# RWIP 17-03
# Workplace diesel exhaust exposure: defining a biosignature to support prevention (DICE study)

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## Acknowledgements

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Additionally, a special thank you to all study participants enrolled in the research study. This study would not be possible without their participation and time commitment.

## Executive Summary

The DICE study (Workplace diesel exhaust exposure: defining a biosignature to support prevention) aimed to establish the effects of differing concentrations of diesel exhaust (DE) on the human body. Through investigation on how these concentrations affect the airways and blood, researchers identified factors that are associated with diesel exhaust exposure levels. This could ultimately lead to health monitoring (biomonitoring) of diesel exhaust exposures in workers through effects on the body.

Study participants completed a telephone and in-person screening visit to ensure that they met the inclusion criteria. These criteria included non-smoking, healthy (no doctor diagnosed asthma), between the ages of 19-49, not taking any inhaled corticosteroids, etc. Consenting participants experienced four randomized, double-blinded exposure conditions separated by at least 4 weeks each. These exposures included a control exposure of filtered air (FA) and three DE conditions in the following particulate matter ($PM_{2.5}$) concentrations: 20ug/$m^3$, 50ug/$m^3$, and 150ug/$m^3$. Data was collected via questionnaires, blood, nasal, lung function tests and exhaled nitric oxide test before, during and up to 24 hours after exposures. The methacholine challenge was also administered to test for airway responsiveness before and 24 hours after exposure.

This study endured the two-year COVID-19 pandemic with recruitment starting prior yet significantly delayed by the human research curtailment put into place in March 2020. Despite the challenges, the team wrapped up recruitment with 15 participants completing all trials in the study. Using minimally invasive methods, the researchers identified concentration-dependent increases in airway inflammation and self-reported symptoms, as well as changes in blood proteins associated with inflammation and cardiovascular disease. These proteins will be used in independent corroboratory studies and additional epidemiology of occupational diseases linked to air pollution exposure.

Findings from this research could be utilized by occupational health practitioners to draft policy to further enhance protection of workers. These minimally invasive methods could be adapted into routine tests to monitor the health of workers routinely exposed to DE. Through community presentations, international research conferences, and peer-reviewed journal publications, the researchers have disseminated findings and seek to translate them into practice and policy.

## Project Overview

Approximately 40,000 employees in Manitoba are inadvertently exposed to diesel exhaust (DE) at work because of the wide use of diesel engines in vehicles and machines used in construction, trucking, forestry, transit, and resource extraction. Although local air monitoring of diesel exhaust exposure exists in some occupational settings, such monitoring depends heavily on surrogate models and may yield a distorted picture of individual exhaust exposure. Thus, a clear exposure limit based on biomonitoring is needed to adequately protect workers. Our research looked at utilizing a very sensitive tool known as "proteomics" to identify the subtle yet complex changes in human proteins in blood that occur with varying degrees of diesel exhaust exposure. Additionally, we aimed to look at airway responsiveness (AR) and inflammation, as well as symptoms linked with air pollution exposure. Our research aims to establish the relationship between exposure concentration and biological effect as an aid to determination of reference ranges for acceptable exposure.

**Hypothesis 1:** DE inhalation elicits a characteristic protein output, in a concentration-dependent manner.
**Aim:** Demonstrate, using a proteomic analysis of plasma, a panel of protein markers (biosignature) that acutely responds to a range of occupationally relevant DE concentrations.
*Rationale*: Having a blood-based marker of exposure to DE will facilitate personalized monitoring and more precise links of exposure to potential adverse effects.

**Hypothesis 2:** DE inhalation alters airway inflammation and function, in a concentration-dependent manner, and that alteration is associated with changes in the proteome**.**
**Aim 2a:** Define level of airway inflammation (FeNO) in response to a range of occupationally relevant DE concentrations.
**Aim 2b:** Determine the concentration-dependent changes in lung function ($FEV_1$).
**Aim 2c:** Determine the methacholine response ($PC_{20}$) following exposure to a range of occupationally relevant DE concentrations.
**Aim 2d**: Correlate changes in FeNO, $FEV_1$ and $PC_{20}$ to changes in blood proteins.
*Rationale*: We and others have demonstrated that airway inflammation[1] and responsiveness[2] are altered by a relatively high concentration of DE (300 µg/m3 $PM_{2.5}$), but it is unknown whether similar effects occur at lower DE concentrations and if such potential effects correlate to blood markers of exposure.

**Hypothesis 3:** DE inhalation induces a concentration-dependent increase in symptoms.
**Aim:** Determine concentration-dependent changes in self-reported symptoms using visual analog scale questionnaires [3].
*Rationale:* Symptoms are reliable indicators of the effects of air pollution on health and quality of life [4].

**Urine metabolites:** We previously planned to investigate the concentration-dependent changes in polyaromatic hydrocarbons (PAH) in urine. We were in discussions with Centre de toxicologie du Québec to complete the assays, but they recently discontinued their PAH work. Following

these technical challenges and further consideration, we will be assessing blood gene expression changes instead. Gene expression will provide deeper insight into the mechanisms underlying proteomic changes and health effects. We have sent the samples to the Heart Lung Institute (UBC) for gene expression quantification and analysis.

