Toxicity of different biodiesel exhausts in primary human airway epithelial cells grown at air-liquid interface.

*Katherine R. Landwehr[1,2], Jessica Hillas[2], Ryan Mead-Hunter[1], Andrew King[3], Rebecca A. O'Leary[4], Anthony Kicic[1,2,5,6], Benjamin J. Mullins[1], Alexander N. Larcombe[1,2], on behalf of WAERP[7,8]

[1]Occupation, Environment and Safety, School of Population Health, Curtin University, PO Box U1987, Perth, 6845, Western Australia, Australia

[2]Respiratory Environmental Health, Wal-yan Respiratory Research Centre, Telethon Kids Institute, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia

[3]Fluid Dynamics Research Group, School of Civil and Mechanical Engineering, Curtin University, Perth, Western Australia, Australia

[4]Department of Primary Industries and Regional Development, Perth, 6151, Western Australia, Australia

[5]Department of Respiratory and Sleep Medicine, Perth Children's Hospital, Nedlands, Perth, 6009, Western Australia, Australia

[6]Centre for Cell Therapy and Regenerative Medicine, The University of Western Australia, Perth, 6009, Western Australia, Australia

[7]Telethon Kids Institute, Centre for Health Research, The University of Western Australia, Nedlands, Perth, 6009, Western Australia, Australia

[8]St. John of God Hospital, Subiaco, Perth, 6008, Western Australia, Australia

Corresponding Author*:

Email: katherine.landwehr@telethonkids.org.au

Post: PO Box 855, West Perth, Western Australia, 6872

Graphical Abstract:

Abstract:

Biodiesel is created through the transesterification of fats/oils and its usage is increasing worldwide as global warming concerns increase. Biodiesel fuel properties change depending on the feedstock used to create it. The aim of this study was to assess the different toxicological properties of biodiesel exhausts created from different feedstocks using a complex 3D air-liquid interface (ALI) model that mimics the human airway. Primary human airway epithelial cells were grown at ALI until full differentiation was achieved. Cells were then exposed to 1/20 diluted exhaust from an engine running on Diesel (ULSD), pure or 20% blended Canola biodiesel and pure or 20% blended Tallow biodiesel, or Air for control. Exhaust was analysed for various physio-chemical properties and 24-hours after exposure, ALI cultures were assessed for permeability, protein release and mediator response. All measured exhaust components were within industry safety standards. ULSD contained the highest concentrations of various combustion gases. We found no differences in terms of particle characteristics for any of the tested exhausts, likely due to the high dilution used.

Exposure to Tallow B100 and B20 induced increased permeability in the ALI culture and the greatest increase in mediator response in both the apical and basal compartments. In contrast, Canola B100 and B20 did not impact permeability and induced the smallest mediator response. All exhausts but Canola B20 induced increased protein release, indicating epithelial damage.

Despite the concentrations of all exhausts used in this study meeting industry safety regulations, we found significant toxic effects. Tallow biodiesel was found to be the most toxic of the tested fuels and Canola the least, both for blended and pure biodiesel fuels. This suggests that the feedstock biodiesel is made from is crucial for the resulting health effects of exhaust exposure, even when not comprising the majority of fuel composition.

Keywords: Exhaust Toxicology, Biodiesel Health Effects, Air-Liquid Interface, Exhaust Exposure, Biodiesel Exhaust, Primary Human Cell Culture

Abbreviations:

B100; 100% biodiesel fuel

B20; Blended mineral diesel fuel with 20% biodiesel

DPF; Diesel particulate filter

FAME; Fatty acid methyl esters

ULSD; Ultra-low sulfur mineral diesel

O_2; Oxygen

CO; Carbon Monoxide

CO_2; Carbon Dioxide

NO_x; Nitrogen oxides

NO; Nitrogen Monoxide

NO_2; Nitrogen Dioxide

PAHs; Polycyclic aromatic hydrocarbons

PM; Particulate matter

SO_2; Sulfur Dioxide

G-CSF; Granulocyte colony-stimulating factor

GM-CSF; Granulocyte-macrophage colony-stimulating factor

IFN-γ; Interferon gamma

IL1-RA; Interleukin 1 receptor antagonist

IL-6; Interleukin 6

IL-7; Interleukin 7

IL-8; Interleukin 8

IL-9; Interleukin 9

IP-10; Interferon gamma-induced protein *10*

MCP-1: Monocyte chemoattractant protein 1

MIP-1β; Macrophage inflammatory protein 1-beta

PDGF-bb; Platelet derived growth factor BB

RANTES; Regulated on activation, normal T cell expressed and secreted

TNF-α; Tumour necrosis factor-alpha

VEGF; Vascular endothelial growth factor

1.Introduction:

Biodiesel is a renewable diesel fuel created through the transesterification of fatty acids found within natural fats and oils into fatty acid methyl esters (FAME) (Knothe et al., 2015). It can be used to replace commercial mineral diesel in many diesel engines including those currently on road (Fontaras et al., 2009). The type of fat or oil used to create the biodiesel alters the FAME profile and other properties of the fuel (Knothe and Steidley, 2005; Ramos et al., 2009). This in turn changes the combustion exhaust composition (Graboski et al., 2003) and the resulting health impacts of exhaust exposure (Landwehr et al., 2021). Global biodiesel production has increased 50-fold since 2000 (EIA,

2020a) and as diesel fuel gets more difficult and expensive to extract, it is likely that usage will increase even further. This is due to the fact that diesel fuel is still heavily used for long distance transporting due to its economical fuel usage (Suppes and Storvick, 2016) and other renewable energy options are not yet capable of replacing diesel engines in this aspect (Amjad et al., 2010; Camuzeaux et al., 2015).

Currently, biodiesel is often used as a mandated blend with mineral diesel in order to increase lubricative properties and address global warming concerns (EU, 2016; EU, 2019; Li et al., 2019). A blend of 20%, labelled B20, is the most common type of blend tested within literature as it is already in use (EERE, 2020; Hamje et al., 2014; International, 2020). Biodiesel made from Canola (Rapeseed) and Soy are some of the most commonly used biodiesel types and are thus also the most commonly tested (Møller et al., 2020; OECD/FAO, 2020), however other types such as Palm, Coconut and animal fats such as Tallow are also currently in use worldwide (ARENA, 2018; OECD/FAO, 2020).

As diesel exhaust is generally inhaled, effects of exposure occur primarily in the respiratory and cardiac systems (Behndig et al., 2011; Giles et al., 2018; Mills et al., 2011; Peters et al., 2017), although effects on other organs such as the brain (Nejad et al., 2015) and bladder (Latifovic et al., 2015) have also been reported. Diesel exhaust contains many toxic/irritating compounds including oxides of nitrogen (NO_x), carbon monoxide (CO), elemental carbon particles and polycyclic aromatic hydrocarbons (PAH) which are known to impact health (Fontaras et al., 2009; Gioda et al., 2016; Graver et al., 2016). However, the majority of toxic effects caused by inhalation of diesel exhaust have been attributed to the ultrafine particle component (<100 nm diameter) (Breitner et al., 2011; Oberdörster et al., 1995). Particles under 35 nm in size make up more than 90% of particles found within diesel exhaust and yet only account for approximately 10% of the mass (Kittelson et al., 2002; Ris, 2007). Ultrafine particles are considered especially toxic as they are capable of bypassing the epithelial barrier of the lungs and directly entering the blood stream (Brook et al., 2010; Goodson et al., 2017). Additionally, diesel exhaust can contain toxic chemicals such as aromatic hydrocarbons,

aldehyde, ketones and heavy metals (Fontaras et al., 2009; Gioda et al., 2016) which are known to readily adsorb/adhere to the surface of these more easily inhaled ultrafine particles (Mullins et al., 2016; Munack et al., 2006).

