Effects of renewable diesel exhaust on lung function and self-rated symptoms for healthy volunteers in a human chamber exposure study

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Abstract

**Background:** Diesel engine exhaust causes adverse health effects. Meanwhile, the impact of renewable diesel exhaust on human health is less known. In this study, nasal patency, pulmonary function, and self-rated symptoms were assessed in 19 healthy volunteers after two separate 3-hour exposures to renewable diesel (hydrotreated vegetable oil [HVO]) exhaust, and exposure to filtered air (FA) for comparison. The HVO exposures were generated with two modern non-road vehicles (2019) having either: 1) no aftertreatment system (HVO\(_{PM+NOx}\)), or 2) an aftertreatment system containing a diesel oxidation catalyst and a diesel particulate filter (HVO\(_{NOx}\)). The exposure concentrations complied with current EU occupational exposure limits (OELs) of NO, NO\(_2\), formaldehyde, polycyclic aromatic hydrocarbons (PAHs), and future OELs of elemental carbon (EC) from 2023.

**Results:** Exposure to HVO\(_{PM+NOx}\) consisted of PM\(_1\) (≈90 µg m\(^{-3}\), 54 µg m\(^{-3}\) EC) and NO\(_x\) (NO 3.4 ppm, NO\(_2\) 0.6 ppm). The average total respiratory tract deposition of PM\(_1\) was 27 µg h\(^{-1}\). The deposition fraction of HVO PM\(_1\) was 40-50% higher compared to diesel exhaust PM\(_1\) from an older vehicle, due to smaller particle sizes of the HVO\(_{PM+NOx}\) exhaust. Exposure to HVO\(_{NOx}\) consisted mainly of NO\(_x\) (NO 2.0 ppm, NO\(_2\) 0.7 ppm) with low level of PM\(_1\) (~1 µg m\(^{-3}\)). Compared to filtered air, exposure to HVO\(_{PM+NOx}\) and HVO\(_{NOx}\) caused higher incidence of self-reported symptoms (78%, 63%, respectively, vs. 28% for FA, p<0.03). Especially, exposure to HVO\(_{PM+NOx}\) showed 40-50% higher eye and throat irritation symptoms. Compared to filtered air, a decrement in nasal patency was found for the HVO\(_{NOx}\) exposures (-18.1, 95%CI: -27.3 to -8.8 L min\(^{-1}\)), and for the HVO\(_{PM+NOx}\) (-7.4 (-15.6 to 0.8) L min\(^{-1}\)). Overall, no change was indicated in the pulmonary function tests (spirometry, peak expiratory flow, forced oscillation technique), except a slight increase in FEV\(_1\)/FVC after exposure to HVO\(_{NOx}\).
**Conclusion:** Short-term exposure to HVO exhaust below the EU OELs did not cause severe pulmonary function changes in healthy subjects. However, an increase in self-rated mild irritation symptoms, and mild decrease in nasal patency after two HVO exposures may indicate irritative effects from exposure to HVO exhaust from modern non-road vehicles below future OELs.
Introduction

Exposure to petroleum diesel engine exhaust is known to cause adverse health effects (1–4), and since 2012, diesel engine exhaust has been classified as carcinogenic to humans (5). Several human exposure studies have linked diesel exposure to acute health effects, among them short-term reduced lung function (6–10), airway inflammatory responses (10–13), irritation symptoms (6,7,14,15), and cardiovascular effects (16–18). Stricter emission standards in recent years and improved emission reduction techniques have reduced the particle and gas emissions from modern diesel vehicles. At the same time the use of renewable diesel fuels has increased rapidly in an effort to reduce net CO₂ emissions. These fuels, such as hydrotreated vegetable oil (HVO) and biodiesel of fatty acid methyl ester (FAME) types, decrease the particulate matter (PM) emissions compared to petroleum diesel (19–22). However, compared to petroleum diesel the toxicity of these emissions is less evaluated. Only a few controlled human exposure studies of FAME type fuels exist (8,23), while there are to the best of the authors’ knowledge, no such studies on HVO.

Compared to FAME fuels, HVO is the preferred fuel for full substitution (pure biodiesel, B100) of petroleum diesel in non-modified diesel engines due to its greater engine compatibility. With all the known adverse effects of petroleum diesel exhaust from older vehicles, the research focus should be directed to investigate the health effects of realistic exposure concentrations from modern renewable fuels used in vehicles with different exhaust aftertreatment systems.

The exhaust emissions of HVO are, on the one hand, similar to petroleum diesel as the combustion generates carbonaceous PM (“soot”), nitrogen oxides (NOₓ), polycyclic aromatic hydrocarbons (PAHs), etc., but on the other hand, they are different in terms of concentration, particle size and chemical composition (24–27). For example, HVO can decrease the PM emission by 20-50% in comparison to petroleum diesel (20,21). It should be noted that similar to diesel, HVO contains no oxygen or feedstock-derived impurities (e.g., metals) that influence emission characteristics, as do FAME-type biodiesels. The aerosol emission characteristics thus differ for HVO and FAME.
compared to petroleum diesel (22,28). The solid PM fraction of the exhaust from diesel and HVO is dominated by soot, which can be measured thermo-optically as elemental carbon (EC). A new EU occupational exposure limit (OEL) for diesel engine exhaust, measured as EC, of 50 µg m⁻³ (29) will be implemented in 2023 to reduce the exposure of the 3.6 million workers within the member states (30). Because estimates of the life-time mortality risk from occupational exposure to diesel for cancer alone (not including myocardial infarction or COPD) indicate that exposure levels need to be kept extremely low (Vermeulen et al. 2014), it is of key importance to identify potential adverse effects of the substitutes. As the substitution of petroleum diesel by renewable diesel is increasing, a considerable number of people will be exposed to its exhaust. It is hence of interest to understand the potential health effects of exhaust exposure from engines running on HVO, while complying with the future OELs.

Exhaust aftertreatment systems are used in diesel vehicles in order to reduce the environmental and health hazardous emissions of PM (both mass and number concentration), CO, NOₓ, and organic compounds such as PAHs. An aftertreatment system can, for example, contain a diesel oxidation catalyst (DOC) that oxidizes CO and organic compounds (31,32), and a diesel particle filter (DPF) that oxidizes soot particles which removes significant amounts of PM (33). Hence use of aftertreatment systems should reduce exposure to such emissions and their associated health effects. However, a recent review assessing the effects of DPFs’ use on health impacts in occupational settings did not present conclusive results (34). It is thus of interest to investigate the health impact from vehicles with different degrees of emission reduction technology.

Due to improved engine operation, modern diesel engines with or without aftertreatment systems generally emit lower concentrations of pollutants (35) and reduce exhaust particle mass and size (36). Particle characteristics such as size and morphology are of key importance in considering possible health effects, as these characteristics determine where in the lungs the particles will
deposit. The deposition pattern in the lung depends on multiple aerosol characteristics and not solely on the respirable PM mass concentration (37).

In the present study we investigate the human health effects from exposure to exhaust from two modern non-road vehicles (wheel loaders). Due to their engine capacity, they fall under different emission standards and were equipped with 1) no external aftertreatment device, or 2) a DOC in combination with a DPF. The different emissions aftertreatment allowed for a comparison of the exposure to NO\textsubscript{x} from diesel engines with and without a particulate fraction and other gaseous pollutants. We aimed to evaluate self-rated symptoms, nasal patency, and pulmonary function after exposure to HVO exhaust from modern non-road vehicles that complied with the EU OELs. Additionally, we investigated the lung deposition of HVO exhaust particles with the deposition of petroleum diesel from an older light-duty vehicle presented by Wierzbicka et al. in a previous study (15).
Results

Main findings

We compared the effects of exposure to exhaust from hydrotreated vegetable oil (HVO) (a renewable diesel fuel) with filtered air (FA). The vehicle without an external aftertreatment system (HVO\textsubscript{PM+NO\textsubscript{x}}) generated emissions of PM, NO\textsubscript{x} and organic compounds. The vehicle with an external aftertreatment consisting of a DOC and DPF (HVO\textsubscript{NO\textsubscript{x}}) emitted NO\textsubscript{x} with only negligible concentrations of particles and measured organic components (hereafter referred to only NO\textsubscript{x}).