In our experimental approach, we utilized a crossover study design that essentially avoids the problem of confounding variables – i.e. genetics and lifestyle difference – that can cloud interpretation of observational studies. Accordingly, we enrolled healthy volunteers (N=20) who were non-smokers, as smoking may confound air pollution effects (though we will test for any incidental exposure cigarette smoke). Participants sat in a room for 4 hours and breathed either clean FA or air that contains pollution at levels similar to adverse conditions in occupational settings that use diesel engines [5]. Our lab has a long track record (over 10 years and hundreds of exposures) performing such exposures safely. A lung specialist physician then assesses volunteer's lung health and takes clinical samples. We then used advanced molecular biology tools at the Manitoba Center for Proteomics and Systems Biology (University of Manitoba) to measure different molecules and compare samples from our volunteer participants following exposure to clean air and diesel exhaust. The following figure (Figure 1) summarizes the study design:

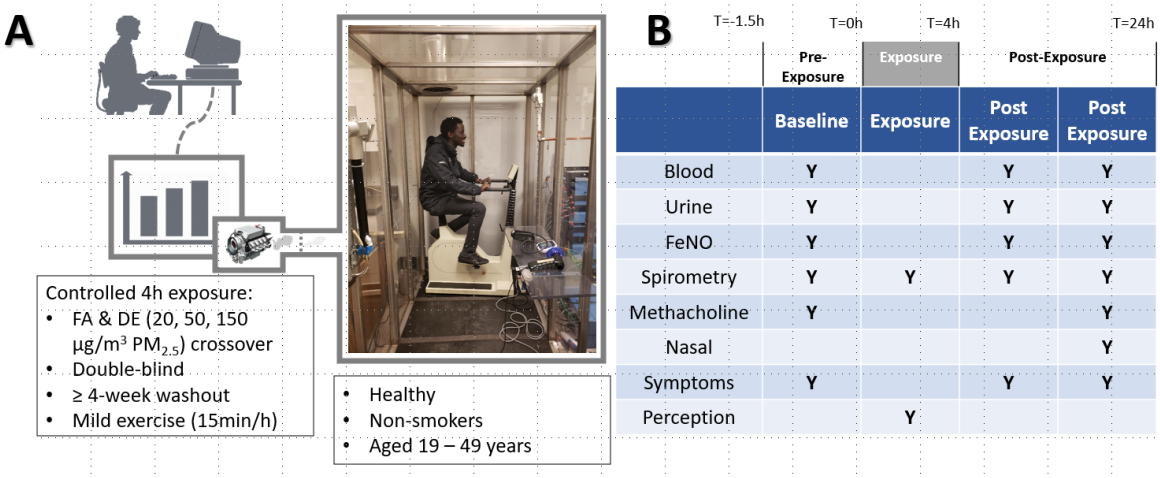



**Figure 1. DICE study design. Exposure setup (A) and sample collection (B).** Telephone and in-person screenings were completed for inclusion criteria. Consenting participants visited the lab over a span of 2 days for each exposure. Data was collected from participants via questionnaires, blood, urine, exhaled nitric oxide test (FeNO), and nasal samples at different timepoints (T). Participants were subjected to a 4-hour (h) exposure to filtered air (FA), and diesel exhaust (DE) at 20, 50, and 150µg/m$^3$ $PM_{2.5}$ with a minimum 4-week washout period between conditions. Airway responsiveness (AR) was assessed on both days using the methacholine test at baseline and 24h post exposure.

Our research aim was to find a simple, clinically relevant strategy that can be used to measure the impact of DE on workers' lung health. This knowledge will empower regulators, companies, and ultimately workers to better manage their health risks. Our research provides specific data to help regulators to make informed decisions about the risks of DE exposure, and assist in creating evidence-based policies to limit occupational DE exposure based on relevant biology.

## Progress and Results

Of the 43 participants screened, 20 participants enrolled in the study (summarized in Table 1). All exposures were well-tolerated and 15 participants completed 4 exposures. Five participants withdrew primarily due to scheduling constraints; 2 completed 3/4 exposures (more than half) and were included in analyses.

**Table 1. Enrolled cohort summary**

| **Characteristics** | **Mean (95% CI)** |
|---|---|
| Sex (M/F) | 11/9 |
| Age (years) | 30.20 (25.84, 34.56) |
| BMI | 24.90 (23.01, 26.79) |
| FEV1 % predicted | 99.57 (95.73, 103.40) |
| FeNO (ppb) | 22.54 (26.14, 18.95) |
| $PC_{20}$ (mg/mL) | >128 |

The team analyzed all the collected data submitted to and published in peer-reviewed journals.

### Proteomics

Using liquid-chromatography-mass spectrometry on plasma isolated from blood samples from this study, we detected 2047 proteins with high confidence. These plasma proteome studies resulted in the following specific outcomes; (1) we identified that specific proteins, MMP and fractalkine, which are risk factors associated with cardiovascular disease, increase following DE exposure even at low concentrations (published in *Environmental Research*, 2022) [6], (2) we identified a panel of proteins that change in plasma in a DE concentration-dependent manner and are enriched in complement activation pathways (Figure 2), of which complement factor I was confirmed using Enzyme-linked Immunosorbent Assays (Figure 3), and (3) we showed that changes in plasma proteins following exposure to DE, even at low concentrations, are different in females compared to males.,. There were more than 90 proteins that changed in response to DE showed sex-related differences. Proteins that showed sex-related differences in response to DE included those related to oxidative stress, inflammatory response elements, cardiovascular disease and antimicrobial peptides. These results suggest that proteins associated with inflammatory response and innate immunity to infections may be differently altered in females and males by traffic-related air pollution.