Biodiesel exhaust is typically similar to mineral diesel exhaust, apart from a few important differences. It generally contains more NO_x and a smaller median particle size (Fontaras et al., 2009; Giakoumis et al., 2012), which has concerning implications for the toxic effects of exhaust exposure. Previous studies on biodiesel exhaust toxicity have provided conflicting results (Larcombe et al., 2015; Madden, 2016; Møller et al., 2020), with some studies finding biodiesel to be more toxic than mineral diesel in terms of cytotoxicity and inflammatory effects (Skuland et al., 2017; Yanamala et al., 2013), others diesel to be more toxic than biodiesel in terms of mutagenicity and vascular effects (Hemmingsen et al., 2011; Mutlu et al., 2015) and yet others finding blended biodiesel/mineral diesel fuels to be more toxic than either of the pure fuels in terms of mutagenicity and oxidative activity (Adenuga et al., 2016; Krahl et al., 2008). A common limitation in previous literature is the tendency to treat biodiesel as the same regardless of the feedstock used during creation, to the point that the type of biodiesel used is not always stated in previous studies (Ackland et al., 2007; Hawley et al., 2014). Since health impacts are known to change depending on feedstock type (Landwehr et al., 2021), this makes attempts to clarify biodiesel toxicity difficult. Furthermore, methodologies used to test toxicity vary greatly with engine configurations (Brito et al., 2010; de Brito et al., 2018; Hemmingsen et al., 2011; Magnusson et al., 2019), exhaust after-treatment technologies (André et al., 2015b; Gioda et al., 2016; Magnusson et al., 2017), exhaust dilutions (de Brito et al., 2018; Douki et al., 2018), mineral diesel reference fuels (Brito et al., 2010; Mullins et al., 2016) and toxicological measurements (Adenuga et al., 2016; Gioda et al., 2016; Mutlu et al., 2015) to the point that meaningful comparisons between different studies are virtually impossible (Larcombe et al., 2015; Møller et al., 2020).

Another limitation of prior literature is the tendency to focus solely on the particle components of the exhaust, ignoring the health impact of the gaseous components entirely (André et al., 2015a; Larcombe et al., 2015). Exhaust particles are generally collected on filters and extracted using solvents to be added directly to the media of cell lines or the Ames bacterial mutagenicity assay to test cytotoxicity and mutagenicity respectively (Bünger et al., 2000; Cervena et al., 2017). A strength of this approach is that the exact deposition amount of the particles added during exposure is known (Cervena et al., 2017), however collecting particles on a filter often removes the ultrafine particles entirely as particles agglomerate to create an artificial particle size spectra (Morin et al., 2008) and the health impact of the gaseous components is removed.

With the aforementioned limitations in mind, the aim of this study was to assess the exhaust toxicity of two different biodiesels and their 20% blends in a 3D primary airway epithelial cell model that accurately mimics human lung formation (Martinovich et al., 2017). We chose Tallow and Canola biodiesel as both feedstock types are amongst the most popular types currently in use (ARENA, 2018; OECD/FAO, 2020) and our previous study found them to be at opposite ends of the toxicity spectrum of six different biodiesel feedstocks, with Tallow being more toxic than ultra-low sulfur diesel (ULSD) and Canola being less (Landwehr et al., 2021). Furthermore, we hypothesised that Tallow would be the most toxic feedstock type and Canola the least and mimic our observations made using monolayer cultures (Landwehr et al., 2021). Collectively, results generated are the first to expose fully differentiated primary human airway epithelial cells to multiple biodiesel exhausts, using exhaust generated from an engine paired with modern exhaust after-treatment devices (both a diesel particulate filter and oxidation catalyst).

2.Material and Methods:

2.1 Fuel Types: Commercial ULSD was obtained from local suppliers (SHELL, WA, AUS, biodiesel free, <10ppm sulfur). Two different biodiesel types and their respective 20% blends within ULSD were also used in this study. Canola and Tallow biodiesel were created in our laboratory using high quality,

food grade commercial oils/fats (Campbells Wholesale Reseller, WA, AUS). All oils were converted to fatty acid methyl esters (FAME) using an established sodium methoxide transesterification process (Knothe et al., 2015).

2.2 Participants: This study was approved by the St John of God Hospital Human Ethics Committee (901). Airway epithelial cells were derived from trans-laryngeal, non-bronchoscopic brushings of the tracheal mucosa of children through an endotracheal tube as previously described (Kicic et al., 2006; Lane et al., 2005). Informed parent/guardian permission was obtained prior to brushings obtained from six healthy, non-atopic volunteers (six total, aged 2-9 years, 3 males) undergoing elective surgery for non-respiratory related conditions. Atopy was determined using a radio-allergo-sorbent test for a panel of common childhood allergens and positive results were excluded. Volunteers clinically diagnosed with bacterial or viral chest infections or any underlying chronic respiratory disease such as asthma were also excluded.

2.3 Tissue Culture: Primary airway epithelial cell cultures and differentiated ALI models were established as previously described (Martinovich et al., 2017) and grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. All cells tested negative for mycoplasma. Cells were passaged weekly in Corning T75 tissue culture flasks (CLS430720, Corning ®, MERCK, NSW, AUS) and used for differentiation before passage 3 in all cases. For differentiation, cells were seeded at 250,000 cells per membrane onto transwell membranes (Corning® Transwell, 12mm with 0.4µm pore polyester membrane, MERCK, NSW, AUS), allowed to reach confluence over a period of three days and then air lifted. Cells were then differentiated for a minimum of 28 days in UNC-ALI media (Table S1, Looi et al., 2018) before use in exposure experiments. Trans epithelial resistance (TER) was tested weekly and the final reading occurred just before exposures began (Figure S1). Cultures that deviated too far from the average TER for each subject were rejected. Media was refreshed prior to all exposures. ALI models were grown in duplicate for every subject and exposure and randomly assigned to exposure conditions to minimise bias.

2.4 Exposure Methodology: All cultures were randomised in layout on the culture plate to avoid bias caused by uneven exhaust dispersion. They were then exposed for one hour to either air as a control or exhaust generated from a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) coupled with a dynamometer and fitted with Euro V/VI after-treatment technology consisting of a diesel particulate filter and oxidation catalyst (Daimler, Germany) (Landwehr et al., 2019). All exhaust exposures were run at cold start to simulate real world exposures, at a constant load of 40% and speed of 2000 rpm. Exhaust was diluted 1:20 with air inside a mixing chamber attached to the exhaust piping and pumped through an isokinetic sampling point at a rate of 10 L/min into a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) maintained at 37^{o}C containing the ALI models. Once the models were exposed, exhaust was vacuumed out for physico-chemical analysis of gas and particle properties. Exposure to air was used as a negative control.

