Biodiesel feedstock determines exhaust toxicity in 20% biodiesel : 80% mineral diesel blends.

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Abstract:

To address climate change concerns, and reduce the carbon footprint caused by fossil fuel use, it is likely that blend ratios of renewable biodiesel with commercial mineral diesel fuel will steadily increase, resulting in biodiesel use becoming more widespread. Exhaust toxicity of unblended biodiesels changes depending on feedstock type, however the effect of feedstock on blended fuels is less well known. The aim of this study was to assess the impact of biodiesel feedstock on exhaust toxicity of 20% blended biodiesel fuels (B20). Primary human airway epithelial cells were exposed to exhaust diluted 1/15 with air from an engine running on conventional ultra-low sulfur diesel (ULSD) or 20% blends of soy, canola, waste cooking oil (WCO), tallow, palm or cottonseed biodiesel in diesel. Physico-chemical exhaust properties were compared between fuels and the post-exposure effect of exhaust on cellular viability and media release was assessed 24 hours later. Exhaust properties changed significantly between all fuels with cottonseed B20 being the most different to both ULSD and its respective unblended biodiesel. Exposure to palm B20 resulted in significantly decreased cellular viability (96.3 ± 1.7%; $p<0.01$) whereas exposure to soy B20 generated the greatest number of changes in mediator release (including IL-6, IL-8 and TNF-α, $p<0.05$) when compared to air exposed controls, with palm B20 and tallow B20 closely following. In contrast, canola B20 and WCO B20 were the least toxic with only mediators G-CSF and TNF-α being significantly increased. Therefore, exposure to palm B20, soy B20 and tallow B20 were found to be the most toxic and exposure to canola B20 and WCO B20 the least. The top three most toxic and the bottom three least toxic B20 fuels are consistent with their unblended counterparts, suggesting that feedstock type greatly impacts exhaust toxicity, even when biodiesel only comprises 20% of the fuel.

Keywords: Exhaust Exposure, Health, *in Vitro* Exposure Model, Vehicle Emissions, Toxicology of Exhaust Emissions

Abbreviations:

