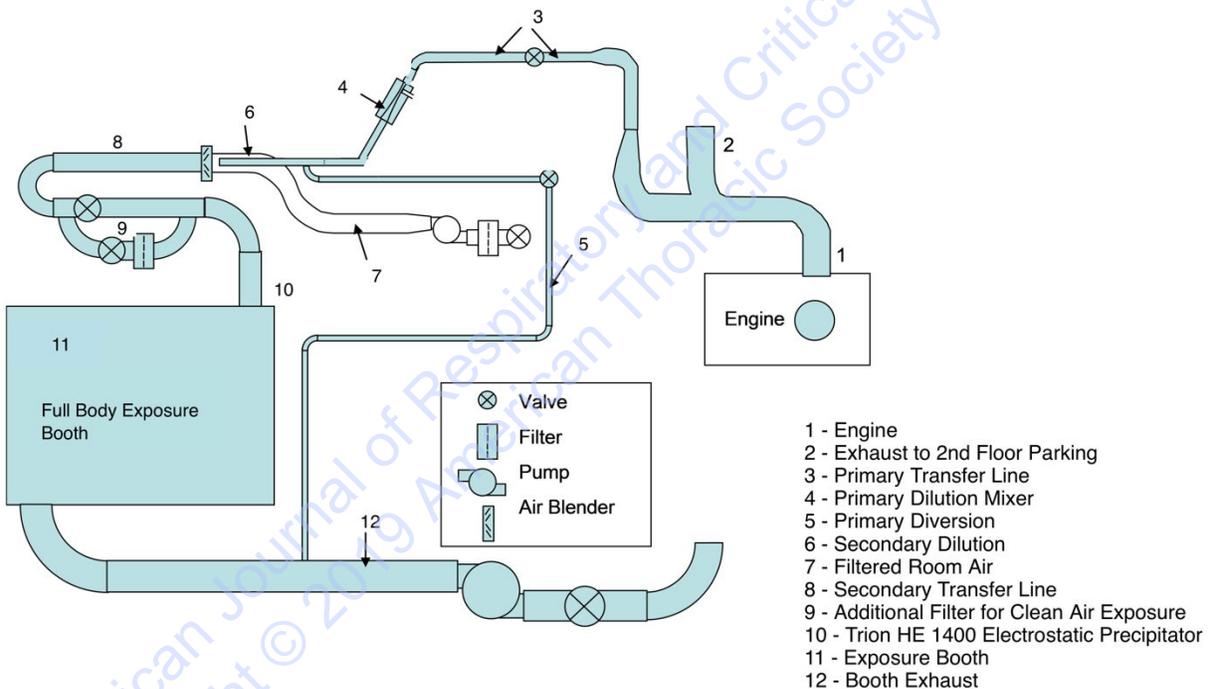


Figure E1. Exposure system schematic for FA, DE, and PDDE exposures.

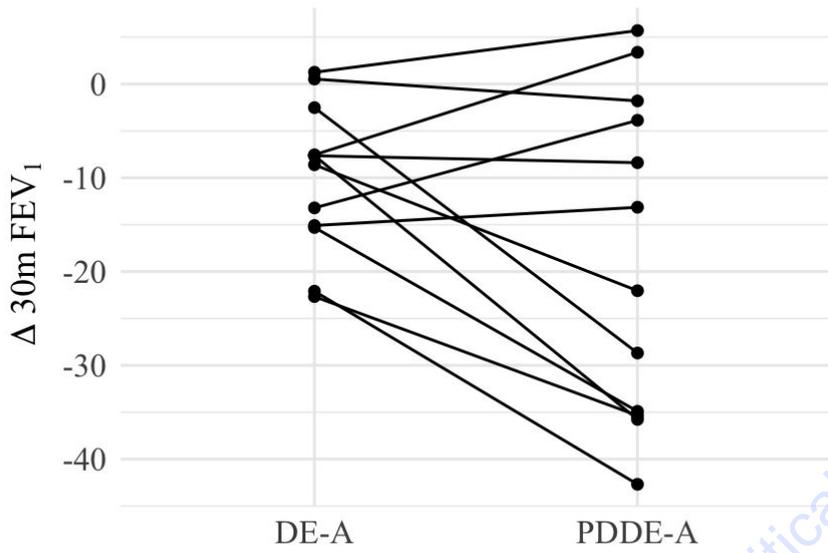


Figure E2. Comparison of individual FEV_1 responses to DE-A and PDDE-A at 30 minutes.

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Table E1. Features included in the genetic risk score and definition of their risk alleles. Risk alleles were defined according to the literature. In absence of a clear definition of risk alleles, the minor allele was used as risk allele. The genetic risk score was calculated as the sum of the number of risk alleles for each participant.