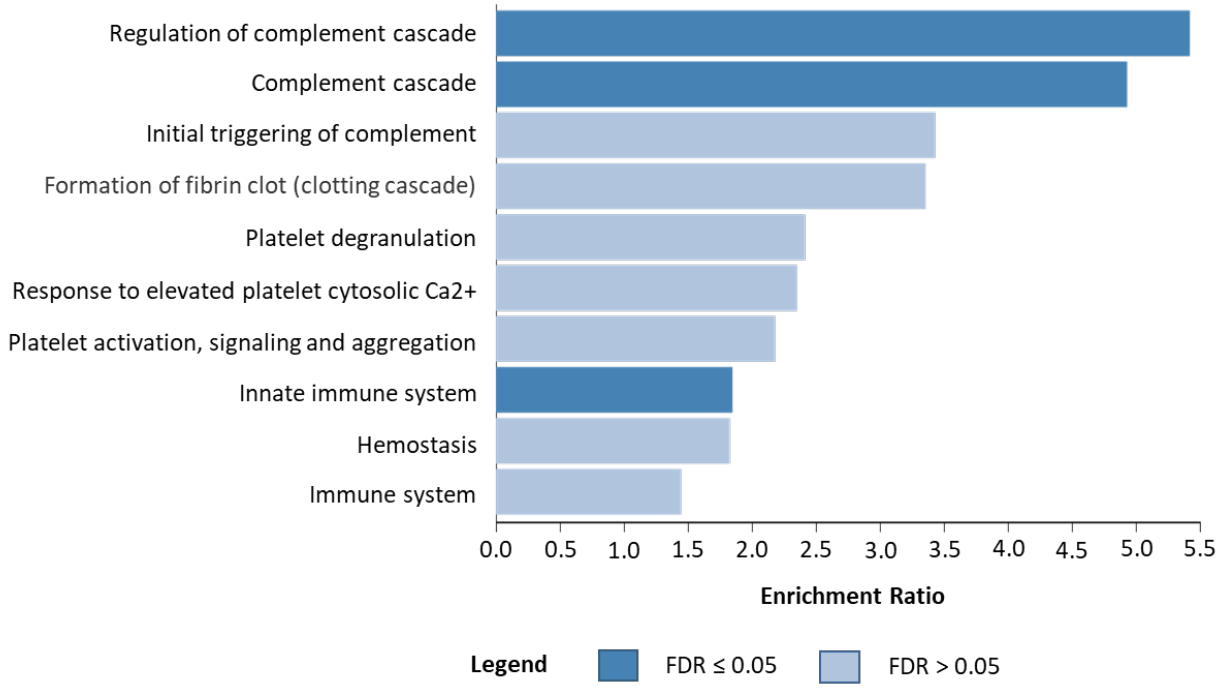



**Figure 2. Pathway enrichment of proteins that exhibit a concentration-response**. Label free mass spectrometry was used to quantify proteins in plasma (at 24h) obtained from 15 healthy non-smokers exposed to filtered air and DE standardized to 20, 50 and 150µg/$m^3$ $PM_{2.5}$ for 4h, separated by ≥4-week washout periods. Forty-five proteins that exhibit a significant ($q<0.05$) concentration-response were identified using linear mixed effects models with a participant-level intercept to account for repeated measures. Pathway enrichment was determined by over-representation analysis using WebGestalt, with Reactome set as the functional database.