FAME; Fatty acid methyl esters

ULSD; Ultra-low sulfur diesel

O2; Oxygen

CO; Carbon Monoxide

CO2; Carbon Dioxide

NOx; Nitrogen oxides

NO; Nitrogen Monoxide

NO2; Nitrogen Dioxide

SO2; Sulfur Dioxide

PM; Particulate matter

ULSD; Ultra-low sulfur diesel

IL-1β; Interleukin 1-beta

IL1-RA; Interleukin 1 receptor antagonist

IL-6; Interleukin 6

IL-8; Interleukin 8

IL-9; Interleukin 9

G-CSF; Granulocyte colony-stimulating factor

GM-CSF; Granulocyte-macrophage colony-stimulating factor

IFN-γ; Interferon gamma

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

MCP-1: *Monocyte chemoattractant protein 1*

MIP-1β; Macrophage Inflammatory Protein 1-beta

RANTES; Regulated on Activation, Normal T Cell Expressed and Secreted

TNF-α; Tumor necrosis factor-alpha

VEGF; Vascular endothelial growth factor

1. Introduction:

Biodiesel use is increasing worldwide (EIA, 2020) due to pressure from the climate change crisis, demand for alternate fuels and the increasing cost of fossil fuel extraction. Currently, the majority of biodiesel use worldwide is in the form of blended fuels where biodiesel is combined with commercial mineral diesel in order to improve the lubricity of low sulfur diesel fuel and manage environmental concerns (EERE, 2020; Li et al., 2019; Peng, 2017). Blend types are normally specified by a label B followed by the percentage of biodiesel blended within the fuel, e.g. B20 for 20% biodiesel fuel with 80% mineral diesel or B100 for 100% biodiesel fuel. The percentage of biodiesel within commercial diesel varies from country to country and is largely dependent on whether biodiesel blending is mandatory or optional (Barros, 2020; Price, 2019). The requirement for biodiesel blends to be labelled as a blend (instead of just diesel) also varies between different countries. For example, in Brazil, B12 became the mandated blend in March 2020 (Barros, 2020), while European Union countries have a legislated maximum amount of B7 (EU, 2016) and the US blend mandates change from state to state with some mandating a blend of B20 with labelling and others with no blending requirements (EERE, 2020; ASTM, 2020a, b). In Australia a maximum blend of B5 is allowed without labelling and B20 can be sold with labelling (ACCC, 2021; Price, 2019). As global climate change concerns increase and air pollution regulations become more stringent, it is likely that mandated blend amounts will increase (EU, 2009; Ragauskas et al., 2006). Thus, most biodiesel research into blended fuel focuses on blends between 20-30% biodiesel, with some testing up to 50% (Larcombe et al., 2015; Møller et al., 2020).

As with studies that investigate the effects of exposure to exhaust generated by the combustion of B100 biodiesels, studies which test blended biodiesel-diesel fuels also produce variable results. This is like due to the use of a wide range of methodologies, and because different studies blend their biodisesl with mineral diesel of varying chemical composition. This makes comparisons between different studies difficult. For example, different engine types (André et al., 2015; Magnusson et al., 2019), different exhaust after-treatment systems (Adenuga et al., 2016; André et al., 2015; Magnusson et al., 2019), whether speed and load are kept constant or a drive cycle is used (Fontaras et al., 2009; Magnusson et al., 2017) and whether particulate matter (PM) is measured by mass and/or particle number (Magnusson et al., 2017; Mullins et al., 2016), all contribute to changing the resulting exhaust outputs. This leads to some studies showing the B20 exhaust contains higher concentrations of more toxic pollutants such as oxides of nitrogen (NO_x) and PM (Brito et al., 2010; Graver et al., 2016) when compared with either diesel or B100 fuels, with other studies showing the opposite relationship (Libalova et al., 2016; Mullins et al., 2016).

Another confounding factor in any attempt to compare different biodiesel blend studies is the fact that different countries have different legislative requirements and standards for the chemical properties of commercial diesel fuel (which is subsequently blended with biodiesel). These differences can significantly impact exhaust physico-chemical properties, and are most easily observed in the amount of biodiesel that may already be present in diesel fuel (without labelling) *prior* to blending and with respect to permitted levels of sulfur. Thus, while biodiesel amounts already present within diesel fuel can range from nothing up to 7% (Magnusson et al., 2019), sulfur levels can also change drastically. For example, some studies use “ultra-low-sulfur-diesel” (ULSD) containing 10 ppm sulfur or less (Mullins et al., 2016), others use fuel containing up to 50 ppm sulfur (André et al., 2015) and some up to 500 ppm

sulfur (Brito et al., 2010). Sulfur levels are known to alter the toxic effects of diesel exhaust exposure with higher sulfur levels resulting in higher mutagenicity (Bünger et al., 2000).

Similarly the toxic effects measured after exposure to exhaust generated from biodiesel blends are also inconsistent, with studies showing biodiesel blends to be more toxic than (in terms of cytotoxicity and oxidative effects (Adenuga et al., 2016; Betha et al., 2012)), similarly toxic (in terms of DNA damage and mediator release (Cervena et al., 2017; Jalava et al., 2012)) or less toxic (in terms of DNA damage, oxidative stree and mediator release (Steiner et al., 2013; Yang et al., 2017)) mineral diesel. Biodiesel blends have also been shown to be more toxic than (in terms of oxidative effects and genotoxicity (Ackland et al., 2007; Adenuga et al., 2016)), similarly toxic (in terms of inflammatory response, DNA damage and gene expression dysregulation (Brito et al., 2010; Cervena et al., 2017; Libalova et al., 2016)) or less toxic than (in terms of cytotoxicity and DNA damage (Mullins et al., 2016; Vogel et al., 2019)) B100 fuels generated from the same feedstock type. The effects of different feedstocks being used to make the biodiesels within the blended fuel is rarely considered (Møller et al., 2020), despite the known associations between fuel characteristics and engine performace (Fontaras et al., 2009; Knothe and Steidley, 2005; McCormick et al., 2001).