2.5 Gas and Particle Analysis: Exhaust exiting the sealed incubator was analysed every 10 minutes for concentrations of common combustion gas products including O_2, CO, CO_2, NO_x (NO and NO_2) and SO_2 using a combustion gas analyser (TESTO 350, Testo, Lenzkirch, Germany). Similarly, exhaust was analysed every 10 minutes for particle concentrations between the sizes of 3 nm-340 nm using a Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany). Particles less than 10nm in size were excluded from further calculations due to high variability of measurements. Count-median particle size was calculated using the number of particles mean. Particle mass was calculated from particle spectra, assuming sphericity and using the 40% load diesel exhaust particle density as previously described (Olfert et al., 2007). Particle number was further separated into two fractions: nucleation mode particles below 23 nm in size and solid particles above 23 nm (Amanatidis et al., 2014). Particles for both B100 fuels and ULSD were collected on quartz filters (47mm, SKC, USA) and sent for PAH analysis using Gas Chromatography Tandem Mass Spectrometry at Queensland Health Forensic and Scientific Services (Queensland, Australia).

2.6 Permeability: After being left to rest for 24 hours, permeability of the ALI models was analysed as previously described (Looi et al., 2016). Briefly, transepithelial electrical resistance was measured using a Epithelial Volt/Ohm (TEER) Meter (EVOM2 with chopstick electrode set, MERCK, AUS) to assess model integrity (Figure S1) before cultures underwent a fluorescent dextran permeability assay. Fluorescein isothiocynate labelled dextran beads (MERCK, NSW, AUS) were dissolved in HEPES buffered Hank's Balanced Salt Solution (HEPES-HBSS) (4 kDa beads, final concentration 2 mg/mL) and 0.5mL was added to the apical compartment of each ALI insert. 1.5 mL of fresh HEPES-HBSS buffer without dextran beads was added to the basal compartment and cultures were placed on an orbital shaker within an incubator at 37°C in an atmosphere of 5%CO_2/95% air for 6 hours to allow agitation to help beads flow from the apical to the basal compartment. Basal compartment samples of 0.75 mL were taken at 0, 0.5, 1, 2, 3, 4, 5 and 6 hours with fresh buffer replacing the sample. Apparent permeability was then calculated using the equation $P_{app} = (dQ/dt) \times (1/AC_0)$, where dQ/dt is the steady-state flux, A is the surface area of the membrane and C_0 is the initial concentration in the basal compartment.

2.7 Histology: After permeability, inserts were randomly allocated to either fixation or lysis for RNA extraction. Fixation occurred in 10% formalin for 30 minutes and inserts stored in 100% ethanol until all samples were collected. Inserts were then embedded in paraffin, and 5-μm thick sections sliced for hematoxylin and eosin staining as per manufacturers protocol (Richard Allan Scientific, Thermofisher Scientific Histology Series Stain).

2.8 Protein Concentration: After being left to rest for 24 hours, protein concentration of the insert lysate and apical and basal supernatant was assessed using a Pierce™ BCA protein assay kit (Thermofisher Scientific, MA, USA). Insert lysate was collected from half the insert after permeability analysis, basal supernatant was collected from the basal compartment of each exposed ALI culture (1.5 mL total) and apical supernatant was collected by performing a 0.5 mL media wash of the apical compartment.

2.9 Oxidative Stress PCR: After resting for 24 hours, RNA was extracted from insert lysate for one insert per ID per exposure, using RNeasy RNA Extraction Kit (Qiagen) as per kit instructions. Reverse transcription and qRT-PCR was performed as previously described (Ling et al., 2020) using TaqMan primers (ThermoFisher) and TaqMan Gene Expression Master Mix (ThermoFisher) as per master mix TaqMan protocol. The experimental primers used were as follows: *SOD1* Hs00533490_m1, *GPX1* Hs00829989_gH, *NOX4* Hs01558199_m1, *NOX5* Hs00225846_m1, *PRDX3* Hs04942082_m1, *CTSB* Hs00947433_m1, *HMOX1* Hs01110250_m1. The primers for the housekeeping gene were *PPIA* Hs99999904_m1. Expression was analysed using the $2^{-\Delta\Delta CT}$ method.

2.10 Mediators: Mediator release was assessed 24 hours after exposure for both the apical and basal compartments using a Bio-Rad 27plx human cytokine kit (Bio-rad, CA, USA) and accompanying software (Bio-Plex Manager, v6.1.1, Bio-Rad, Tokyo, Japan). The 27 mediators analysed can be further split into mediators that affect the innate and adaptive immune systems or act as regulators (Dayer et al., 2017; Duffy et al., 2013; Holdsworth and Gan, 2015; Sokol and Luster, 2015). Normalisation was performed to adjust supernatant concentrations to 1mL and all mediator concentrations were normalised to total protein lysate for each exposure group.

2.11 Statistical Analysis: Data are presented as mean ± standard deviation where indicated. All statistical analyses were performed using R statistical software (v3.4.3) (R Team, 2018) loaded with the packages “lme4” and “mgcv”. P-values less than 0.05 were considered significant. All statistical analyses excluding gas concentration data were completed using multivariate general linear modelling methodologies with the families “Gamma(inverse/log)” and “gaussian(log)” as best fit the data, applying a backwards elimination approach to remove insignificant predictive variables. For combustion gas analysis a separate General Additive Model (GAM) file was fitted to each gas measurement with concentration as the response variable and time as the predictor, thus allowing for non-parametric fits.

3.Results:

3.1 Gas Analysis: Mean values and standard deviations of gas components for each fuel are shown (Table 1), with the exception of CO which shows only the highest reading at 10 minutes due to the cold start effect on the performance of the catalytic converter. Trends over time were also captured (Figure S2). All fuels displayed similar trends over time with NO_x (NO and NO_2), CO_2 and SO_2 increasing rapidly in the first half of the exposure, O_2 decreasing rapidly in the first 20 minutes and CO peaking in the first 10 minutes before decreasing rapidly to undetectable concentrations. Of the four fuels tested we found Canola B20 to be the most different to ULSD with significantly increased O_2 and significantly decreased SO_2 and NO_x in the form of NO and NO_2 (Table 1: $p<0.05$). Canola B20 was also the most different to its B100 counterpart with four significant differences compared to Tallow B20's one (Table 1: $p<0.05$).

3.2 Particle Analysis: Particle spectra between the sizes of 10 nm-340 nm were obtained for each exhaust (Figure S3). All fuels displayed small peaks in particle size at approximatley 100 nm. In terms of particle number concentration, no significant differences were found between any of the fuels (Figure S3). Median particle size and particle mass were also calculated from the particle spectra and found to be similar (Table S2). Of the 28 PAHs tested in filter collected particulate matter, only 3 were found at concentrations above the limit of detection (Table S3).

3.3 Histology, Permeability and Protein Concentration: The ALI cultures used in this experiment were fully differentiated, as shown by the multiple layers and ciliated cells (Figure 1, representative images). No gross morphological differences in the airway epithelium were observed between Air and exhaust exposed inserts. Exposure to the exhaust of Tallow B100 and Tallow B20 resulted in significantly increased permeability of the ALI cultures, (1.33±0.67 and 1.52±0.41 fold change respectively) compared to that of Air exposure ($p<0.05$) (Figure 2). Tallow B20 exhaust also significantly increased permeability in comparison to ULSD and Canola B20 ($p<0.01$).