SNP	Risk allele	Reference
GSTT1	Null	PMID: 29074540
GSTM1	Null	PMID: 15528218
rs6726395	G	risk allele as defined in PMID: 21774808
rs1800629	A	risk allele as defined in PMID: 16865291
rs2284367	G	risk allele as defined in PMID: 20049130
rs4880	C	risk allele as defined in PMID: 22531667
rs4646903	G	minor allele
rs1800566	T	risk allele as defined in https://www.snpedia.com/index.php/Rs1800566
rs1051740	C	minor allele
rs1695	G	risk allele as defined in https://www.snpedia.com/index.php/Rs1695
rs2917669	A	minor allele
rs8191438	G	minor allele
rs28362491	deletion	https://www.snpedia.com/index.php/Rs28362491
rs689452	C	minor allele
rs2364722	G	minor allele
rs1138272	T	risk allele as defined in PMID: 24465030

Table E2. DNA Sequences used to perform GSTM1 and GSTT1 genotyping.

Gene	Forward Primer (5'>3')	Reverse Primer (5'>3')
GSTM1	CTTGGAGGAACTCCCTGAAAAG	TGGAACCTCCATAACACGTGA
GSTT1	GTGCAAACACCTCCTGGAGAT	AGTCCTTGGCCTTCAGAATGA

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Table E3. DNA Sequences used to perform targeted pyrosequencing.

SNP ID	Alleles	Strand	Coordinate	Gene	Forward Primer (5'>3')	Reverse Primer (5'>3')	Sequencing Primer (5'>3')
rs6726395	A/G	+	2:177238501	NFE2L2	<i>Bio tin</i> -GGGTTTGATGGCTTTAGCT	ACGTGCCTCATTGATCCACTA	TTTGATCCACTAACATCTG
rs1800629	A/G	+	6:31575254	TNF	<i>Bio tin</i> -CAATAGGTTTGGAGGGGCATG	GCCACTGACTGATTTGTGTGTAG	GGCTGAACCCCGTCC
rs2284367	A/G	-	11:34462995	CAT	<i>Bio tin</i> -GCATTTTGCTTTTCATGTAGATGTGAC	CAGGAGTGGCCAGAAACATATTA	GTAGGGGAAGGAAGAAC
rs4880	C/T	-	6:159692840	SOD2	AGTCGTAGGGCAGGTCCG	<i>Bio tin</i> -GGGCTGTGCTTCTCGTC	GCCCAGATACCCAA
rs4646903	C/T	-	15:74719300	CYP1A1	TGCGGCCCAACTACTCA	<i>Bio tin</i> -TGAGACAGGGTCCCCAGGT	GGAGAATCGTGTGAGC
rs1800566	C/T	-	16:69711242	NQO1	<i>Bio tin</i> -TGGAGTGTGCCAATGCTAT	GCATTTCTGTGGCTTCCAAGTCT	TGGCTTCCAAGTCTTAG
rs1051740	C/T	+	1:225831932	EPHX1	ACTGGAAGAAGCAGGTGGAGATT	<i>Bio tin</i> -TTCTCATGACATACATCCCTCTCTG	GGTGGAGATTCTCAACA
rs1695	A/G	+	11:67585218	GSTP1	AGGACCTCCGCTGCAAAAT	<i>Bio tin</i> -CTGGTGCAAGTCTCACATAG	CCTCCGCTGCAAAATA
rs2917669	C/T	-	16:69725317	NQO1	AGGTCCACAGGGAAGGAAATT	<i>Bio tin</i> -TGTTTCCAAATAATCTCACTCTG	CAGGGAAGGAAATTACAA
rs8191438	CG	+	11:67583625	GSTP1	TGCGGTCTCTTCTGCTG	<i>Bio tin</i> -TGAGTCCCGGAGTCGC	AGGCTTCCCGGCCAG
rs28362491	-/ATTG	+	4:102500998	NFKB1	<i>Bio tin</i> -GGGCTATGGACCGCATGAC	AAGGGCTGGAGCCGGTAG	CGCTGCCGGCCCAA
rs689452	N	-	16:69718561	NQO1	<i>Bio tin</i> -AGCCAGTACGATCAGTCTTCT	TGAAGGTTGCTGTTGGTAAT	GTTTGGTAATGGGTTTT
rs2364722	A/G	+	2:177260059	NFE2L2	CGTCTCAAAAAAAAAAGCCATCTGT	<i>Bio tin</i> -CTGCTTTTAAATAAGCGCCAAACA	TGAATAAATCTTGGCCTA
rs1138272	C/T	+	11:67586108	GSTP1	AGGGATGAGAGTAGGATGATACATG	<i>Bio tin</i> -GGGTCTCAAAGGCTTCAGTT	GGTGTCTGGCAGGAG

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