Finally, in previous studies investigating the potential health effects of exposure to biodiesel blend exhaust, whole exhaust, or diluted exhaust are rarely used. Instead, most studies only consider the toxic effects of the exhaust particles extracted from filters using either the Ames assay or an immortalised cell line (André et al., 2015; Larcombe et al., 2015; Surawski et al., 2011). This generates an artifical particle spectra as exhaust particles easily agglomerate to larger sizes when collected using this method (Morin et al., 2008). As such, both the gaseous component of the exhaust, and the particle size spectrum are generally ignored (André et al., 2015; Landwehr et al., 2019; Larcombe et al., 2015).

Thus, while there are some published data on the health effects of exposure to exhaust generated from different biodiesel blends, it is impossible to draw any firm conclusions regarding which base-oils may be more or less harmful in blend form (compared with mineral diesel and/or B100). This means that there is an urgent need for a comparative assessment of exhaust exposure health effects of biodiesel blends made from different feedstocks, in which exhaust is generated, and exposures performed in a consistent way. This would allow assessment and direct comparison of the toxicity of different feedstock types and could allow identification of lower toxicity feedstocks before higher biodiesel concentratated diesel blends become mandated in more countries. This comparison needs to be done in such a way that methodological setup including engine paramaters and endpoint measures are kept as consistent as possible. To address this, we exposed primary airway epithelial cells to diluted exhaust generated by an engine running on ULSD or a 20% blend of biodiesel within that same ULSD fuel. Blends were made from several different biodiesel feedstocks representing those commonly used worldwide (Eea, 2013; OECD/FAO, 2020) including soy, canola, waste cooking oil (WCO), tallow, palm and cottonseed. The pure biodiesel exhaust exposure were also assessed alongside the B20 exhaust exposures (Landwehr et al., 2021b) so comparisons between each blend type and its matched B100 could be made. Fuel characteristics (such as fatty acid methyl ester profiles) were measured and exhaust physico-chemical properties for each blend type were recorded. Twenty-four hours after exposure health outcomes including cellular viability and mediator release were analyed. Based on the

published literature, and our own previous research, we hypothesised that exposure to blended biodiesel exhaust would cause more severe and a wider variety of toxic health effects than exposure to ULSD and B100 exhausts and that the different blended exhausts would cause a spectrum of health impacts, with some being more toxic than others. The results of this study will confirm if biodiesel feedstock type impacts exhaust toxicity, even when it only makes up 20% of the total fuel.

2. Materials and Methods:

2.1 Fuel Types: Six different blended biodiesel fuels (B20) were used in this study. Soy, canola (rapeseed), tallow, palm and cottonseed biodiesel were created using high-quality, food-grade, commercial oils (Campbells Wholesale Reseller, WA, AUS and Range Products, WA, AUS). Waste cooking oil was obtained as used cooking fryer waste from a restaurant in Perth, Western Australia. All oils were converted to fatty acid methyl esters (FAME) using an established sodium methoxide transesterification process (Knothe et al., 2015) also used in our previous studies (Landwehr et al., 2021b; Landwehr et al., 2019). Commercial ULSD was obtained from a local supplier (SHELL, WA, AUS, biodiesel free). All blended fuels used in this study were obtained by blending 20% B100 from each biodiesel feedstock type with 80% ULSD.

2.2 Participants: Approval for this study was obtained from the St John of God Hospital Human Ethics Committee (901). Proof of approval is available upon request. Primary airway epithelial cells were obtained from trans-laryngeal, non-bronchoscopic tracheal mucosa brushings through an endotracheal tube (Kicic et al., 2006; Lane et al., 2005). With informed parent/guardian permission, cells were obtained from eight healthy, non-atopic volunteers (2-4yrs, four males) undergoing elective surgery for non-respiratory related conditions. Positive results for atopy, assessed using a radio-allergo-sorbent test for a panel of common childhood allergens, or clinician diagnosis of chest infection or any underlying chronic respiratory disease such as asthma resulted in cells samples being excluded from this study.