The two exposures to HVO caused mild self-rated irritations symptoms. 44% reported eye irritation symptoms during the HVO\textsubscript{PM+NO\textsubscript{x}} exposure. The number of volunteers who reported throat irritation symptoms was a factor 4.5 and 4 higher for HVO\textsubscript{PM+NO\textsubscript{x}} and HVO\textsubscript{NO\textsubscript{x}} respectively, compared to FA. In comparison to FA exposure, nasal obstruction (lower PNIF) occurred for the HVO\textsubscript{NO\textsubscript{x}} exposure. In the first of the following two sections we describe the exposure aerosol characteristics and the HVO exhaust particle deposition in the airways in relation to diesel exhaust particles. In the second, we describe the health effects in terms of self-rated symptoms and airway function.

Exposure and lung deposition

Exposure aerosol characteristics

A summary of the average aerosol exposure concentrations and characteristics are presented in Table 1. The average aerosol exposure characteristics during the three exposure scenarios are presented in Fig. 1. The average PM\textsubscript{1} concentration during the HVO\textsubscript{PM+NO\textsubscript{x}} exposures was 93±13 µg m\textsuperscript{-3} and the average particle number (PN) concentration was 3.0 \cdot 10\textsuperscript{5} ± 0.3 \cdot 10\textsuperscript{5} cm\textsuperscript{-3} (Table 1). In contrast, during HVO\textsubscript{NO\textsubscript{x}} and FA exposures, the average exposure concentrations of PM\textsubscript{1} were ~1 µg m\textsuperscript{-3} and PN <100 cm\textsuperscript{-3} (Table 1). For HVO\textsubscript{PM+NO\textsubscript{x}}, the elemental carbon (EC) fraction of total carbon was 66±3% corresponding to an average EC concentration of 54±6 µg m\textsuperscript{-3} (Table 1). The
two vehicles were operated in a similar load/idle sequence, which is seen as increasing (during load) and decreasing (during idle) PM1 mass (Fig. 1a) and NO concentrations during HVO\textsubscript{PM+NO}\textsubscript{x} exposure, and increasing/decreasing NO\textsubscript{2} levels for the HVO\textsubscript{NO}\textsubscript{x} exposures (Fig. 1b).

The NO and NO\textsubscript{2} concentrations were comparable for the two HVO exposures (no vehicle had external NO\textsubscript{x} removal devices, such as selective catalytic reduction [SCR]) and on average below the 8-hour OELs. During the HVO\textsubscript{NO}\textsubscript{x} exposure, the average NO\textsubscript{2} was slightly higher and the average NO lower compared to HVO\textsubscript{PM+NO}\textsubscript{x} (Table 1). The variations in NO and NO\textsubscript{2} from the load/idle operation as described above can be seen in Fig. 1b. In addition, NO increased rapidly after the cold start at the start of the HVO\textsubscript{NO}\textsubscript{x} exposure and decreased shortly thereafter when the diesel oxidation catalyst (DOC) of the vehicle has reached the operating temperature and started to convert NO to NO\textsubscript{2} more efficiently.

During the HVO\textsubscript{PM+NO}\textsubscript{x} exposure scenario (with the vehicle without aftertreatment) gas-phase organic compounds were quantified, but below the OELs, among them PAHs (889±53 ng m\textsuperscript{-3}), BTEX (11.6±3.0 µg m\textsuperscript{-3}), and formaldehyde (51±6 µg m\textsuperscript{-3}). These emissions were low or below limit of detection (LOD) during the other exposure scenarios. Full PAH (33 native and alkylated, 10 oxy- and 17 nitro-PAHs) and BTEX analyses are presented in Additional file B and C, respectively.

The particle number and mass size distribution of HVO\textsubscript{PM+NO}\textsubscript{x}, together with the effective density of the soot agglomerates, are shown in Fig. 2a. The effective density decreased with increasing mobility size, as the soot agglomerates became more open in their structure, and the diesel soot power law function described by Park et al. (38) could be fitted to the experimental data (Fig. 2a). The mass mobility exponent (D\textsubscript{fm}) was on average 2.3 (where 3 corresponds to perfect spheres). An example of the soot agglomerates generated during the HVO\textsubscript{PM+NO}\textsubscript{x} exposure is imaged by
transmission electron microscopy (TEM) in Fig. 2b. The average primary particle diameter of the soot agglomerates of $\text{HVO}_{\text{PM+NOx}}$ was 24.5 ± 7.3 nm and an example is marked in Fig. 2b.

Table 1. Summary of the average aerosol concentrations and characteristics during exposures in the chamber.

|                      | $\text{HVO}_{\text{PM+NOx}}$ | $\text{HVO}_{\text{NOx}}$ | FA |
|----------------------|-----------------------------|----------------------------|----|
| **Particle phase**   |                             |                            |    |
| PM1 (µg m$^{-3}$) (Gravimetric) | 93 ± 13                     | ~1$^a$                      | ~1$^a$ |
| PM1 Total Carbon (TC, µg m$^{-3}$) | 82 ± 10                     | <1                          | <1  |
| EC/TC (%)            | 66 ± 3                      | -                           | -   |
| OC/TC (%)            | 34 ± 3                      | -                           | -   |
| PM1 (µg/m$^3$) (SMPS and $\rho_{eff}$) | 81 ± 9                      | 0.0 ± 0.0                   | 0.5 ± 0.5 |
| PM2.5 (µg m$^{-3}$) (extrapolated fit) | 81 ± 9                      | -                           | -   |
| GMD$_{\text{mass}}$ (nm) | 114                         | -                           | -   |
| GSD$_{\text{mass}}$ (nm) | 1.48                        | -                           | -   |
| PN (cm$^{-3}$)       | 3.0 · 10$^5$                | 9.0 · 10$^5$                | 7.1 · 10$^1$ |
| PN std. dev          | 0.3 · 10$^5$                | 4.1 · 10$^1$                | 4.3 · 10$^1$ |
| GMD$_{\text{PN}}$ (nm) | 71                          | 47.2                        | - |
| GSD$_{\text{PN}}$ (nm) | 1.64                        | 1.46                        | -   |
| Surface area (cm$^2$ cm$^{-3}$) | 9.5 · 10$^{-5}$           | -                           | -   |
| Surface area std. dev (cm$^2$ cm$^{-3}$) | 1.4 · 10$^{-5}$        | -                           | -   |
| Particle phase PAHs$^b$ (ng m$^{-3}$) | 43 ± 3                      | 0.3 ± 0.7                   | 0.2 ± 0.2 |
| **Gas phase**        |                             |                            |    |
| Gas phase PAHs$^b$ (ng m$^{-3}$) | 850 ± 48                    | 97 ± 11                     | 116 ± 29 |
| Formaldehyde (µg m$^{-3}$) | 51 ± 6                      | <8                          | <8  |
| Sum BTEX (µg m$^{-3}$) | 7.9 ± 2.5                   | 1.7 ± 0.3                   | 1.3 ± 0.1 |
| VOCs (ppb)           | 333 ± 69                    | 11 ± 5                      | <10 |
| NO (ppm)             | 3.4 ± 0.1                   | 2.0 ± 0.1                   | <0.001 |
| NO$_2$ (ppm)         | 0.57 ± 0.04                 | 0.70 ± 0.04                 | <0.001 |
| NO/NO$_2$ ratio      | 5.7                         | 2.9                         | -   |
| CO$_2$ (ppm)         | 1344 ± 143                  | 1332 ± 135                  | 813 ± 140 |

All values are the average of all exposures (n=5-6) of a given type with ±1 std. dev. Full compound analyses of PAHs and BTEX are found in Additional file B and C, respectively.  
$^a$ There were large uncertainties in the gravimetric mass analysis at low/no mass concentrations. The mass concentrations were in the range of the blank filters (-1±3 µg).  
$^b$ 33 native and alkylated, 10 oxy- and 17 nitro-PAHs were included in the analysis.