Figure 1: Representative morphological images of ALI cultures. Cultures these images were obtained from have been exposed to a) Air, b) ULSD, c) Canola B100 and d) Tallow B100. No differences in morphology were found after any of the exposures. Note: Scale bar: 50 µM.

Figure 2: Permeability measurements of ALI cultures after exhaust exposure. Permeability is measured as apparent permeability (Papp Coefficient) and normalised to fold change compared to Air for each fuel (*=p value<0.05, **=p value<0.01, n=12 for ULSD, Canola B20 and Tallow B100, n=11 for Air, Canola B100 and Tallow B20).

Exposure to both Tallow B100 and Canola B100 also resulted in significantly increased protein concentration in both the basal and apical compartments ($p<0.05$) (Figure 3). Exposure to Tallow B20 resulted in increased protein concentration only in the apical supernatant and exposure to ULSD resulted in increased protein concentration only in the basolateral supernatant ($p<0.01$).

Figure 3: Protein concentrations in apical and basal compartments of the exposed ALI cultures. Measured protein concentration in a) apical wash and b) basal supernatant (*= $p<0.05$, **= $p<0.01$).

3.4 Reactive Oxygen Species (ROS): Transcription of various oxidative stress response genes was analysed using quantitative polymerase chain reaction and the $2^{-\Delta\Delta CT}$ method (Figure 4 and Supplementary Figure S4). Of the seven genes tested, two were found to be significantly different to Air controls: *NOX4* and *PRDX3*. The expression of *NOX4* was significantly decreased after exposure to Canola and Tallow B100 exhausts, whereas *PRDX3* expression was significantly increased after exposure to Tallow B20 exhaust ($p<0.05$ in all cases).

Figure 4: Transcription of oxidative stress genes in the exposed ALI cultures. Mean (standard deviation) fold change in expression compared to Air exposed controls for the oxidative stress response genes a) *CSTB*, b) *HMOX1*, c) *NOX4* and d) *PRDX3* (*= $p<0.05$, **= $p<0.01$, n=6 in all cases except *PRDX3* ULSD and Canola B20 where n=5). The remaining three markers can be found in the supplementary (Figure S4).

3.5 Mediator Release: Mediator release was measured for both the apical and basal supernatants (Tables 2 and 3). Of the 27 mediators analysed, 16 were released at levels above the limits of detection, 15 for each compartment. PDGF-bb was only measured above the limit of detection in the apical supernatant, whereas VEGF was only measured above the limit of detection in the basal supernatant. The 15 mediators released in the apical compartment can be translated as local mediator release (Floreth et al., 2011) and significant differences primarily impacted the innate immune system with differences found in 3 regulatory (IL-1RA, IL-7 and PDGF-bb), 5 adaptive (IL-5, IL-9, IFN-γ, IP-10 and RANTES) and 7 innate mediators (IL-6, IL-8, G-CSF, GM-CSF, MCP-1, MIP-1β and TNF-α) ($p<0.05$) (ElKassar and Gress, 2010; Holdsworth and Gan, 2015; Sokol and Luster, 2015). In comparison to Air exposed controls, Tallow B20 was the most immunogenic locally with significantly altered release of 11 mediators, followed by 10 for Tallow B100 whereas Canola B100 was the least with only two altered mediators being produced.

The mediator release in the basal compartment (Table 3) can be interpreted as systemic mediator production (Floreth et al., 2011) and was also found to primarily impact the innate immune response with 3 regulatory (IL-1RA, IL-7 and VEGF), 5 adaptive (IL-5, IL-9, IFN-γ, IP-10 and RANTES) and 7 innate mediators (IL-6, IL-8, G-CSF, GM-CSF, MCP-1, MIP-1β and TNF-α) ($p<0.05$) (ElKassar and Gress, 2010; Holdsworth and Gan, 2015; Sokol and Luster, 2015). Systemic inflammation was impacted more than local with more exhaust exposures significantly altering mediator production; both IL-1RA and IL-6 were significantly released in all exhaust in the basal compartment compared to Air exposed controls. Tallow B20, Tallow B100 and Canola B20 exhaust exposures induced the greatest mediator responses with the significant increase of 7 mediators each compared to Air exposed controls, although Canola B20 impacted the regulatory response more with significantly increased release of VEGF compared to Tallow B20 and B100 which impacted the innate response more with the increased release of TNF-α ($p<0.05$).

4.Discussion:

Results of this study show that exposure to highly diluted biodiesel or diesel exhaust elicits a range of health impacts in a primary human airway epithelial ALI exposure model. Of the two biodiesels tested we found Tallow to cause the most negative health impacts, with both the 20% blend and the pure biodiesel exhausts inducing increased permeability of the epithelial barrier, increased protein concentrations in the apical and/or basal compartment (suggesting epithelial cell damage) and the broadest range in mediator release. These findings were unexpected considering that ULSD exhaust contained the highest concentrations of exhaust gas components and no differences were found between any of the different fuel types for exhaust particle characteristics. Subsequently, we found Canola biodiesel to have the least negative health effects of the tested fuels, with no effect on permeability and the smallest impact on mediator release.

In terms of exhaust characteristics, Canola B20 was the most different to diesel exhaust, with three of the six gases tested being significantly different. All B20 and B100 exhausts were found to have decreased NO_2 concentrations (compared with ULSD), and Canola B100 and Canola B20 exhausts displayed a decrease in NO levels. This is in contrast to previous studies which have found NO_x levels to be increased in the exhaust of biodiesel when compared to mineral diesel (de Brito et al., 2018; Graver et al., 2016), although reports on biodiesel blends are contradictory with studies showing both more and less NO_x (Graver et al., 2016; Mullins et al., 2016). This difference to previous literature observed in our study could be attributed to some effect of biodiesel on exhaust after treatment devices, which are known to impact exhaust NO_x concentrations (Ko et al., 2019), as many of the previous studies that assessed biodiesel health effects used old technology engines not equipped with exhaust after-treatment devices (Larcombe et al., 2015). For example, use of biodiesel in an engine equipped with a diesel particulate filter (DPF) has been found to lower particle loading and shorten regeneration time compared to ULSD, however, biodiesel also reacted more readily with the lubricating oil which in turn caused a slower rise in DPF inlet temperature (Pechout et al., 2019). All these effects would impact the concentrations of various exhaust components.

We found no significant differences in the particle characteristics between any of the tested fuels in the range of 10-340 nm, likely because of the high dilutions used. Previous studies have found differences in particle mass concentrations to be subtle enough that a 1/20 dilution would negate any differences between fuels (de Brito et al., 2018,(Valand et al., 2018). This suggests that the increased toxicity observed after Tallow biodiesel exhaust exposures was not caused by an increase in ultrafine particles, as has been suggested by previous studies (Lankoff et al., 2017; Mullins et al., 2016). Since all exhausts had similar fine particle concentrations and the combustion gas concentrations were highest in ULSD, this suggests that Tallow biodiesel exhaust and Tallow biodiesel blend exhaust toxicity is associated with an exhaust component that has not been broadly tested for in this study, such as PAH's or heavy metals (Fontaras et al., 2009; Kowalska et al., 2017). Although we attempted to analyse PAH concentrations for Diesel and Canola and Tallow B100, collected particle deposits were so low than only 3 of the 28 tested PAH's were found at concentrations above the limit of detection (Table S3). These concentrations were highest in Tallow B100 exhaust however an analysis of 3 PAHs cannot be considered comprehensive and previous studies have found tallow biodiesel to contain lower levels of non-volatile organic compounds and particulate semi-volatile organic compounds than other biodiesel types (Cheng et al., 2017; Schirmer et al., 2016).