2.3 Tissue Culture: Airway epithelial cells were reconditioned and established using a well described methodology (Martinovich et al., 2017). Reconditioned cells were then passaged weekly and grown at 37°C in an atmosphere of 5%CO_2/95% air under aseptic conditions. All cells used for exposures were below passage 6. Prior to exposure, cells were seeded into 5 dishes per volunteer per exposure at 400,00 cells per cell culture dish (Eppendorf 35 x 10 mm cell culture dish) and maintained in Basal Epithelial Basal Media supplemented with growth additives (BEGM®; LONZA, Switzerland).Twenty-four hours before exposure media was changed to BEGM without epithelial growth factor. Only 4 of the dishes were exposed with the fifth used to make sure cell numbers were consistent between exposures.

2.4 Exposure Methodology: A diagram of our exposure set up can be found in the supplementary methods, Figure S2. Exhaust was generated using a single cylinder, 435cc design Yanmar L100V engine (Yanmar, Italy) fitted with Euro V/VI after-treatment equipment (diesel particulate filter and diesel oxidation catalyst, (Daimler, Germany)) and coupled with a dynamometer. The engine was run at a constant speed of 2000 rpm and load of 40%, selected based on load distributions used in previous studies (Olfert et al., 2007) and engine manufacturer guidelines. Cold start was included for all exposures and exhaust was diluted 1/15 with air. Dilution ratio was measured by comparing exhaust characteristics before and after dilution, with the final dilution settings chosen to simulate real-world

relevant levels of exhaust. Immediately after dilution, to minimise differences in exhaust particle deposition between raw and diluted exhaust, it was pumped into a sealed incubator (Model 1535, Sheldon Manufacturing, OR, USA) set at 37°C containing the cells. Cell culture dishes were randomly allocated and placed within a custom designed baffle plate holding up to 36 dishes to help ensure even exhaust distribution, the exact details and diagrams of which are described in (Landwehr et al., 2021a). Exposure lasted one hour and cells were left to rest for 24-hours at 37°C in an atmosphere of 5%CO_2/95% air prior to post exposure biological analysis. These timepoints were chosen based on previous data that found a one-hour exposure caused the most toxic effects (Landwehr et al., 2019), and that waiting 24 hours after the initial exhaust exposure generated the greatest inflammatory cytokine response (Mullins et al., 2016).

2.5 Gas and Particle Analysis: After exposure, exhaust exiting the incubator was analysed at a sampling rate of 1 L/min every 10 minutes for combustion gas concentration (oxygen (O_2), carbon monoxide (CO), carbon dioxide (CO_2), nitrogen oxides (nitrogen monoxide (NO) and nitrogen dioxide (NO_2)) and sulfur dioxide (SO_2) (TESTO 350, Testo, Lenzkirch, Germany)) and particle concentration between the sizes of 3 nm-340 nm (Universal Scanning Mobility Particle Sizer (U-SMPS 1700 Palas, Karlsruhe, Germany) capable of measuring up to 10^8 particles/cm^3). The lower limit of detection of the gas analyser was 0.1 %/ppm depending on the gas being analysed (1 ppm for SO_2). Due to the high air dilution, the upper limit of detection was never reached. For the particle spectra averaged over the one-hour exposure, particles less than 10 nm in size were excluded from further calculations due to high variability of measurements between duplicate exposures at that size. Count-median particle size was calculated using the number of particles mean and particle mass was calculated assuming sphericity and using the 40% load diesel exhaust particle density as described (Olfert et al., 2007). Particle number was either analysed as the total number of particles or separated into two fractions: liquid particles below 23 nm in size and solid particles above 23 nm. The separation of particle sizes into above and below 23 nm diameter was chosen based on the approximate size of the divide between solid and liquid particles within diesel exhaust, around the nucleation mode size (Amanatidis et al., 2014). The unit, $(dN/dlogD_p)/cm^3$, refers to the normalised concentration for the number of particles (dN) within the log of the measurement channel width ($dlogD_p$) per cubic centimetre (cm^3).