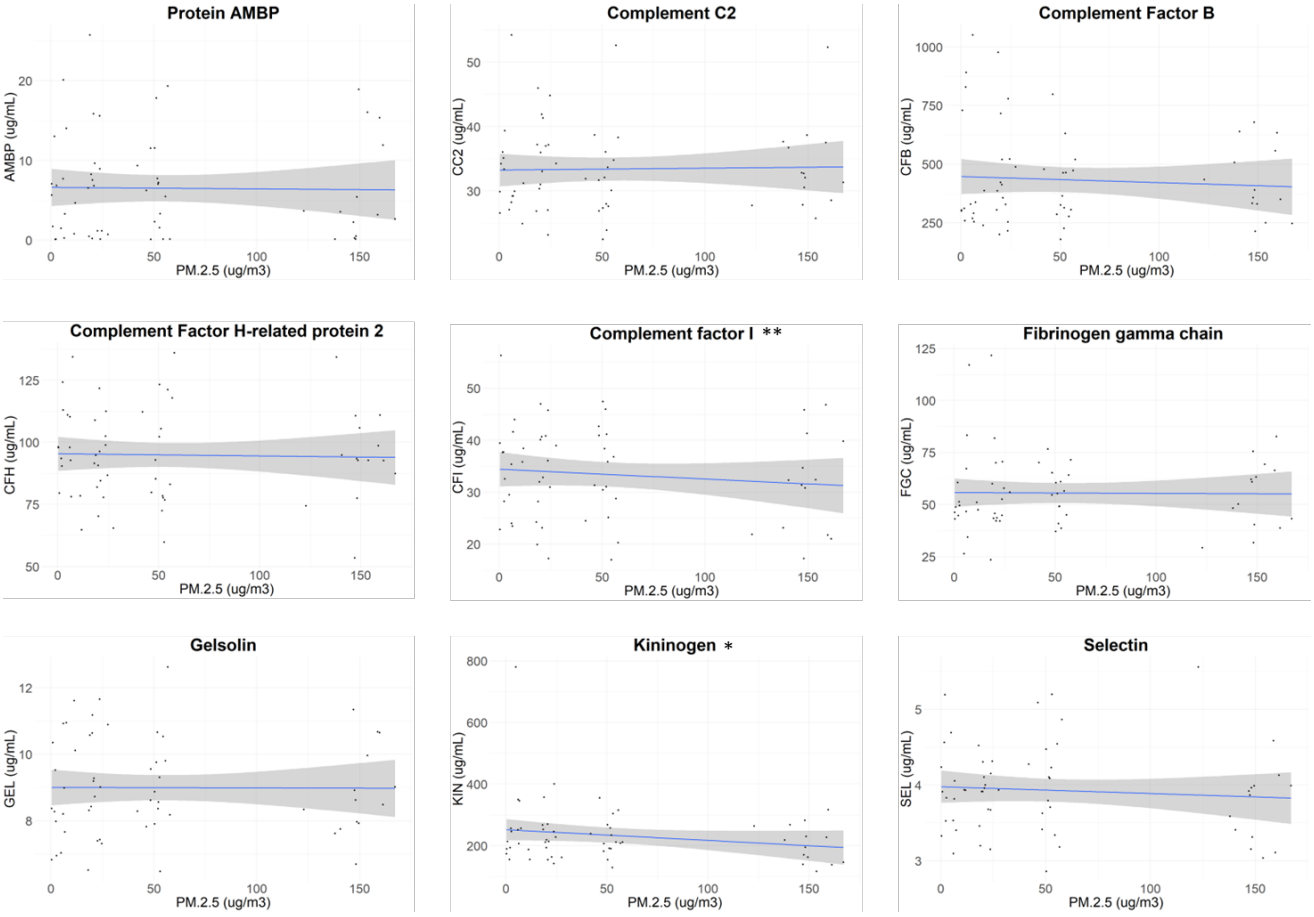



**Figure 3. Diesel exhaust (DE) concentration-responses for top protein candidates.** Enzyme-linked immunosorbent assays were used to quantify proteins in blood (at 24h) from 15 health non-smokers exposed to filtered air and DE standardized to 20, 50 and 150µg/m$^3$ $PM_{2.5}$ for 4h, separated by ≥4-week washout periods. Y axes show protein concentration (µg/mL), while X axes show $PM_{2.5}$ µg/m$^3$. Shaded grey regions are 95% confidence intervals. Linear mixed effects models were used with a participant-level intercept to account for repeated measures: **p<0.05, * p=0.051-0.1.

## Lung Function

DE exposure did not have any significant effects on lung function (Figure 4) and airway responsiveness (Figure 5), likely due to the resilience of this healthy young population and relatively low exposure level and duration of the study.

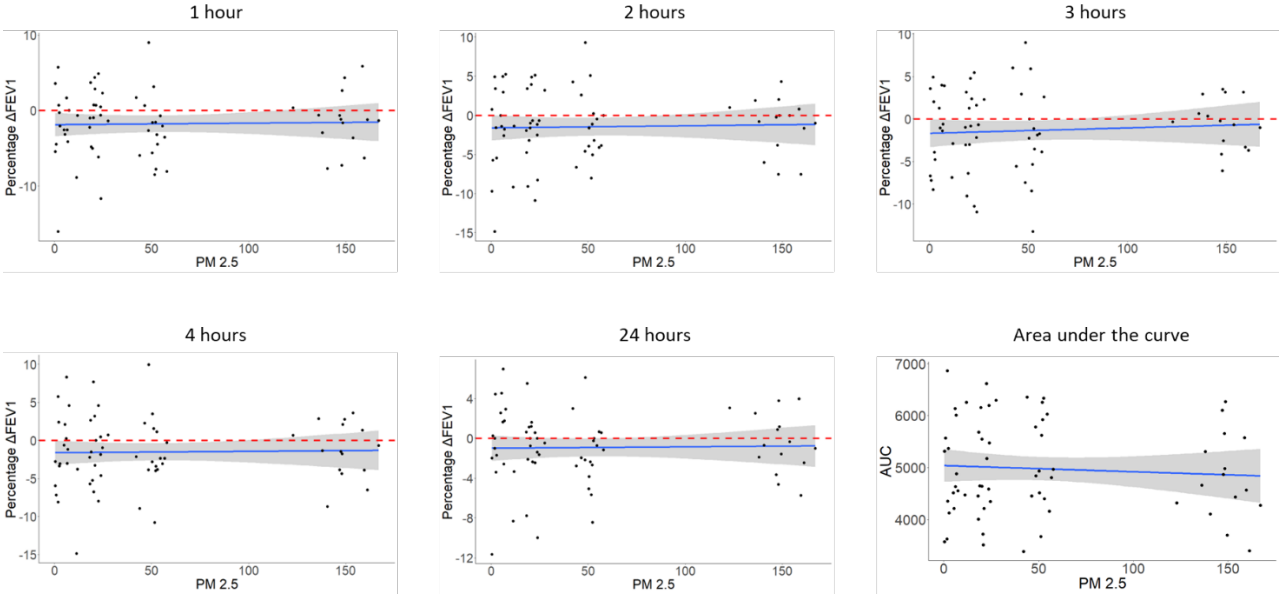



**Figure 4. Concentration-response of diesel exhaust (DE) and forced expiratory volume in 1 second ($FEV_1$).** Spirometry data were collected from participants before, during and up to 24h after exposure to filtered air (FA) and DE standardized to 20, 50 and 150µg/$m^3$ $PM_{2.5}$. The Y-axis shows percentage change in $FEV_1$ from baseline or $FEV_1$ area under the curve (AUC), while the X-axis shows $PM_{2.5}$ values. The dashed line represents 0 (no change from baseline) and grey zones indicate 95% confidence intervals. Statistical analysis was conducted using linear mixed effects models, with participant ID as a random effect.