Inhaled deposited fraction and dose
The calculated average inhaled deposited particle mass from nasal breathing during the HVO_{PM+NOx} exposure in the tracheobronchial and alveolar regions, from airway generation 1 to 24, is presented in Fig. 3a. Deposited doses are given as deposited mass (µg) and as deposited mass per lung tissue area (ng cm⁻²). The mass dose is largest in the distal airways (airway generations >10), but when expressed as deposited mass per lung tissue area the largest dose is found in the upper tracheobronchial region (airway generation <10). No deposition was analyzed for HVO_{NOx} due to low PM and PN concentrations (Table 1).

A comparison is presented in Fig. 3b of the total deposited particle mass doses in the different airway regions in the current study and in a previous petroleum diesel exposure study (6,15). The comparison was made for both the original diesel mass concentration in the past study (276 µg m⁻³, light grey bars) and with the same mass concentration as in this study (93 µg m⁻³, dark grey bars) using the original aerosol characteristics of the petroleum diesel (MMD 195 nm, GSD 1.65). The deposited mass of the diesel aerosol was higher in comparison to HVO_{PM+NOx}, but lower when using the same exposure mass concentrations. The deposited fractions of the HVO_{PM+NOx} aerosol mass (i.e., the fraction of the deposited mass compared to the total inhaled mass concentration) were around 40-50% higher compared to petroleum diesel in the tracheobronchial and alveolar regions. The average accumulated deposited dose in the respiratory tract was 82 µg for HVO_{PM+NOx}, corresponding to an hourly average of 27 µg h⁻¹.

The inhaled deposited dose depends on multiple lung parameters (FRC, tidal volume, breathing pattern, etc.). For additional comparison with petroleum diesel, the same deposition model (oral breathing, [39]) was used as in Wierzbicka et al. (15). Table 2 presents the deposited HVO_{PM+NOx} particle dose expressed as mass, number and surface area compared to the petroleum diesel exposure (using the original exposure concentrations). Please note the three times higher particle mass concentration in the case of diesel in comparison to HVO when looking at values of deposited...
dose by mass. The mass (and surface area) deposition fraction was a factor 1.5 higher for HVO_{PM+NOx} than diesel. Additionally, the deposited dose in terms of particle number was a factor 1.2 higher for HVO_{PM+NOx}. As the particle number concentration was higher for HVO_{PM+NOx}, despite the lower mass concentration, this led to a higher number of particles deposited in the lungs for HVO compared to diesel. The deposited mass fractions differ slightly from the multiple-path particle dosimetry (MPPD) model due to model characteristics, see Additional File D for a comparison of the deposition fraction depending on particle size for the two models.

Table 2. The average lung deposition (oral breathing) fractions of PM1 of HVO_{PM+NOx} and petroleum diesel.

|                          | HVO (PM1) | Diesel (PM1), Wierzbicka et al. 2014 |
|--------------------------|-----------|--------------------------------------|
| Mass concentration (µg m^-3) | 93 ± 13   | 276 ± 56                              |
| Particle number concentration (µg m^-3) | 3.0 · 10^8 ± 0.3 · 10^8 | 3.9 · 10^8 ± 0.5 · 10^8* |
| Surface area concentration (µg m^-3) | 9.5 · 10^{-5} ± 1.4 · 10^{-5} | 3.5 · 10^4 ± 0.7 · 10^4 |

The average lung deposition (oral breathing) fractions of PM1 of HVO_{PM+NOx} was calculated with the model presented by Rissler et al. (39) and compared to the calculated deposited doses of petroleum diesel from Wierzbicka et al. (15). The average mass, particle number and surface area concentrations of the respective exposures are given. All values are presented as mean ± 1 std. dev.

*PM0.5

Health effects

Self-rated symptoms
The number of volunteers reporting symptoms from eye, throat, nose, and chest during the exposures are shown in Table 3. Exposure to HVO (HVO_{PM+NOx} and HVO_{NOx}) caused significantly higher incidences of reported symptoms compared to FA (78%, 63% vs. 28%, p<0.03 for both).

The proportion of volunteers who reported throat irritation was a factor 4.5 and 4 higher for HVO_{PM+NOx} and HVO_{NOx}, respectively, compared to FA. The difference was statistically significant for HVO_{PM+NOx} (p=0.011) and with borderline significance for HVO_{NOx} (p=0.062). The proportion of reported eye irritation symptoms was around a factor 2.5 higher for HVO_{PM+NOx} compared to FA with a borderline significance (p=0.07). No volunteers reported chest tightness during the FA exposure, while a few individuals did so during the HVO_{PM+NOx} and HVO_{NOx}, respectively.

However, it should be noted that the reported symptom scores were generally low (mostly below 10 in a 0-100 VAS) for all categories.

### Table 3: Descriptive table of the reported symptoms during each exposure scenario.

|                | Any reported symptom | Eye a | Throat b | Nose c | Chest d |
|----------------|----------------------|-------|----------|--------|---------|
|                | Yes/total           | %     | P (χ²-test) | Yes/total | %     | P (χ²-test) | Yes/total | %     | P (χ²-test) | Yes/total | %     | Yes/total | %     |
| FA             | 5/18                 | 28%   | -         | 3/18    | 17%   | ref       | 2/18     | 11%   | ref       | 1/18     | 6%     | 0/18     | 0%     |
| HVO_{NOx}     | 12/19               | 63%   | 0.031     | 7/19    | 37%   | 0.27 e    | 8/19     | 42%   | 0.062 e   | 2/19     | 11%   | 1/19     | 5%     |
| HVO_{PM+NOx}  | 14/18               | 78%   | 0.003     | 8/18    | 44%   | 0.07 e    | 9/18     | 50%   | 0.011     | 5/18     | 28%   | 3/18     | 17%    |

Descriptive table of the number of volunteers reporting any type of symptoms, symptoms categorized by type, and χ²-tests for any reported symptoms, eye and throat symptoms. The χ²-tests are compared to the FA exposure. χ²-tests were not performed for nose and chest symptoms due to the low number of reported symptoms. The number of subjects was 19 for HVO_{NOx} and 18 for FA and HVO_{PM+NOx}.

a Itching, running and/or sore eyes. b Sore/dry/irritated throat. c Running nose and/or nose congestion. d Chest tightness/breathlessness. e P values obtained from Fisher exact test.

### Airway function

**Peak nasal inspiratory flow (PNIF) and peak expiratory flow (PEF)**

The changes in ΔPNIF and ΔPEF at each time point during the exposure are shown in Fig. 4, and absolute values are presented in Additional file E. For both PNIF and PEF, there was an increasing trend throughout the FA exposure while no such increase was seen for neither of the two HVO
exposures. The differences between average changes in PNIF and PEF measurements (ΔPNIF and ΔPEF) during the two HVO exposure scenarios compared to FA are presented in Table 4. Although no decrease in absolute PNIF values was found for the HVO exposures, we observed a statistically significant decrement in ΔPNIF during HVO\textsubscript{NOx} exposure compared to the FA exposure (-18.1 L min\textsuperscript{-1}, p≤0.001), and a borderline significant decrement during HVO\textsubscript{PM+NOx} exposure (-7.4 L min\textsuperscript{-1}, p=0.08). No difference in ΔPEF was found between the HVO exposures and FA.