2.6 Cellular Viability: Viability was measured using the ThermoFisher Live/Dead staining kit (ThermoFisher). Briefly, cells were suspended in 1x Annexin staining buffer and stained with a 1/40 dilution of Annexin V, Alexa Fluor™ 488 conjugate solution and 1 µg/mL propidium iodide before undergoing flow cytometry analysis. Annexin V -ve/PI -ve populations were counted as viable cells, Annexin V +ve/PI -ve as early apoptotic, Annexin V as late apoptotic and Annexin V -ve/PI +ve as necrotic (Filograna et al., 2015).

2.7 Mediator Release: Mediator release was analysed as per kit protocol 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). Of the 27 mediators tested, 14 were found to be released within measurable concentrations; IL-1 β, IL-1RA, IL-6, IL-8, IL-9, G-CSF, GM-CSF, IFN-γ, IP-10, MCP-1, MIP-1β, RANTES, TNF-α and VEGF. Results were first normalised to protein content and then background air exposure readings were subtracted for each subject.

2.8 Statistical Analysis: Data are presented as mean ± standard deviation and majority of biological data contains results for all volunteers (n=8), with the exception of cottonseed B20 (n=7) and palm B20 (n=6). All statistical analyses were completed using R statistical software (V3.4.3)(R Team, 2021) loaded with the packages "mgcv" and "lme4". P-values less than 0.05 were considered significant. Gas measurements were analysed by fitting a General Additive Model (GAM) file with concentration as the response variable and time as the predictor, allowing for non-parametric fits. All other statistical analyses were completed using multivariate general linear modelling methodologies with the families "gaussian(identity/log)" and "Gamma(inverse/log)" as best fit the data, applying a backwards elimination approach to remove insignificant predictive variables (Landwehr et al., 2021a; Landwehr et al., 2021b). See supplementary Tables S1 and S2 for univariate linear regression analysis of exhaust properties and biological outcomes.

3. Results:

3.1 Exhaust gas analysis: Mean exhaust gas concentration for each fuel over the 60-minute exposure period are shown (Table 1), with the exception of CO, for which the peak measurement (at the 10-minute mark) is shown. This is due to engine cold-start effects whereby CO concentrations peak rapidly, before zeroing by the 20-30-minute mark.

All blends showed similar trends in combustion gas production (Table 1, Supplementary Figure S1) throughout the 60-minute exposures. Most combustion gases increased rapidly within the first half of the exposure before levelling out around the 30-minute mark. The exceptions were O_2, which instead rapidly decreased until levelling out at ~30 minutes, and CO which peaked ~10 minutes after engine start, before rapidly returning to zero. This is likely caused by the cold start effect. Cottonseed B20 was the most different to ULSD with significantly increased mean O_2 and significantly decreased CO_2 and NO_x in the form of decreased NO ($p<0.01$ in all cases). In contrast, WCO B20 and soy B20 were found to be the least different to ULSD with only NO_2 and CO_2 respectively being significantly different to ULSD exhaust ($p<0.05$ in all cases).

3.2 Particle Analysis: Average particle spectra were obtained for each exhaust between the sizes of 5 nm and 340 nm (Figure 1) and key exhaust particle characteristics were analysed (Table 2). Canola B20, tallow B20 and palm B20 were found to be significantly different to ULSD in terms of total particle number concentration, with canola B20 and palm B20 increasing and tallow B20 decreasing ($p<0.05$ in all cases). All fuels showed peaks in particle concentrations between the sizes of 80-100 nm. This peak was largest in canola B20 and WCO B20 with significantly increased particle number concentrations over 1.4 times that of ULSD ($p<0.05$ in all cases).