One of the more concerning implications of this study is that we found considerable toxic health effects despite exhaust parameters being within Australian Work Standards, which is also used as a guideline for European standards (EU OSHA, 2013) and is equal to or stricter than the US Occupational Safety and Health Administration standards (US OSHA). The Safe Work Australia standards for various exhaust components are time weighted 8 hour averages of 3 ppm for NO_2 (with concentrations not exceeding 5 ppm over a 15 minutes average), 25 ppm NO, 2 ppm SO_2 (with concentrations not exceeding 5 ppm over a 15 minutes average), 30 ppm CO and 5,000 ppm CO_2 (with concentrations not exceeding 30,000 ppm over a 15 minute average) (SWA, 2019). Oxygen must not fall below "safe levels" of 19.5% (SWA, 2018). It is recommended that in Australia,

particulate matter exposure from diesel exhaust not exceed 100 µg/m^3 elemental carbon, although this is not a hard limit (AIOH, 2017). In America, the limit for a non-coal mining setting is set at 160 µg/m^3 total carbon (MSHA, 2016) and the European Union has set a recent occupational exposure limit of 50 ug/m^3 elemental carbon (EU, 2019; EU, 2004). The diluted exhaust used in this study meets all these limits.

Despite the exhaust used in this study being "safe" in terms of work standards, we measured increased airway epithelial barrier permeability after just one hour of exposure to Tallow B100 and B20 in comparison to Air controls. The airway epithelium is designed to act as a first line of defence against insults from viruses, bacteria and other environmental insults such as diesel exhaust (Celebi Sözener et al., 2020; Faber et al., 2020; Looi et al., 2018). Increased permeability compromises this function and allows these insults to invade the underlying lung tissue (Faber et al., 2020), providing a potential mechanism for entrance into the cardiovascular system (Brook et al., 2010; Cho et al., 2018; Neophytou et al., 2019). Since previous biodiesel exhaust studies have found indications of cytotoxicity in submerged cell line cultures, this indication of increased permeability could provide a functional consequence of that cytotoxicity (Agarwal et al., 2018; Bünger et al., 2000). As ultrafine particles are capable of bypassing this barrier to enter the bloodstream directly (Brook et al., 2010; Celebi Sözener et al., 2020), an increase in epithelial barrier permeability would only amplify this effect and likely contribute to even worse health outcomes.

In addition, if ULSD exposure at higher concentrations also causes increased barrier permeability then this could help explain why previous studies, including those by Gowdy et al (2010), Zarcone et al (2017) and Shears et al (2020), have found dual insults of diesel exhaust exposure and respiratory pathogens such as influenza, non-typeable *H influenzae* and *S pneumoniae* to increase the severity of disease (Gowdy et al., 2010; Shears et al., 2020; Zarcone et al., 2017). Increased permeability would help the virus or bacteria infiltrate the airway epithelium, potentially facilitating infection and increasing disease severity (Fukuoka et al., 2016; Looi et al., 2018; Shears et al., 2020). Alvarez-Simón

et al 2017 have successfully used diesel exhaust to sensitise mice to soy protein to simulate an allergic asthma model (Alvarez-Simón et al., 2017). Increased permeability would also facilitate passage of allergens across the epithelial barrier which would be a crucial process in driving allergic responses (Celebi Sözener et al., 2020). Since only small amounts of Tallow biodiesel exhaust is needed to induce increased barrier permeability, this has concerning implication for human exposure in areas where Tallow biodiesel is already in use (ARENA, 2018; EIA, 2020b; Flach et al., 2019; Toldrá-Reig et al., 2020). This is especially true during the current COVID-19 pandemic, where increased permeability could potentially increase infection severity (Ali and Islam, 2020; Pozzer et al., 2020).

Although we measured the expression of seven different oxidative stress response genes, only two were found to be differentially expressed compared with Air controls. The expression of *NOX4*, or NADPH oxidase 4, is associated with physiological signalling as an oxygen sensor and it catalyses the reduction of O_2 into various species of ROS (Schröder et al., 2012). It was decreased in response to both B100 exhaust exposures and previous studies have shown that inhibited expression helps to attenuate some species of ROS (Hollins et al., 2016; Kuroda et al., 2010). *PRDX3* or Peroxiredoxin 3, belongs to a family of peroxidases that function as antioxidant enzymes and thus help to protect against the damage caused by ROS (Rebelo et al., 2021). We found this gene to be more highly expressed after exposure to Tallow B20 exhaust. Overall, these changes suggest oxidative stress may be ongoing within the exposed cells, however the small change in expression, combined with the response being observed mostly in the more toxic exposures, suggests that either the highly diluted exhaust used in our exposures does not result in high amounts of oxidative stress or that the 24-hour timepoint chosen is not optimal to best measure a ROS response.

We also found altered mediator release in both the apical and basal compartments after exhaust exposure. The mediators measured have a variety of effects and can impact both the innate and adaptive immune responses (Holdsworth and Gan, 2015). As the ALI culture models human lung

formation, mediator changes in the apical compartment can be interpreted as changes more relevant to local inflammation of the airway lumen, whereas changes in the basal compartment can be interpreted as relevant to the basement membrane of the airway and thus a more systemic reaction (Floreth et al., 2011). Of the 27 mediators tested, we found 15 were released above measurable concentrations in both the apical and basal compartments. These 15 cytokines mostly overlapped, however VEGF (which helps promote angiogenesis and lung injury repair (Boussat et al., 2000)) was released only in the basal compartment and PDGF-bb (which helps promote wound repair but is also connected with airway hyperresponsiveness (Kardas et al., 2020)) was released only in the apical compartment. Of the 5 exhausts tested, Tallow B20 and Tallow B100 were the most immunogenic in the apical compartment and Tallow B20, Tallow B100 and Canola B20 were the most immunogenic in the basal compartment. Canola B100 was the least immunogenic in both compartments, which supports our previous work (Landwehr et al., 2021).

The differences seen between apical and basal mediator release, where more mediators are released for all 5 exhaust exposures in the basal compartment, are indicative of the mediator response to exhaust being driven mostly through systemic inflammation and potentially through different cell types such as basal epithelial cells, with local airway lumen inflammatory responses in the apical compartment only occurring after exposure to the more inflammatory exhausts. This is understandable as many immune cells, such as neutrophils, would need to be recruited to the site of insult (Sokol and Luster, 2015) and the local inflammation mediators released apically for the more inflammatory Tallow B20 and Tallow B100 exposures primarily impact the innate immune response (Holdsworth and Gan, 2015; Sokol and Luster, 2015). Previous studies looking into the impact of diesel exhaust exposure on workers have found indications of systemic inflammation (Wang et al., 2017), and our results indicate that exposure to any of the pure biodiesel or blended exhausts would likely result in similar, or worse, responses.