Table 4: The average changes in ΔPNIF and ΔPEF (L min\textsuperscript{-1}) during each exposure scenario and compared to the FA exposure.

| Scenario        | Estimated mean (PNIF) (95%CI) | Beta (PNIF) (95%CI) | p-value | Estimated mean (PEF) (95%CI) | Beta (PEF) (95%CI) | p-value |
|-----------------|-------------------------------|---------------------|---------|-------------------------------|-------------------|---------|
| FA              | 10.3 [4.1, 16.6]              | Ref                 | ref     | 2.2 [-5.1, 9.5]              | Ref               | ref     |
| HVO\textsubscript{NOx} | -7.7 [-14.4, -1.1]          | -18.1 [-27.3, -8.8] | <0.001  | -2.5 [-10.2, 5.3]             | -4.6 [-15.6, 6.3] | 0.40    |
| HVO\textsubscript{PM+NOx} | 2.9 [-3.4, 9.1]            | -7.4 [-15.6, 0.8]  | 0.08    | 4.7 [-2.4, 11.9]             | 2.5 [-7.1, 12.2]  | 0.60    |

Estimated average changes in ΔPNIF and ΔPEF (L min\textsuperscript{-1}) during each exposure scenario (estimated mean) and differences between the two HVO exposures and FA exposure (beta). The beta values (L min\textsuperscript{-1}) and significance (p-value) are based on the linear mixed model with exposure order correction. Values within brackets are the 95% CI.

**Spirometry**

The result of the FVC (forced vital capacity), FEV\textsubscript{1} (forced expiratory volume in one second) and FEV\textsubscript{1}/FVC (in L, z-score and as % of predicted) is presented in Additional file F. FEV\textsubscript{1} and FVC showed minimal and statistically insignificant differences after all exposure scenarios (Additional file F1). A minimal (from 0.81 to 0.82) but statistically significant (p<0.05) increase in mean FEV\textsubscript{1}/FVC was found after the HVO\textsubscript{NOx} exposure. Compared to FA, no significant changes were found after the HVO exposures (Additional file F2).

**Oscillometry parameters**
The results of the oscillometry parameters reflecting reactance \( (X_5, A_X, F_{RES}) \) and resistance \( (R_5, R_{19}) \) are presented in Table 5. There was no statistically significant difference before and after any exposure for any parameter (median). Weak statistical evidence \( (p=0.084) \) was found for a decrease in reactance \( (X_5) \) after HVO\(NO_x\); however, similar trends were not seen for the related parameters of \( A_X \) and \( F_{RES} \) which downplays the probability of a physiological effect on the lung.

Some volunteers had baseline oscillometry values deviating from the normal range \((40, 41)\) but with normal spirometry measures; thus, the volunteers were further categorized into a “typical” and “atypical” group based on their oscillometric measures (Additional file A). The atypical group showed a higher proportion of having a history of symptoms and atopy \((80\% \text{ vs. } 54\%, p<0.05)\).

They were hypothesized to be more sensitive and have a different lung reaction to the exposures than the typical group. However, no significant interactions between the typical/atypical groups and PNIF or PEF were found. Neither were any statistically significant changes found for any oscillometry parameters of the atypical/typical groups after the HVO exposures in comparison to FA.

Table 5. The average lung reactance \( (X_5, A_X) \), resistance \( (R_5, R_{19}, R_{5-19}) \) and resonant frequency \( (F_{RES}) \) before and after exposure and the paired test.

| Exposure | Before exposure | After exposure | Related-Samples Wilcoxon Signed Rank Test |
|----------|-----------------|----------------|-----------------------------------------|
|          | median (25%, 75%) | mean (Std. D) | median (25%, 75%) | mean (Std. D) | p-values |
| \( R_5 \) (cmH\(2\)O s L\(^{-1}\)) | FA | 3.56 (3.28, 4.16) | 3.66 (0.87) | 3.86 (3.17, 4.31) | 3.82 (1.07) | 0.215 |
|         | HVO\(NO_x\) | 3.75 (2.86, 4.74) | 3.98 (1.33) | 3.87 (3.13, 4.86) | 4.17 (1.67) | 0.494 |
|         | HVO\(PM+NO_x\) | 3.61 (3.1, 4.68) | 4.06 (1.67) | 3.6 (2.93, 5.22) | 4.10 (1.84) | 0.845 |
| \( R_{19} \) (cmH\(2\)O s L\(^{-1}\)) | FA | 3.09 (2.63, 3.77) | 3.11 (0.73) | 3.24 (2.67, 3.71) | 3.20 (0.71) | 0.286 |
|         | HVO\(NO_x\) | 3.12 (2.56, 3.96) | 3.21 (0.74) | 3.33 (2.68, 3.88) | 3.28 (0.83) | 0.355 |
|         | HVO\(PM+NO_x\) | 3.14 (2.61, 3.82) | 3.17 (0.86) | 3.03 (2.54, 3.97) | 3.26 (1.00) | 0.215 |
| \( R_{5-19} \) (cmH\(2\)O s L\(^{-1}\)) | FA | 0.38 (0.2, 0.72) | 0.54 (0.51) | 0.51 (0.16, 0.68) | 0.63 (0.69) | 0.5 |
|         | HVO\(NO_x\) | 0.79 (0.18, 1.11) | 0.77 (0.80) | 0.72 (0.24, 0.98) | 0.88 (1.08) | 0.212 |
|         | HVO\(PM+NO_x\) | 0.49 (0.22, 1.07) | 0.89 (1.05) | 0.5 (0.27, 1.15) | 0.84 (1.04) | 0.948 |
We present the effects of the first controlled human chamber exposure to exhaust from renewable diesel fuel, namely, hydrotreated vegetable oil (HVO). Modern non-road vehicles with or without an aftertreatment system were used to investigate the health effects from two different but realistic exposures: 1) PM combined with NO\textsubscript{x} and, 2) NO\textsubscript{x} alone, both of which were compared to filtered air (FA) exposure. The exposure levels were designed to be close to, but below the current EU 8-hour OELs for NO, NO\textsubscript{2}, BTEX and PAHs (naphthalene and benzo(a)pyrene), and the future EU OEL for elemental carbon (EC, from 2023).

We found that exposures to HVO exhaust caused mild irritation symptoms compared to FA. Additionally, the observed PNIF patterns indicate that nasal obstruction occurred for both HVO exposures compared to FA. However, no overall changes in pulmonary function measured as PEF, spirometry or forced oscillation technique (FOT), were observed after 3 hours of exposure to HVO exhaust. Our findings indicate that exposure to HVO exhaust from modern non-road vehicles at relatively low exposure levels (below the future OELs) during a short period (3 hours) can cause irritative symptoms.

**Self-rated symptoms**

Exposure to HVO\textsubscript{PM+NO\textsubscript{x}} caused a significantly higher number of reported symptoms compared to FA, and some evidence of a similar trend for symptoms of eye and throat irritation (Table 3).
addition to PM and NOx, the HVO<sub>PM+NOx</sub> exposure also contained low concentrations of VOCs such as formaldehyde, BTEX and PAHs, which were not detected for HVO<sub>NOx</sub>. Formaldehyde exposure is a known irritant, causing eye and respiratory tract irritation (42), however, strong responses are generally found at much higher concentrations (>200 µg m<sup>-3</sup>, (15,43)) than used in this study (HVO<sub>PM+NOx</sub>: 51±6 µg m<sup>-3</sup>). The mild symptoms of irritation in this study could hence be an effect of the formaldehyde, despite the low concentration, in combination with the other VOCs and the PM. However, the effect of NO or NO<sub>2</sub> alone cannot be disregarded since there were symptoms reported for the HVO<sub>NOx</sub> exposure as well but to a lower extent. Symptoms similar to the ones observed in this study have earlier been reported by Mudway et al. (14) who found nasal, throat, and eye irritation as well as bronchoconstriction in healthy volunteers exposed to petroleum diesel at levels similar to the ones used in this study.