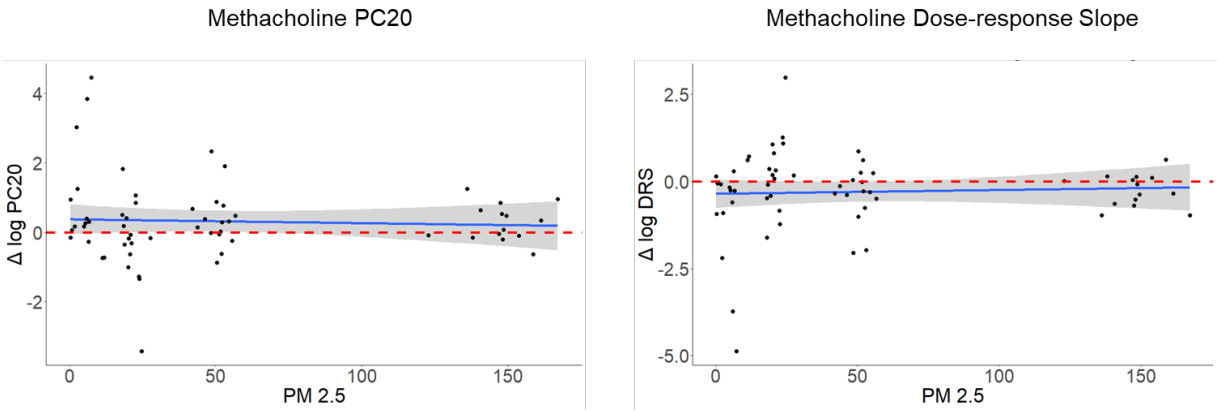



**Figure 5. Concentration-response for methacholine provocation concentration causing a 20% drop in $FEV_1$ ($PC_{20}$) and dose-response slope (DRS).** Spirometry data were collected from participants before and 24h after exposure to filtered air (FA) and diesel exhaust (DE) standardized to 20, 50 and 150µg/$m^3$ $PM_{2.5}$. The Y-axis shows change (delta) in log $PC_{20}$ (left) and DRS (right) from baseline, while the X-axis shows $PM_{2.5}$ values. The dashed line represents 0 (no change from baseline) and grey zones indicate 95% confidence intervals. Statistical analysis was conducted using linear mixed effects models, with participant ID as a random effect.

## Airway inflammation

After adjusting for multiple comparisons, we observed a concentration-dependent increase in FeNO at 4h (0.16% per µg/m$^3$, p=0.01) from initiation of exposure (Figure 6A). Compared to FA, this increase was significant at all 150 µg/m$^3$ while increasing trends were observed at 20 and 50µg/m$^3$ (Figure 6B). These effects were resolved at 24h. Of the nasal proteins we assessed, we observed a trend towards increasing IL-6 (Table 2).