Previous studies that have exposed ALI cultures to biodiesel exhaust have several limitations. One key issue is that they often use undifferentiated cell lines grown on transwell membranes, which is limited in how accurately it can model human tissue and negates effects caused by genetic variability (Kicic et al., 2006; Barraud et al., 2017; Steiner et al., 2013). Additionally these cell lines are cultured at ALI conditions for the minimal time possible for the experiment (<12 hours) (Barraud et al., 2017; Steiner et al., 2013), due to the cell lines inability to survive extended time outside of liquid, which further limits how accurately they can model real world exposures. Other studies only expose the cultures to one type of biodiesel and attempt to extrapolate that data to represent biodiesel as a whole (Hawley et al., 2014; Vaughan et al., 2019) when our data shows that biodiesel feedstock type greatly impacts health effects. Previous studies also use exhaust concentrations that are too high to be entirely relevant to real world conditions, with either particle mass (>200 $\mu g/m^3$) or NO_2 (>9 ppm) concentrations being much higher than many occupational exposure limits permit (SWA, 2018, 2019; (AIOH, 2017; EU, 2019; MSHA, 2016; EU, 2004). Previous studies that assess the health impacts of diesel and biodiesel exhaust without the use of an ALI culture focus almost exclusively on the particulate matter components of exhaust, generally ignoring the gaseous components entirely (André et al., 2015b; Larcombe et al., 2015). In addition, they use filters to collect these particles and then expose submerged cultures directly to extracted particle solutions (Cervena et al., 2017; Gioda et al., 2016). While this method allows for accurate dosing and easier comparison between exposures, it removes both the effects of the gaseous components and the ultrafine particles which agglomerate on the filter leading to skewed particle size spectra (Morin et al., 2008). We exposed our cultures directly to dilute exhaust and found significant health effects after exposure to Tallow biodiesel even though the particle size spectra between the different exhausts did not change. This means that the ALI cultures in each exposure group were likely exposed to similar dosages of particles. This in turn means that the increased toxicity in the Tallow B100 and B20 exposure groups is a direct effect of the different exhaust components being more toxic than those of ULSD, not something that can be attributed to just having more particles within one exposure group. The same

can be said for Canola, which we found to be less toxic than ULSD despite having similar exhaust particle profiles.

There are several limitations to our study. We used highly diluted exhaust concentrations in order to simulate real world exposure events, meaning that the health impacts observed are relatively small. Using more concentrated exposures may allow more differences between treatments to be identified but at the detriment to losing this "real-world" applicability. However, the primary strength of the current study was that toxicological differences were observed despite using occupational exhaust concentrations. Our study also lacks a comprehensive particle chemistry analysis, in part because the exhaust concentrations used were so low that we could not collect enough particles for more than one type of analysis, and a direct cytotoxicity analysis, mainly due to an inability to force the cells into single cell suspension for flow cytometry without significantly lowering baseline viability. Furthermore, using the ALI airway-epithelial cell model, we focused primarily on the toxicological effects of exposure on the lungs, missing the potential effects of exposure (primary or secondary) to other biological systems. We also used primary cells obtained from "healthy" patients, meaning that those with underlying respiratory conditions or diseases could have different health impacts to what was found in our study. Finally, we used two different first-generation biodiesel types chosen based on current biodiesel usage, when in future biodiesel will likely be created from oil crops that do not compete with food prices.

5.Conclusion:

In conclusion, this study is the first to use differentiated epithelial cells grown at ALI to assess the exhaust toxicity of more than one type of biodiesel or biodiesel blend. It is also one of the first studies to use exhaust diluted to real-world exposure concentrations to assess biodiesel and biodiesel blend toxicity, as well as the first to use a transepithelial permeability assay to assess exposure impacts for either diesel or biodiesel exhaust. We found exposure to Tallow biodiesel exhaust, both B100 and B20, to be the most toxic with increased permeability and the greatest

mediator response. This was followed by ULSD and then Canola B100 and B20 exhaust, showing that even when biodiesel does not comprise of the majority of the fuel, the feedstock type used to make it still significantly impacts exhaust toxicity. These results support our previous study (Landwehr et al., 2021) into the toxic effects of different biodiesel exhaust exposures where we also found Tallow biodiesel to be the most toxic and Canola the least using a submerged culture experimental design which incorporated some additional endpoint measurements. This suggests that a less complicated submerged model can be used to assess the basic toxicity of different biodiesel fuels so long as whole exhaust is used, however for a more comprehensive assessment into the mechanisms of toxicity a more complicated model such as ALI or *in vivo* animal models is needed.

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Authors' contributions: KRL, AKicic, BJM and ANL designed the experiments. KRL and JH grew and maintained the cultures. KRL performed the exposures and all downstream analysis with help from ANL. AKing and BJM designed and helped build the exposure set-up. KRL and RM-H created the fuels. KRL and BJM maintained the engine. RO'L advised on statistical analyses. Interpretation and discussion of data was performed by KRL, AKicic, BJM and ANL. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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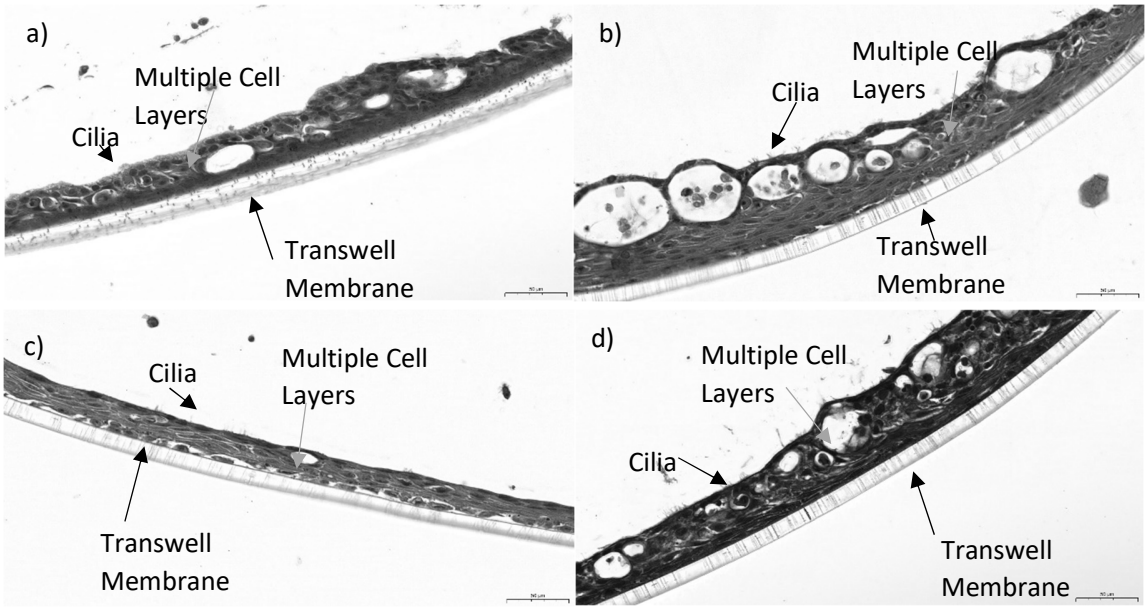


Figure 1: Representative morphological images of ALI cultures. Cultures these images were obtained from have been exposed to a) Air, b) ULSD, c) Canola B100 and d) Tallow B100. No differences in morphology were found after any of the exposures. Note: Scale bar: 50 µM.