No one exhaust particle characteristic was consistently different between ULSD and all B20 exhausts, suggesting that differences observed may be feedstock specific. Only cottonseed B20 and palm B20 showed a peak in the blended fuels between the sizes of 20-35 nm. Hence palm B20 and cottonseed B20 exhaust contained significantly more particles compared with ULSD at this size and WCO B20 contained significantly fewer ($p<0.05$).

3.3 Cellular Viability: Only exposure to palm B20 exhaust resulted in a significant reduction in cellular viability compared with air exposed controls (96.3 ± 1.7%; $p<0.01$) (Figure 2). Exposure to exhaust from the remaining five B20 fuels did not significantly alter viability

compared to air. ULSD exhaust exposure resulted in a significant decrease in viability when compared with both tallow B20 and cottonseed B20 ($p<0.01$).

Cell death mechanisms were assessed post exposure, and effects of B20 exhaust exposure were compared with both ULSD and air exposed controls (Figure 3). Compared to air exposed controls a significant increase in early apoptotic cell death was observed in cells exposed to ULSD, WCO B20, tallow B20, palm B20 and cottonseed B20 ($p<0.05$). ULSD exhaust exposure also significantly increased early apoptotic cell death when compared with soy B20 and canola B20. Late apoptotic cell death was significantly decreased in tallow B20 exposed cells when compared to both air and ULSD exposed cells ($p<0.05$). Necrotic cell death was decreased when compared to air in both WCO B20 and cottonseed B20 exposures. No consistent pattern was observed in cell death mechanisms between all B20 exhausts and ULSD, suggesting health impacts are likely feedstock specific.

3.4 Mediator Release: Of the panel of 27 mediators tested, 14 were measured at concentrations above the limit of detection (Table 3, Supplementary Table S3). Most of these 14 mediators primarily impacted the innate immune response (IL-1β, IL-6, IL-8, G-CSF, GM-CSF, MCP-1, MIP-1β and TNF-α), with IL-9, IFN-γ, IP-10 and RANTES primarily impacting the adaptive inflammatory immune response. Only TNF-α was significantly increased in all exposures compared to air ($p<0.05$ for all treatments), however G-CSF, GM-CSF and MCP-1 were all significantly increased for at least four of the exposures. In comparison to air, soy B20 exhaust induced the largest immune impact with significant differences in the concentrations of 9 mediators post exposure, followed by palm B20 exhaust and tallow B20 exhaust (Table 3). WCO B20 and canola B20 exhaust induced the fewest significant differences compared with air. This suggests that the inflammatory effect on the cells may be feedstock specific and changes between different biodiesel types.

4. Discussion:

The results of this study show that exposure to 20% blended biodiesel exhaust elicits a range of toxic effects on airway epithelial cells and that these changes vary when compared to both ULSD and between different types of B20. The exhaust properties of canola and cottonseed B20 were found to be the most different to ULSD, whereas the exhaust properties of cottonseed B20 were also found to be the most different to its respective unblended biodiesel fuel. Importantly, the three most toxic (tallow, soy and palm) and three least toxic (cottonseed, canola and WCO) biodiesel exhaust types are consistent between B100 and B20 fuels (Landwehr et al., 2021b). This suggests that feedstock type alters resulting exhaust toxicity even when biodiesel content only makes up 20% of the total volume fuel. No one exhaust characteristic can be found that is different in only the three most toxic or only the three least toxic (Table 1&2, Figure 1), suggesting that any observed toxic effects are due to a range of exhaust components instead of one component alone, possibly including some not measured in this study (Fontaras et al., 2009). More data are needed before health effects can be accurately attributed to individual exhaust components, or potentially toxic interactions between multiple exhaust components.