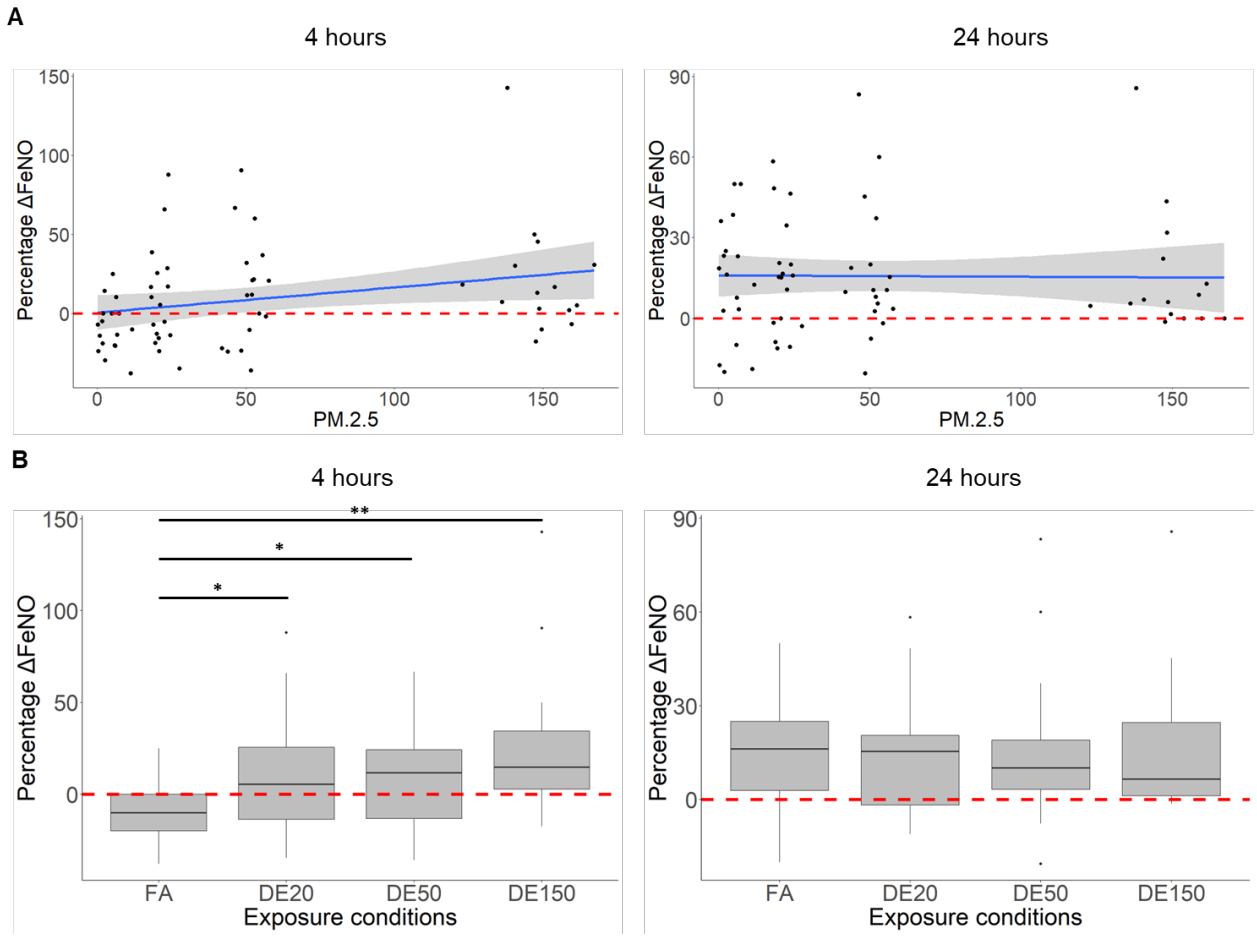



**Figure 6. Diesel exhaust (DE) effect on fractional exhaled nitric oxide (FeNO) summarized by concentration-response (C-R) and nominal exposure category.** FeNO data were collected from participants before, during and up to 24h after exposure to filtered air (FA) and DE standardized to 20, 50 and 150µg/m$^3$ $PM_{2.5}$. Panel A shows the C-R for FeNO; the Y-axis shows percentage change in FeNO from baseline at 4h (left) and 24h (right), while the X-axis shows $PM_{2.5}$. Panel B shows the DE effect on FeNO summarized by nominal exposure category; the Y-axis shows percentage change in FeNO from baseline at 4h (left) and 24h (right), while the X-axis shows nominal exposure category. Statistical analysis was conducted using linear mixed effects models, with participant ID as a random effect: **p<0.05, *p=0.051-0.1.

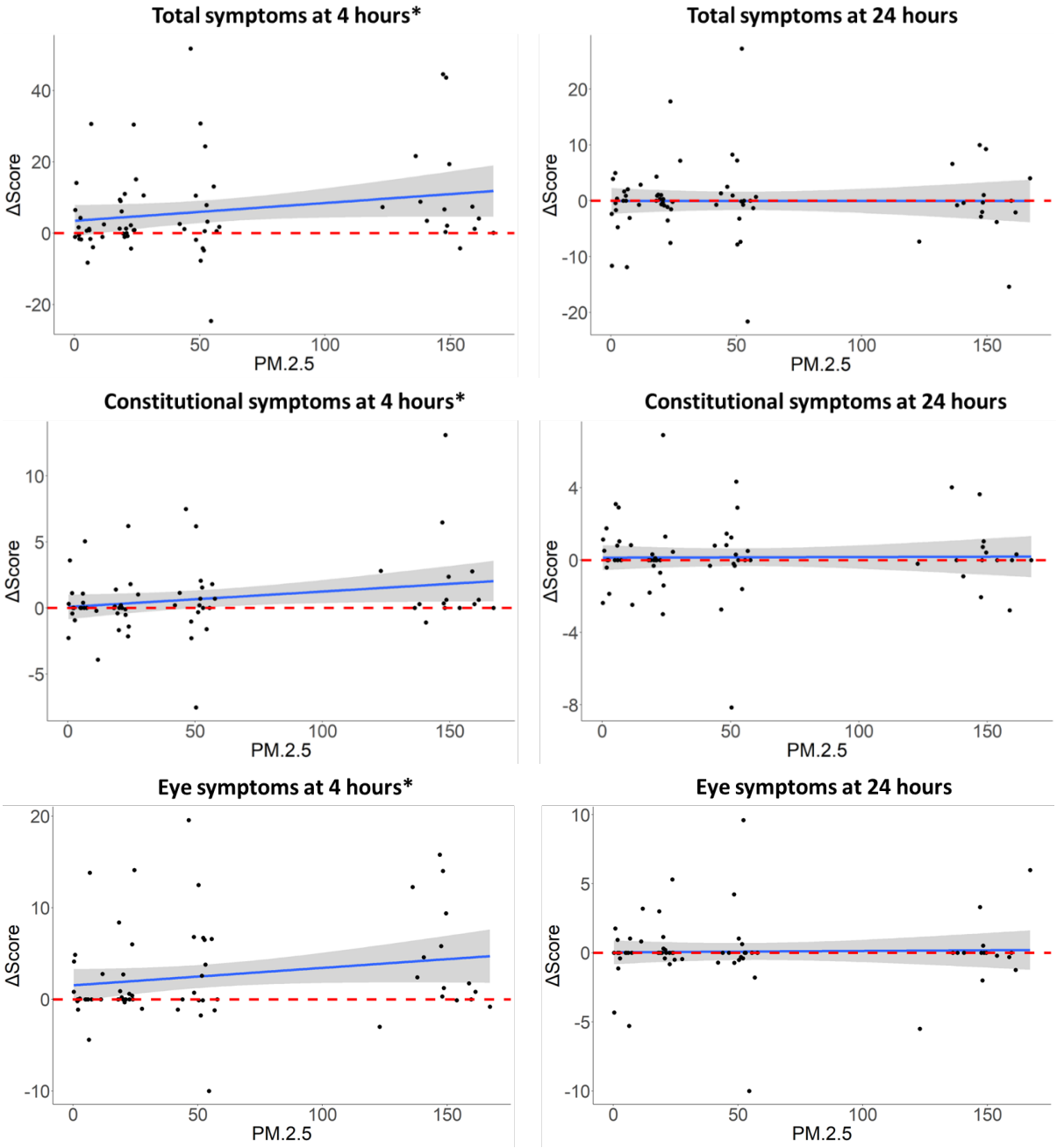
**Table 2.** Concentration-responses for cytokines in nasal epithelial lining fluid at 24h.