Respiratory function

Different patterns in PNIF values were found for the HVO and FA exposures (Fig. 4). In contrast to FA exposure, PNIF did not increase during exposure to the HVO exhaust, hence indicating a nasal obstruction during the two HVO exposures. The lack of an increase in PNIF was seen already 55 minutes into the exposure (Fig. 4). The decrements of ΔPNIF were larger during the HVO<sub>NOx</sub> exposure than during HVO<sub>PM+NOx</sub> in comparison to FA (Table 1). As the HVO<sub>NOx</sub> exposure did not contain any PM fraction (PN<100 cm<sup>-3</sup>, PM~1µg m<sup>-3</sup>), the effects are attributed to the NO and NO<sub>2</sub> exposure. In addition, the larger impact on the nasal patency may be related to the NO<sub>2</sub> rather than the NO concentration since NO was lower during the HVO<sub>NOx</sub> exposure than during HVO<sub>PM+NOx</sub>. The 3-hour average NO<sub>2</sub> concentration was similar for the two HVO exposures but fluctuated more for HVO<sub>NOx</sub> and this caused short periods with higher concentrations (Fig. 1b). However, the impact of NO<sub>x</sub> on nasal patency is not known, and the uptake of NO<sub>2</sub> generally occurs deeper down in the lungs and causes effect on the small airways (44) and asthma-related respiratory effects (reviewed in [45]). In this study no overall changes in lower airway function.
(assessed with PEF, spirometry and FOT) were seen, while, for example, reduction in PEF has been reported after petroleum diesel exhaust exposures of both higher (6) and lower (9) PM and NO₃ exposure concentrations. However, in studies with NO₂ exposures alone at similar concentrations as in this study (1-3h, 0.1-4 ppm), NO₂ has not caused any significant effects on lung function (assessed by spirometry) in healthy subjects (46–49).

Another cause for the increased nasal obstruction after the two HVO exposures could potentially be due to local or pulmonary vasodilation induced by the NO exposure. NO is a known pulmonary and systemic vasodilator, and when clinically administered it causes preferential pulmonary vasodilation, which is used, for example, to treat hypoxemia and acute respiratory distress syndrome (5-80 ppm) (50,51). However, as the decrement in PNIF (compared to FA) was lower for HVO₉PM+NOx, which contained higher NO than HVO₉NOx, we cannot attribute the effect solely to NO. In addition, we cannot exclude that there is an interaction effect of the PM and gases, as the changes in nasal patency were less pronounced during HVO₉PM+NOx than HVO₉NOx.

The temporary changes in measured airway functions were small and unlikely to have clinical importance in healthy persons from short-term exposure. Nevertheless, we cannot exclude a risk from either short-term or long-term exposure on more sensitive persons, for example older people or those with pre-existing lung or cardiovascular disease. The long-term effect of renewable diesel exhaust is unknown, but the short-term responses in this study indicate that even exposure below the future OELs is not completely without risk for negative health effects. Reduced PNIF is not a measure of lung function, but clinically a long-term nasal obstruction caused by occupational exposure could be considered indicative for the development of irritation asthma (52).

In previous studies, oscillometry measurements found early manifestations of lung disease before these were measurable with spirometry (53,54). The subjects with baseline oscillometric values
just outside the normal range ("atypical group", Additional file A) were hence hypothesized to have a different lung reaction than persons within the normal range. Differences between the typical/atypical groups were investigated for oscillometric parameters, PNIF and PEF but due to the small sample size, no clear conclusions can be drawn. The subjects with atypical oscillometry measures showed a higher proportion of having a history of symptoms and atopy (80% vs. 54%, p<0.05) from the initial medical assessment, which indicates that they may be more sensitive to pollutants and allergens. However, studies with larger numbers of subjects need to be carried out in order to draw any conclusions. It should also be noted that this group did not show any indications of anomalies in the spirometry and for future controlled exposure studies, the oscillometry measurement may increase the possibility to investigate small differences in lung function. In addition, oscillometry may potentially be valuable in the assessment of lung function effects related to occupational exposures for early detection and disease prevention.

**Aerosol characteristics, deposited dose and occupational exposure limits**

The total deposited mass dose of $\text{HVO}_{\text{PM}+\text{NOx}}$ during the 3-hours exposure ($82 \pm 32 \, \mu g$, Fig. 3b) was comparable to the hourly mass dose for people working outdoor in relatively polluted cities during a similar time interval ($50 \, \mu g \, \text{PM2.5 h}^{-1}$, (55)). Compared to a previous exposure study on petroleum diesel (15), the HVO particles generated by the modern diesel engine in this study had a smaller mobility size which caused higher deposition fractions in terms of mass, surface area and number. The difference in deposition is due to the higher deposition fraction (Additional file D) of smaller particles which dominated $\text{HVO}_{\text{PM}+\text{NOx}}$ emissions (MMD 108nm) in comparison to petroleum diesel (MMD 195 nm). It means that in the case of $\text{HVO}_{\text{PM}+\text{NOx}}$, two times more particles will deposit due to their smaller size in comparison to the compared petroleum diesel particles.

Modern diesel engines utilize improved combustion parameters of, for example, increased fuel injection pressure and nozzle design, which reduce the size (but not necessarily the number
concentration) of the soot particles, which in turn reduces the soot mass emissions (36). As the upcoming OEL for the EC from diesel engines is only expressed as mass (50 µg EC m⁻³, from 2023), it may be more efficient in mitigating the inhaled and deposited dose of older diesel engine emissions, but not necessarily as efficient in reducing the deposited dose from renewable fuels and modern diesel engines without DPFs. For example, even though the PM1 mass concentration (93 µg m⁻³) was 3 times lower for HVO in this study, compared to the previous exposure study to petroleum diesel (15) with PM1 276 µg m⁻³, the average deposited mass (Table 2) was only a factor 0.8 lower (i.e., only 20% lower). Despite the lower PM1 mass, the number concentration was similar and the surface area even higher due to the reduced particle size. OELs in terms of particle number concentration can hence be more efficient in reducing the exposure to particle emissions from renewable fuels and modern diesel engines similar to the ones used in this study. The particle size distributions and number concentrations need to be assessed in exposure studies and not only the mass in order to understand the deposition and dose dependent effects.

Even though the EU emission standards are continuously becoming more stringent with lower allowed exhaust emissions, modern non-road vehicles lacking full emission aftertreatment systems, like the two vehicles used in this study, will still be in use and pose a risk for hazardous exposure. No vehicle in this study had an external NOₓ reduction unit, and the implementation of one could potentially have reduced the NOₓ emissions and related health effects. From the results in this short-exposure study, we cannot exclude the potential risk of short- and long-term effects from exposure to renewable diesel exhaust from modern non-road vehicles that comply with the latest emission standards and the future OELs.

**Conclusion**

We investigated the effects on airway function after exposure to exhaust from renewable diesel fuel HVO from modern vehicles in comparison to filtered air. The vehicles were manufactured in 2019.
and complied with the current non-road engine EU emission standards. The exposure levels were kept below the EU OELs. Mild irritations symptoms (self-rated) were reported during the two HVO exposures, with a slightly higher incidence number during the exposure from the vehicle without an aftertreatment system (HVO$_{PM+NOx}$). The data also suggested that some individuals might be affected by exposure to HVO exhaust from modern work vehicles below the future EU OELs. Compared to older diesel exhaust exposures, the deposited fraction in the respiratory tract of HVO exhaust PM was higher, in terms of mass, number and surface area. The increase in deposition was due to the smaller soot particle size.

In this study, the focus was on nasal patency and pulmonary function assessments. However, to understand the full potential health effects of HVO exposures, additional analyses of the inflammatory and cardiovascular effects need to be performed. Additionally in this study, only the effects of short-term exposure to HVO exhaust were explored. This means that any conclusions about the long-term effects and effects on potentially sensitive groups need to be addressed in future studies.

Although the HVO fuel is more sustainable from a climate perspective, our study indicates that from a health perspective, the exposure levels need to be as carefully controlled as they are for petroleum diesel.