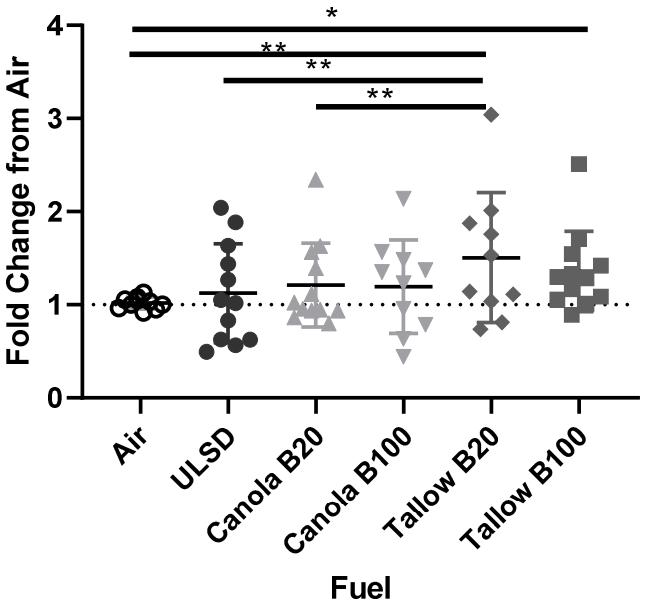


Figure 2: Permeability measurements of ALI cultures after exhaust exposure. Permeability is measured as apparent permeability (Papp Coefficient) and normalised to fold change compared to Air for each fuel (*=p value<0.05, **=p value<0.01, n=12 for ULSD, Canola B20 and Tallow B100, n=11 for Air, Canola B100 and Tallow B20).

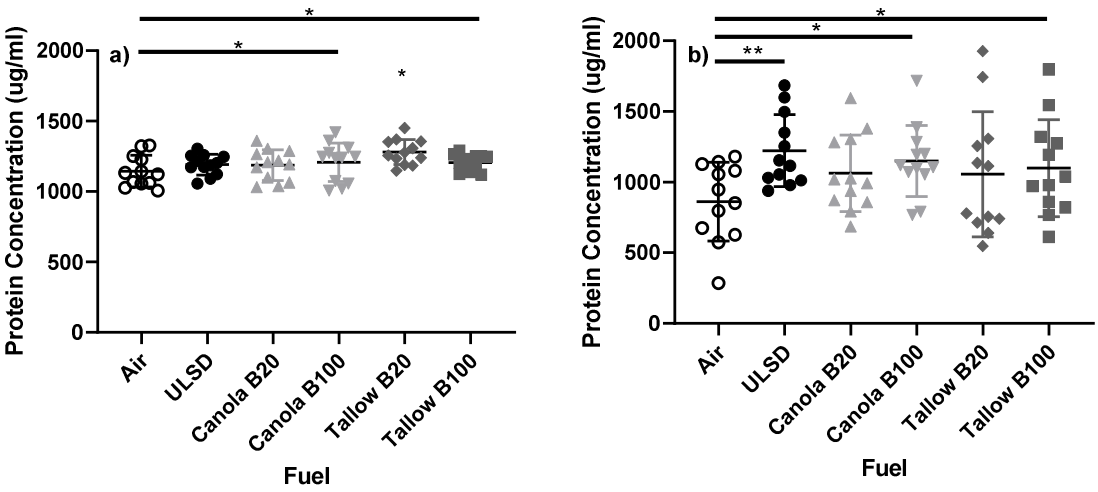


Figure 3: Protein concentrations in apical and basal compartments of the exposed ALI cultures.

Measured protein concentration in a) apical wash and b) basal supernatant (*= $p<0.05$, **= $p<0.01$).

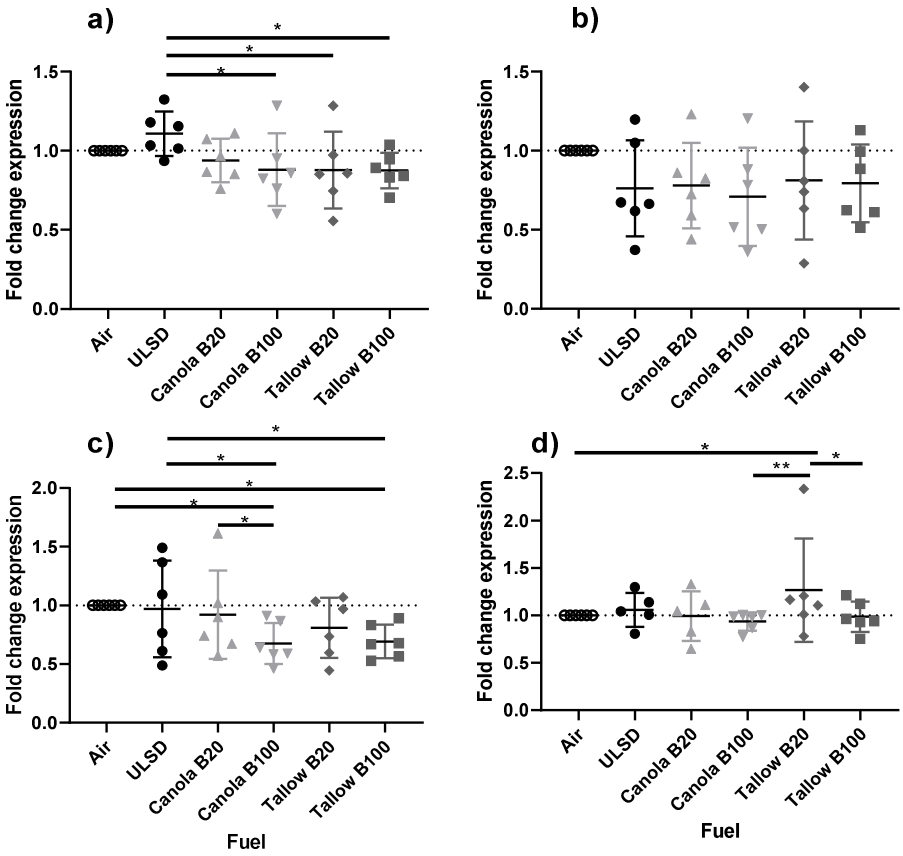


Figure 4: Transcription of oxidative stress genes in the exposed ALI cultures. Mean (standard deviation) fold change in expression compared to Air exposed controls for the oxidative stress response genes a) *CSTB*, b) *HMOX1*, c) *NOX4* and d) *PRDX3* (*= p<0.05, **= p<0.01, n=6 in all cases except *PRDX3* ULSD and Canola B20 where n=5). The remaining three markers can be found in the supplementary (Figure S4).

Table 1: Mean (standard deviation) gas measurements for all exhausts. All significances displayed are compared to ULSD.