| Cytokine | Percent difference (LCI, UCI) | Adjusted p value |
|---|---|---|
| IFN-γ | 0.29 (-0.31, 0.88) | 0.50 |
| IL-1ß | 0.09 (-0.38, 0.58) | 0.78 |
| IL-2 | 0.20 (-0.13, 0.53) | 0.44 |
| IL-4 | 0.12 (-0.23, 0.47) | 0.64 |
| IL-6 | 0.88 (0.08, 1.70) | 0.08 |
| IL-8 | 0.04 (-0.19, 0.27) | 0.78 |
| IL-10 | 0.22 (-0.22, 0.66) | 0.50 |
| IL-12p70 | 0.15 (-0.32, 0.62) | 0.64 |
| IL-13 | 0.12 (-0.13, 0.37) | 0.50 |
| TNF-α | 0.16 (-0.23, 0.56) | 0.56 |

Abbreviations: IFN = interferon; IL = interleukin; LCI = lower 95% confidence interval; TNF = tumor necrosis factor; UCI = upper 95% confidence interval. Concentration-responses were estimated using linear mixed effects models, with participant ID as a random effect. Cytokine concentrations (pg/mL) were log-transformed for analysis but are presented as percentage difference per unit increase in $PM_{2.5}$.

## Symptoms

DE exposure induced a concentration-dependent increase in total symptoms (Est=0.05±0.05, p=0.04), largely due to constitutional (0.01±0.01, p=0.03) and eye (0.02±0.02, p=0.05) symptoms (Figure 7). Participants primarily reported itching and stinging in the eyes (p=0.03) and itchiness or dryness of the skin (p=0.06). Compared to FA, DE at 150 µg/m$^3$ induced an increase in total (8.45±7.69, p=0.04) and eye (3.18±3.03, p=0.05) symptoms, and trends in the nose (1.71±1.88, p=0.08) and constitutional (1.49±1.68, p=0.09) symptoms. These effects were absent at 24 hours.