**Method**

**Study design**

In total 19 volunteers (9 f /10 m, age 20-55 years) were exposed to the two types of engine emissions and particle free air during three separate 3-hour long sessions at least one week apart. The exposure scenarios discussed in this publication were: 1) emissions from a wheel loader without exhaust aftertreatment operated with HVO (HVO$_{PM+NOx}$), 2) emissions from a wheel
loader with an aftertreatment system operated with HVO (HVO\textsubscript{NOx}), and 3) filtered air (FA). We would like to point out that the whole study also included an additional exposure scenario, namely, exposure to aerosolized dry NaCl, the analysis and comparisons of which will be presented separately. The exposures took place in a 22 m\textsuperscript{3} stainless steel chamber with controlled relative humidity, temperature and ventilation. The study was double-blind and a maximum of four participants were exposed at the same time. All exposures took place on Tuesdays, Wednesdays and Thursdays, between 9-12 a.m., with a minimum of one week between each exposure. Before and immediately after each exposure, the participants went through medical examinations that included spirometry and used the forced oscillation technique (FOT). Self-rated symptoms, PEF (peak expiratory flow) and PNIF (peak nasal inspiratory flow) were registered four times: one time before and three times during the exposure (Table 7).

Table 7. Scheduling and time points of the reported measurements and self-rated symptoms.

| Item | Before exposure | During exposure (time after exposure start in minutes)* | Immediately after exposure |
|------|-----------------|--------------------------------------------------------|---------------------------|
|      | Time point      | 1 | 2 | 3 | 4 | 5 |                      |
| Self-rated symptoms | X | 35 min | 95 min | 155 min | |
| Peak Nasal Inspiratory Flow (PNIF) | X | 55 min | 115 min | 175 min | |
| Peak Expiratory Flow (PEF) | X | 55 min | 115 min | 175 min | |
| Spirometry | X |  |  |  | X |
| Airway Oscillometry (Forced Oscillation Technique, FOT) | X |  |  |  | X |

* The exposure lasted 3 hours (180 minutes).

**Study population**

Volunteers were recruited via Lund University online channels and posters. Of the 25 volunteers who underwent the initial medical examination, 19 fulfilled the inclusion criteria and were consecutively selected for the study. The inclusion criteria were the following: men or non-pregnant women; 20-65 years old; no known lung disease; no asthma diagnosis; normal standard ECG reading; no medication that would affect the monitored parameters; non-smoker the last
three years. All except one of the selected participants underwent all three exposures. One person only participated in the exposure to HVO$_{\text{NOx}}$. Characteristics of the study group are summarized in Table 8. The study was approved by the Swedish Ethical Review Authority (registration no. 2019-03320) and performed in accordance with the Declaration of Helsinki.

Table 8. The participants’ medical history and results from the initial medical examinations before commencement of the study.

| Subjects (N=19) |               |
|----------------|--------------|
| Age (median, min-max) | 29 (20-55) |
| Female (N, %) | 9 (47%) |
| Previous smoker (N, %)* | 7 (37%) |
| **Medical history** |               |
| History of any symptoms last 12 months (N, %) | 7 (37%) |
| - Eye symptoms (N, %) | 1 (5%) |
| - Nasal symptoms (N, %) | 3 (16%) |
| - Dry cough (N, %) | 0 |
| History of chronic bronchitis (N, %) | 0 |
| History of bronchial hyperreactivity (N, %) | 5 (26%) |
| **History of childhood atopy** |               |
| Atopic dermatitis/Childhood eczema (N, %) | 3 (16%) |
| Allergic rhinitis/Hay fever (N, %) | 1 (5%) |
| Urticaria (N, %) | 1 (5%) |
| Physician-diagnosed asthma during childhood (N, %) | 1 (5%) |
| **Atopy** |               |
| Phadiatop positive (N, %) | 7 (37%) |
| **Baseline spirometry (prior to bronchodilation)** | FVC % pred. (median, min-max) | 92 (70-106) |
| FEV$_1$ % pred. (median, min-max) | 94 (73-108) |
| FEV$_1$/FVC % pred. (median, min-max) | 100 (90-108) |
| **Baseline spirometry (after bronchodilation)** | FVC % pred. (median, min-max) | 95 (72-107) |
| FEV$_1$ % pred. (median, min-max) | 97 (79-109) |
| FEV$_1$/FVC % pred. (median, min-max) | 100 (90-112) |

*All test subjects were currently non-smokers.

FVC = forced vital capacity, FEV$_1$ = forced expiratory volume in one second.
**Aerosol generation**

The exposures took place in a 22 m³ stainless steel chamber with an air exchange rate of 4 exchanges/hour. The supply air used for dilution was filtered from particles with a HEPA (high-efficiency particulate absorbing) filter and from gases with an active carbon filter. The temperature was kept at 26±1°C and the relative humidity at 33±4%.

**Renewable diesel exposure**

The renewable diesel exhaust exposure scenarios were generated with two types of modern off-road diesel vehicles with different net power (kW). Both vehicles were manufactured in 2019 and complied with the current EU emission legislation. The smaller vehicle, net power 23 kW, followed emission standard Stage IIIa (2007), and the larger vehicle with a net power of 55.4 kW followed Stage V (2019). The smaller vehicle was not equipped with any external exhaust aftertreatment (hereafter denoted HVO_PM+NOx), while the larger vehicle was equipped with a diesel oxidation catalyst (DOC) and a diesel particulate filter (DPF) (hereafter denoted HVO_NOx).

The vehicles were operated switching between load (lifting the fork and applying increased power) and idle with 15-minute intervals. During load, the vehicles were kept running around 1800-1900 rpm and during idle around 900 rpm. Both vehicles were run on 100% HVO. The exhaust was extracted from the exhaust pipe of the vehicle, transported in heated tubing and diluted in two steps: first to approximately 1:20-1:30 (heated to 30°C), and then to a total dilution ratio of 1:160 in the chamber. The setup was previously described by Wierzbicka et al. (15).

**Filtered air**

The filtered air (FA) exposure was obtained by provision of air that passed through a HEPA filter and an active carbon filter. The particle number concentration in the size range 0.02-2.5 μm was on average 71±43 cm⁻³, and the volatile organic compound (VOC) concentration <10 ppb. The FA was used for comparison as a reference exposure.
**Emission characterization**

**Online characterization**

The concentrations of PM2.5 mass, NO, and NO\(_2\) were monitored online during the exposures to keep levels below the pre-determined exposure limits of 150 µg m\(^{-3}\), 1 ppm and 2.5 ppm, respectively. The online PM2.5 mass concentration was monitored by an ambient particulate monitor (TEOM series 1400a, Rupprecht & Patashnick Co., N.Y., U.S.A.). NO and NO\(_2\) was measured with a chemiluminescence analyzer (CLD 700 AL, ECO PHYSICS AG, Switzerland). The raw gas emissions were measured with a flue gas analyzer (Testo 350, Testo AG, Germany) in order to monitor the operation of the vehicles and to ensure exposures below hazardous CO exposure levels (exposure averages were kept below 3 ppm). The CO\(_2\) concentration in the chamber was monitored with a non-dispersive infrared CO\(_2\) analyzer (LI-8020, LI-COR, Lincoln, NB, U.S.A.) and kept below 1500 ppm. The total VOC concentration (range 10-20 000 ppb) was measured by an online photo-ionization technique (VelociCalc, model 9565-P, probe 986, TSI Inc., U.S.A.).

**Particle number size distribution and number concentration**

The particle number size distributions in the range 9.8-430 nm (HVO exposures) or 19-914 nm (FA exposures) were measured with a scanning mobility particle sizer (SMPS), including an electrostatic classifier (TSI model 3082) and condensation particle counter (CPC, model 3775, TSI). The aerodynamic size distribution of 0.5-20 µm was monitored with an aerodynamic particle sizer (APS, model 3321, TSI) during the exposures to ensure that the particle number size distributions maxima were captured with the SMPS.