Fuel	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
O_2 (%)	20.63 (0.105) a	20.74 (0.062) ***,b,c	20.64 (0.146) a	20.67 (0.082) a	20.64 (0.077)
CO (ppm)	0.80 (0.20) d	1.23 (0.67)	1.27 (1.19)	0.80 (0.20) d	1.07 (0.50) *,c
CO_2 (%)	0.35 (0.098)	0.25 (0.145)	0.33 (0.068)	0.31 (0.112)	0.34 (0.101)
NO_x (ppm)	11.47 (2.26) a,b,c	7.82 (1.78) ***,b,c,d	7.75 (1.55) ***,a,c,d	10.43 (2.22) * a,b	10.44 (2.21) a,b
NO (ppm)	7.09 (1.52) a,c	4.94 (1.02) ***,c,d	5.51 (1.18) *** c,d	6.83 (1.41) a,b	7.34 (1.62) a,b
NO_2 (ppm)	4.39 (1.06) a,b,c,d	2.89 (0.83) ***,b,c	2.25 (0.41) ***,a,c,d	3.59 (0.90) ***,a,b,d	3.10 (0.68) ***,b,c
SO_2 (ppm)	0.83 (0.38) b	0.89 (0.32) b	0.50 (0.51) ***,a,d	0.94 (0.23)	0.94 (0.24) b

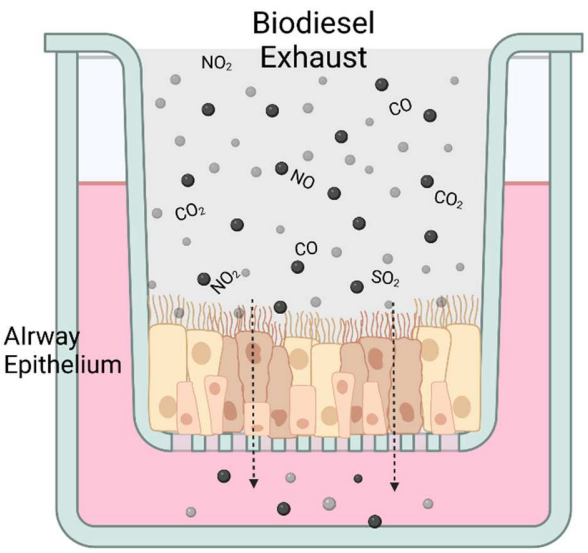
Measurements are shown as the mean concentration for the entire exposure, with the exception of CO which is shown as the peak measurement.
* Significantly different to ULSD (*=p <0.05, **=p <0.01, ***=p <0.001)
a=significantly different to Canola B20 (p<0.05)
b=significantly different to Canola B100 (p<0.05)
c=significantly different to Tallow B20 (p<0.05)
d=significantly different to Tallow B100 (p<0.05)

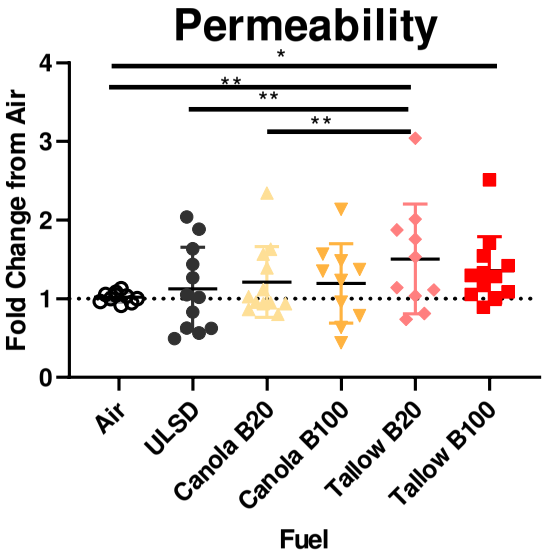
Table 2: Mean mediator release in the apical compartments. Mean (standard deviation) mediator release for the 15 cytokines released above the limits of detection for the apical supernatant samples.

Mediator Concentration (pg/mL/mg protein)	Fuel					
	Air	ULSD	Canola B20	Canola B100	Tallow B20	Tallow B100
IL-1RA	659.37 (987.96)	1264.47 (2113.32)****	1210.17 (1556.21)** d	841.87 (1161.45) ## c,d	1432.94 (1913.46)**** b	1461.20 (2172.96)**** a,b
IL-5	7.23 (8.77)	8.23 (11.56)	10.46 (9.70) b,c	15.76 (23.00)** ## a,c,d	21.91 (37.89)**** ### a,b,d	13.56 (16.31) b,c
IL-6	89.93 (68.02)	130.79 (62.73)	140.25 (79.56)	159.47 (194.00)	234.04 (234.52)*	218.19 (131.65)*
IL-7	23.93 (4.11)	24.30 (6.53)	30.96 (6.72)** # b,d	18.73 (6.88) # a,c	27.59 (8.49) b	22.81 (8.68) a
IL-8	3744.90 (1655.76)	5717.74 (2311.66)*	6311.03 (4263.62)** b	3796.56 (1167.34) # a,c,d	6984.61 (4318.23)** b	7232.75 (4919.93)** b
IL-9	44.04 (7.91)	59.07 (11.55)**	60.27 (16.84)** b	44.24 (11.44) ## a,c,d	59.20 (20.41)** b	60.44 (14.73)** b
G-CSF	40.96 (64.41)	93.08 (107.09)**	61.56 (62.05) d	35.80 (42.43) ## c,d	140.31 (175.58)*** b	130.54 (88.98)*** a,b
GM-CSF	7.17 (5.48)	9.79 (8.34)	8.25 (5.40)	6.18 (4.82) # d	8.55 (6.49)	9.54 (10.42) b
IFN-γ	3.62 (6.35)	8.30 (8.34)	8.32 (5.40)**	5.62 (4.82)	9.52 (6.49)**	9.06 (10.42)*
IP-10	486.74 (276.17)	326.96 (159.21)*	484.45 (250.72) # b	311.37 (292.31) a	403.84 (293.32)	360.41 (163.04)
MCP-1	8.70 (5.38)	11.66 (8.03)	11.70 (7.22) c,d	14.09 (15.07)	22.48 (18.53)** # a	24.25 (29.85)** # a
PDGF-bb	26.57 (26.64)	18.85 (14.41)	44.03 (37.02)** ### b,c,d	13.12 (13.70)* a	9.54 (5.29)* a	14.38 (16.20)* a
MIP-1β	10.95 (2.31)	14.66 (2.28)***	15.53 (3.92)*** b	11.16 (2.89) ## a,c,d	14.40 (4.33)** b	15.31 (4.01)*** b
RANTES	13.80 (8.82)	14.34 (11.47)	12.54 (8.83)	9.80 (5.83)	8.69 (5.48) #	12.00 (7.05)
TNF-α	15.15 (11.88)	24.24 (18.87)*	21.34 (16.19)	16.27	26.01 (18.27)** b	24.14 (21.99)** b

				(10.84) c,d		

=significantly different to Air (=p <0.05, **=p <0.01, ***=p <0.001)
#=significantly different to ULSD (#=p <0.05, ##=p <0.01, ###=p <0.001)
a=significantly different to Canola B20 (p<0.05)
b=significantly different to Canola B100 (p<0.05)
c=significantly different to Tallow B20 (p<0.05)
d=significantly different to Tallow B100 (p<0.05)


Biodiesel
Exhaust
NO2
CO
NO
CO2
CO2
CO
NO2
SO2
Airway
Epithelium


Permeability
Fold Change from Air
0
1
2
3
4
*
**
**
**
Air
ULSD
Canola B20
Canola B100
Tallow B20
Tallow B100
Fuel