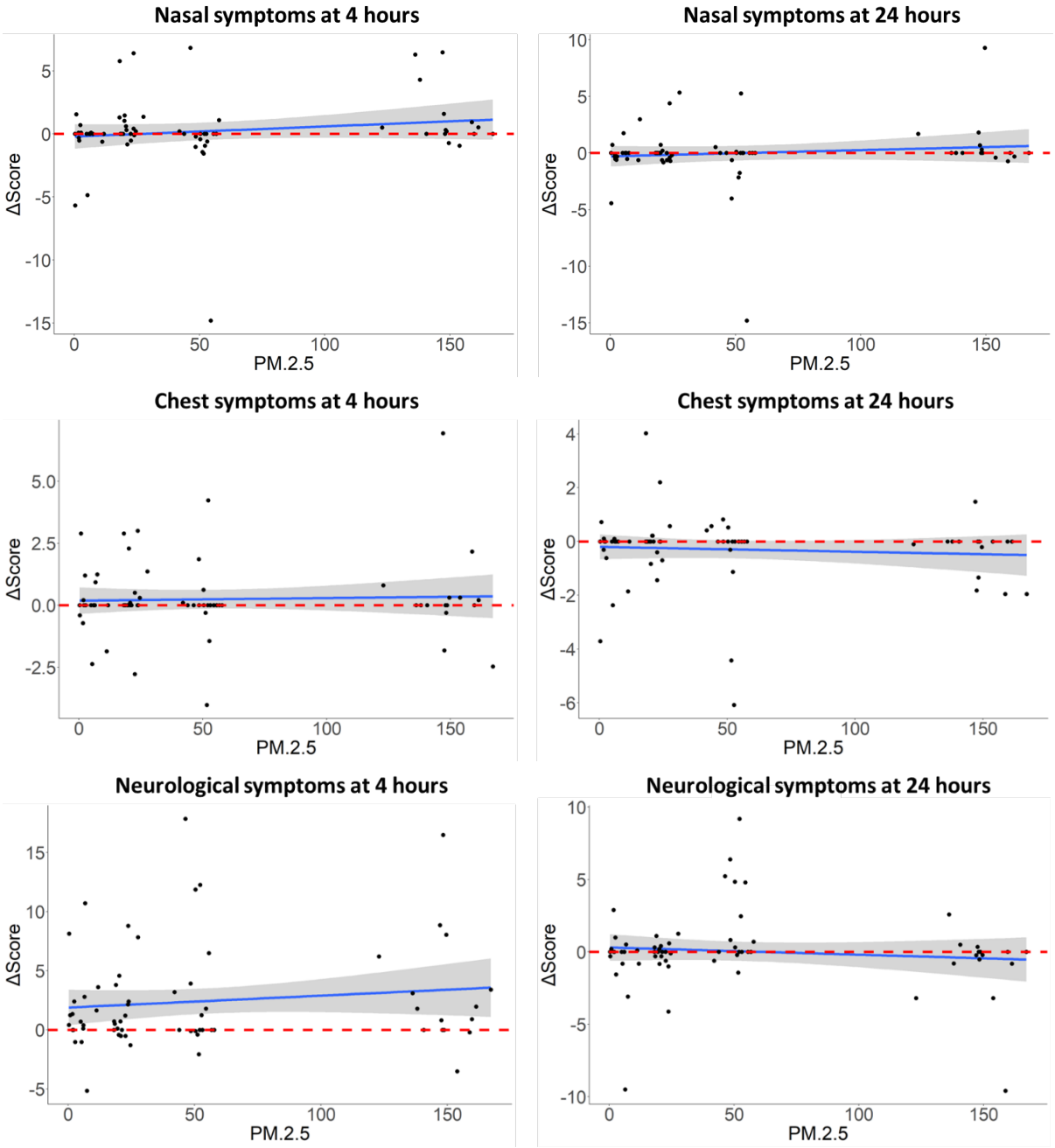



**Figure 7. Diesel exhaust (DE) concentration-response for symptom categories.** Symptoms were recorded before, and at 4 and 24 hours after the start of exposures to filtered air and diesel exhaust (DE) standardized to 20, 50 and 150µg/m$^3$ $PM_{2.5}$. X axes show change in symptom scores from baseline, while Y axes show $PM_{2.5}$ concentrations (µg/m$^3$). Shaded grey regions represent 95% confidence intervals, and the horizontal dashed lines represent 0 (no change from baseline). Linear mixed effects models were fitted with participant ID as a random effect: *p≤0.05.

Following the completion of pending analyses, our findings need to be further confirmed in independent studies, ideally using muti-institutional and international cohorts. The utility of biomarkers identified in this study and those that are confirmed as mentioned above can be

translated for biomonitoring in occupational environments with increased traffic-related or industry-related DE exposures. Additionally, the concentration-response insight from these findings may be crucial for setting exposure guidelines for occupational environments.

## KT efforts

The Carlsten research group has recently published the airways and symptom results above in peer-reviewed journals as *Controlled Diesel Exhaust Exposure Induces a Concentration-dependent Increase in Airway Inflammation: A Clinical Trial* [7] and *Concentration-dependent increase in symptoms due to diesel exhaust in a controlled human exposure study* [8], respectively. Previously, we submitted abstracts *Diesel Exhaust Inhalation During Controlled Human Exposure Induces a Concentration-Dependent Increase in Airway Inflammation (FeNO)* [9] and *Traffic-Related Air Pollution Induces a Concentration-Dependent Increase in Symptoms in a Controlled Human Exposure Study*, which were accepted for the 2020 and 2022 American Thoracic Society International Conferences respectively. Our abstract *Plasma proteomics analysis reveals sex-related differences in response to diesel exhaust* was accepted and published at the 2022 European Respiratory Society International Conference. The manuscript *Concentration-dependent Health Effects of Air Pollution in Controlled Human Exposures* [10] was published in a peer reviewed journal (Environment International, 2021). Juma, a PhD student under Dr. Carlsten's supervision, has previously presented work from this study in the UBC 3MT Thesis competition, where he was the Runner Up for UBC. Additionally, he has presented findings to BC's occupational health stakeholders at WorkSafe BC.

Combined, the Mookherjee and Carlsten research groups have published some of the above discussed results in Environmental Research (2022) [6], have given invited talks at national and international meetings highlighting this work (with acknowledgement to Workers Compensation Board Manitoba) such as conferences organized by The Canadian Respiratory Research Network, Canadian National Proteomics Network, American Thoracic Society, and European Respiratory Society.

The researchers and their trainees have also participated in public lectures, TV interviews such as CTV, CBC, GlobalTV, etc. to discuss the significance of air pollution research. Recent examples include a segment for CBC French in a show called Tout inclus which aired in Summer 2022. This research was also highlighted in multiple international newspapers via press release by The European Respiratory Society in 2022. For example, there was a research feature and interview published by the science correspondent of The Daily Mail (UK) 'Car fumes could pose higher risk to women as they cause increased levels of proteins linked to hardened arteries, research suggest' in 2022. Previous examples include CBC's Nature of Things – Something in the Air episode, and PBS ReInventors by KCTS9 in an episode called "Would you lock yourself in a box of smog – for science?". The knowledge and data acquired from this study is being used further to plan future infrastructure projects, specifically the AirSAFE project, which will strongly benefit further air pollution research being conducted within the province of Manitoba. Collectively, these knowledge dissemination activities have extended our research to stakeholders such as occupational health authorities, clinical practitioners and research scientists.

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