**Effective density (DMA-APM)**
The particle effective density was assessed using an aerosol particle mass analyzer (APM 3600, Kanomax) in combination with a differential mobility analyzer (DMA, TSI Inc., U.S.A.) and a condensation particle counter (CPC, model 3075, TSI Inc., U.S.A.) (56). The effective density was measured at five DMA-selected particle mobility diameters: 50, 70, 100, 150 and 300 nm. Mobility size \(d_p\) selection was performed with the DMA. The APM measured the mass distribution of the selected monodisperse aerosol by stepping the voltage for a constant rotating speed. The effective density, \(\rho_{\text{eff}}\), was derived from the arithmetic mean of the measured APM voltage-number distribution and polystyrene latex spheres (PSL, Polymer Microspheres, Duke Scientific Corporation) reference data as described by McMurry et al. (56), shown in Eq. (1):

\[
\rho_{\text{eff}} = \rho_{\text{PSL}} \frac{V_{\text{APM}}}{V_{\text{APM,PSL}}} \tag{1}
\]

Where \(\rho_{\text{PSL}}\) is the density of the PSL reference particles, \(V_{\text{APM}}\) is the measured arithmetic mean voltage of the sampled particles for a given mobility diameter and RPM, and \(V_{\text{APM,PSL}}\) is the theoretically calculated arithmetic mean voltage of the PSL reference particles for a given mobility diameter and RPM. The DMA-APM system was calibrated with spherical PSL particles with a known density of 1.05 g cm\(^{-3}\).

The effective density of the HVO\(_{\text{PM+NOx}}\) (soot particles) was fitted assuming a power law function, Eq. (2) (57), where \(C''\) is a constant and \(D_{\text{fm}}\) the mass-mobility exponent.

\[
\rho_{\text{eff}} = C'' d_p^{D_{\text{fm}}} m^{-3} \tag{2}
\]

The mass-mobility relationship was used to extrapolate a power law function for the mobility equivalent particle diameters below 50 nm (up to the inherent material density of soot of 1.8 g cm\(^{-3}\)) and above 300 nm.

Particle mass and surface area size distribution
Mass size distributions were calculated by following Eq. (3), utilizing the particle number size distribution (from the SMPS) and the experimentally determined effective density ($\rho_{\text{eff}}$, from the APM) as a function of electrical mobility size ($d_p$).

$$\frac{dM}{d\log d_p} = \frac{\pi d_p^6}{6} \rho_{\text{eff}}(d_p) \frac{dN}{d\log d_p}$$ (3)

Lognormal distributions were fitted to the mass size distribution up to 1 µm (PM1).

The surface area (SA) distributions were calculated using the model described by Rissler et al. (39), which is based on DMA-APM measurements. From the DMA-APM, the mass of individual agglomerates as a function of mobility particle size can be extracted if the effective density follows the soot power law function (Eq. 2). The surface area of individual agglomerates is then calculated by division of the mass of the agglomerate by the primary particle mass and surface area

$$SA_{pp} = 6/\left(\rho_{pp}d_{pp}\right)$$ (39). The primary particle size ($d_{pp}$) is obtained from TEM images, and the inherent material density of soot (1.8 g cm$^{-3}$) used for the primary particle density ($\rho_{pp}$). From the surface area of individual agglomerates as a function of mobility particle size, the particle number distribution (from the SMPS) can be converted to a particle surface area distribution. This method accounts for the agglomerated soot structure and is described in more detail by Rissler et al. and Wierzbicka et al. (15,39).

**Offline characterization**

**Gravimetric analysis**

The PM1 mass concentration was determined by gravimetric analysis performed by the Division of Occupational and Environmental Medicine at Örebro University, Örebro, Sweden. The samples were collected during the entire duration of the exposure (180 minutes) using a PM1 cyclone pre-separator on 37 mm Teflon filters (Zefluor, pore size 1.0 µm) with a flow rate of 5 L min$^{-1}$. The
filters were conditioned for 48 hours at 50±3% RH and 20±1°C and weighed before and after collection.

*Thermal optical carbon analysis and TEM imaging*

Samples for the thermal optical analysis of organic carbon (OC) and elemental carbon (EC) were collected on quartz filters (47 mm, Pallflex Tissuequartz) and analyzed with a thermal optical analyzer (DRI Model 2001 OC/EC Carbon Analyzer, Atmoslytic Inc., U.S.A.) using the NIOSH NMAM 5040 diesel exhaust protocol. The limit of detection for EC (LOD) was 0.06 µg C cm⁻². Two samples were collected in parallel, where one filter collected particle-free air after a Teflon filter (Zefluor, pore size 1.0 µm) which was used to account for gas adsorption artifacts of the filter. Both samples were collected after a PM₁ cyclone at a flow rate of 5 L min⁻¹ during the entire exposure duration (180 min) and stored refrigerated (+6°C) until analysis.

To analyze the soot particle aggregate structure (morphology and primary particle size), samples were collected with electrostatic precipitation using a nanometer aerosol sampler (model 3089, TSI) on lacey carbon coated Cu-grids and analyzed with a transmission electron microscope (TEM, JEOL 3000F). The TEM was operated at 300kV and equipped with a Schottky FEG and 2x2k CCD. An overview of the samples was first imaged at 10,000X magnification in order to ensure that the sample was reasonably homogenous. The TEM images of HVO_PM+NOx were analyzed for primary particle size determination with the ImageJ software (58). The diameters of the clear primary particles without overlap at the edges of the soot agglomerates were measured in TEM images with a magnification minimum of 25,000X. The diameters of 81 primary particles were measured from 10 agglomerates.

*PAH analysis*

Samples for particulate PAH analysis were collected at a flow rate of 2 L min⁻¹ during the entire exposure duration (180 minutes) on Teflon filters (diameter 37 mm, pore size 2 µm (Teflo, Pall
Corporation, Port Washington, N.Y., U.S.A.). These filters were followed by XAD-2 tubes (SKC Inc.) for sampling of gaseous PAHs. The samples were stored at -18°C prior to analysis. The samples were analyzed for 33 native and alkylated PAHs (including the 16 U.S. EPA priority PAHs), 17 nitrated, 10 oxygenated PAHs (nitro-PAHs and oxy-PAHs), and 6 dibenzothiophenes (DBTs), as described by Gren et al. 2020 (22). In short, prior to extraction two labelled internal standard mixtures containing 16 deuterated U.S. EPA priority PAHs were spiked to the filters and XAD-2 adsorbent, respectively. Samples were extracted with 3 mL dichloromethane, cleaned using silica columns and concentrated to a final volume of approximately 30-40 µL. Target compounds were separated on an Agilent 5975C mass spectrometer (MS) coupled to a 7890A gas chromatograph (GC, Agilent Technologies, Santa Clara, CA, U.S.A.). The MS was operated in selected ion monitoring mode (SIM), and electron impact ionization (EI) was performed for PAHs and alkylated PAHs.

Formaldehyde and BTEX analysis

Accredited formaldehyde analysis was performed by the Division of Occupational and Environmental Medicine at Örebro University, Örebro, Sweden. The samples for formaldehyde analysis were collected with a flow rate of 0.2 L min⁻¹ during the entire duration of the exposures (180 min) on Sep-Pak DNPH-silica cartridges (Waters). 2,4-dinitrophenylhydrazine (DNPH) formed derivates with aldehydes and were extracted in acetonitrile. The extracted samples were analyzed with liquid chromatography ultraviolet mass spectrometry (LC-UV/MS) at wavelength 360 nm. The samples were stored at -18°C prior to analysis.

An accredited analysis of benzene, toluene, ethyl benzene, m+p xylene and o-xylene (BTEX) was performed by the IVL Swedish Environmental Research Institute, Gothenburg, Sweden. n-butyl acetate, n-octane and n-nonane were also analyzed but not included in the total BTEX concentration. The samples were collected on thermal desorption tubes (TENAX TA) and analyzed
by a thermal desorption GC-MS method. The sorbent tubes were heated to 250°C under a helium flow for 5 minutes. The emitted compounds were refocused with a cold trap (-30°C) and then quickly heated to 300°C in the thermal desorption instrument (Unity2 and Ultra, Markes) and injected into the GC-MS (ThermoFisher Scientific). Target compounds were separated on a non-polar capillary column (TraceGold, TG-1MS, ThermoFisher Scientific) coupled to a mass spectrometer (ISQ LT, ThermoFisher Scientific).

Model for particle deposition in the respiratory tract

Regional respiratory tract particle deposition fractions from nasal breathing were calculated for the inhaled aerosols with the multiple-path particle dosimetry model (MPPD model version 3.04, (59)). The input parameters are summarized in Table 9.

Table 9. The input parameters for the MPPD model used for estimating the respiratory tract particle deposition.

| MPPD model input data                  | HVO<sub>PM+NOx</sub> | Diesel (Wierzbicka et al. [2014]) |
|----------------------------------------|------------------------|-----------------------------------|
| Model                                  | Yeh/Schum Symmetric    |                                    |
| Functional residual capacity<sup>a</sup> (mL) | 3200 (2680-3750)       | 50                                 |
| Upper respiratory tract volume (mL)   |                        |                                    |
| Particle properties*                   |                        |                                    |
| Density at MMD (g cm<sup>-3</sup>)    | 0.84                   | 0.42                               |
| PM1 mass (µg m<sup>-3</sup>)           | 93 ± 13                | 276 ± 56                           |
| Mass Median Diameter (MMD) (µm)       | 0.108                  | 0.195                              |
| GSD                                    | 1.48                   | 1.65                               |
| Exposure scenario                     |                        |                                    |
| Inhalability adjustment               | No                     |                                    |
| Acceleration of gravity (m s<sup>-2</sup>) | 9.81                   |                                    |
| Body orientation                      | Upright                |                                    |
| Breathing frequency<sup>b</sup> (min<sup>-1</sup>) | 17.1 (13.3-24.9)       |                                    |
| Tidal volume<sup>c</sup> (mL)         | 875 (440-1500)         |                                    |
| Inspiratory fraction                  | 0.5                    |                                    |
| Breathing scenario                    | Nasal                  |                                    |

The aerosol characteristics from a previous diesel exposure study (15) were included and used in the MPPD model to compare the respiratory deposition of HVO and petroleum diesel. The MPPD model’s reference values from ICRP (60) for upper respiratory tract volume and inspiratory fraction were used. * Properties from the exposure aerosol characterization. <sup>a</sup> Calculated by height, age and sex following the guidelines of the European Respiratory Society (61). <sup>b</sup> Measured with a Noxturnal 5.1 breathing belt (Nox...
Medical assessment

Nasal patency and pulmonary function measures

Assessment of nasal patency was performed with measurements of peak nasal inspiratory flow (PNIF) using an inspiratory flow meter (In-check, Clement Clarke International Ltd., U.K.) according to the manufacturer’s instructions. Three recordings at each time point (Table 7) were performed and the highest value was used for analysis (62). The PNIF measurements were compared to the baseline value before the exposure on an individual basis, and a decrease in PNIF indicated an increased nasal obstruction (63).

Lower airway function was assessed with measurements of peak expiratory flow (PEF) measured with a MINI Wright Flow Meter (Clement Clarke International Ltd., U.K.), measuring range of 60-800 L/min. Three recordings at each time point (Table 7) were performed and the highest value was used for analysis (64). The PEF measurements were compared to the baseline value before the exposure on an individual basis, and a decrease in PEF was used as an indication of lower airway obstruction.

Spirometry was performed with SPIRARE 3 (DIAGNOSTICA, Oslo, Norway) according to the European Respiratory Society Guidelines (64). Forced vital capacity (FVC), forced expiratory flow
in the first second (FEV₁), FEV₁/FVC, and z-scores were obtained and compared according to the reference of the Global Lung Initiative (65).

Measures of oscillometry (resistance [R], reactance [X]) were obtained in a frequency range of 5-19 Hz by the forced oscillometric technique (FOT), with a Tremoflo (THORASYS, Thoracic Medical System Inc., Canada) according to the manufacturer’s instructions (40). The resistance at 5 and 19 Hz (R₅, R₁₉), reactance at 5 Hz, area under the reactance curve from 5 to 19 Hz (Aₓ), and the resonant frequency (F_res) before all exposures were averaged and summarized for all volunteers in Additional file A. The individual oscillograms were evaluated by a trained physician. Some volunteers had FOT values deviating from the normal range (40,41) but with normal spirometry measures, thus the volunteers were further categorized into a “typical” and “atypical” group based on their oscillometric measurements. The criteria were as follows: R₅₋₁₉ ≥ 0.8 cmH₂O s L⁻¹, X₅ ≤ -1.8 cmH₂O s L⁻¹, and Aₓ ≥ 14 cmH₂O L⁻¹ (Additional file A). These criteria were based on characteristics of healthy and asthmatic subjects as published by Eddy et al. (41). The atypical group showed a higher proportion of having a history of symptoms and atopy (80% vs. 54%, p<0.05). They were hypothesized to be more sensitive and to have a different lung reaction than the typical group, which was explored further in the analyses.

Self-rated symptoms

Similar to a previous study (6), self-rated symptoms of eye irritation, nose irritation (including runny nose and nasal congestion), throat irritation and chest tightness/breathlessness were rated by the volunteers themselves on a visual analog scale (VAS) (range 0 to 100 millimeter) before exposure, and at 35, 95 and 155 min into the exposure during each exposure session (Table 7).

Statistical analysis
For each self-rated symptom (eye, nose, throat, and chest), when a volunteer gave a higher score than before the exposure at any time during the exposure, this person was recoded as “reported symptoms”, otherwise, “no reported symptoms”. A person was then recoded as “reported any symptom” if he/she reported any of the four symptoms during the exposure. The calculation was performed for each exposure scenario separately. Descriptive analysis was used to count the number of persons and corresponding proportion of persons with reported symptoms during exposure for each exposure scenario. An $\chi^2$-test was used to investigate the difference in proportion between the given exposure scenarios (each of the two exposure scenarios) in comparison to FA exposure when applicable.

For PNIF and PEF measurements that were performed three times during each exposure, absolute changes from before exposure were calculated on the individual level for each exposure scenario at each time point. Linear mixed models were used to analyze the average changes in the selected outcomes at given exposure scenarios versus changes at FA exposure. Subject ID, the exposure scenarios and time points (1-4) were used to identify repeated measurements, and all models included a random slope. The models further included exposure order (e.g., first or second time in the chamber) as an adjustment since the order was imbalanced and a learning effect on the measurement performance might have occurred.

For spirometry and FOT measurements, which were only performed before and after exposure, the Wilcoxon signed-rank test was used to compare the differences between before and after exposure at each exposure scenario.

Additionally, interaction terms between exposure scenarios and typical/atypical groups were tested for PNIF, PEF and with the FOT in the linear mixed models described above, to see if the atypical group with FOT measurements outside the normal range (Additional file A) showed different exposure-related changes in nasal patency and pulmonary function. If the interaction
terms had a p-value <0.05, further stratified analyses were performed in each group for selected outcomes.

All statistical analyses were performed using IBM SPSS Statistics 26. For all tests, p-values <0.1 were considered as weak indications, while P values <0.05 were generally accepted as significant.

**Declarations**

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**Authors’ contributions**

Original project idea and obtaining funding: AG and AW. Exposure scenarios design: AW and LG. Project management and coordination of exposures: AW. Aerosol generation: LG, JP, AW, AG. Initial test and selection of vehicles: LG, JP. Aerosol instrument data collection: LG, FM. Aerosol instrument data analysis and lung deposition analysis: LG. TEM images and analysis: LG. Carried out the experiments: LG, KD, EA, JN, FM. Vehicle operation: FM. PAH analysis: AMK, BS. OCEC analysis: KL. Medical assessment and measurements: KD, EA, JN. FOT data analysis and interpretation: LG, KD, PW. Statistical analysis: YX. Health effects/medical data interpretation: LG, KD, MK, MA, JN, AW, AG, JL. LG wrote the manuscript and conceived the figures. All co-authors contributed to critical revisions of the manuscript. All co-authors will have approved the final manuscript.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
The study was approved by the Swedish Ethical Review Authority (registration no. 2019-03320) and performed in accordance with the Declaration of Helsinki and included obtaining informed written consent.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Abbreviations
APM aerosol particle mass analyzer
BTEX benzene, toluene, ethyl benzene, m+p xylene, o-xylene
CI confidence interval
DMA differential mobility analyzer
DPF diesel particle filter
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