We found soy B20, palm B20 andtallow B20, to be the most toxic fuel types and canola B20 and WCO B20 to be the least (Figure 2&3, Table 3). Previous fatty acid methyl ester (FAME) profile analysis of B100 equivalent fuels (Landwehr et al., 2021b) showed that WCO was mostly canola oil, thus it is unsurprising that the toxic effects of these two B20

fuels are similar. As the conversion process of fatty acids from the feedstock fat/oil into biodiesel FAMEs conserves a lot of the fatty acid structure (Knothe et al., 2015; Graboski et al., 2003), it would be unsurprising if the composition of the oil (such as the number of double bonds and length of the fatty acid chain) greatly altered fuel chemistry and resulting exhaust properties. We previously reported that soy biodiesel had the highest proportion of double-bonded unsaturated FAME molecules and thus the highest predicted iodine number whereas palm and tallow biodiesels contained the highest proportion of saturated FAME molecules and thus the highest predicted cetane numbers. Palm B20 induced decreased viability but it was soy B20 that induced the widest range of mediator responses, followed by palm B20 and then tallow B20. As both iodine and cetane numbers increase, exhaust composition is affected, especially PM and NO_x concentrations (Cardone et al., 2002; Fontaras et al., 2009; McCormick et al., 2001). An increased cetane number is associated with more complete combustion (Bamgboye and Hansen, 2008; Knothe and Steidley, 2005) whereas higher iodine number is associated with a more reactive and unstable fuel (McCormick et al., 2001; Miller and Bowman, 1989). Higher iodine numbers generally trend towards higher NO_x concentrations which could potentially increase exhaust toxicity, however the impact of higher cetane numbers changes from study to study (Fontaras et al., 2009; McCormick et al., 2001). As the ULSD we used had a cetane number of ~49 (SHELL and Australia, 2018), the higher cetane numbers of tallow and palm biodiesel and the more unstable properties of soy biodiesel indicate a drift from the physical properties of conventional ULSD, which diesel engines are designed use. This will likely alter engine performance and exhaust characteristics (Fontaras et al., 2009; Karavalakis et al., 2011) and thus impact the toxicological effects of exhaust exposure. Additionally, altering speed and load settings for the engine would further impact exhaust components and thus exhaust toxicity (Bünger et al., 2007; Fontaras et al., 2009), although the impact is likely to be consistent across the different fuels and thus will not overly impact relative toxicity.

A key finding of this study was that early apoptotic cell death was significantly increased 24 hours after exposure to 4 out of 6 B20 exhausts (and ULSD) (Figure 3). Previous studies have mostly found increases in necrotic and/or late apoptotic cell death (Jalava et al., 2012; Lankoff et al., 2017; Wang et al., 2017). This suggests that toxic effects in our study may be ongoing 24 hours after a single exposure as the change from early to late apoptotic cell death is quick to occur (Elmore, 2007). This is supported by previous literature showing that mice display effects of exhaust exposure up to 7 days after PM exposure (Yanamala et al., 2013). While we acknowledge that these increases are relatively small, it is important to note that we used diluted exhaust and a very short exposure time period in order to mimic a realistic acute exposure. This means that even small changes could be important for populations exposed regularly to dilute exhaust, or once-off to more concentrated exhaust, such as those who live near busy roads or work with diesel-powered equipment (Rynning et al., 2019; Zhang et al., 2009).

Exposure to B20 exhaust also elicited alterations in mediator concentrations (Table 3), most of which were related to innate and adaptive immune responses (Dayer et al., 2017; Duffy et al., 2013; Holdsworth and Gan, 2015; Sokol and Luster, 2015). Only TNF-α (which is primarily involved in the innate acute inflammatory response (Holdsworth and Gan, 2015)), was significantly released after every exposure, while two others (G-CSF and MCP-1), were significantly increased after the majority of B20 exposures. These mediators stimulate innate

neutrophilic and macrophage inflammatory responses (Cox et al., 1992; Holdsworth and Gan, 2015; Lloyd, 2002; Mazzon and Cuzzocrea, 2007), with previous diesel exhaust exposure studies in animals and humans showing macrophages and/or neutrophils increase after exposure (Behndig et al., 2011; Karthikeyan et al., 2013; Tong et al., 2014; Yanamala et al., 2013). Similarly, GM-CSF, which is also associated with the innate macrophage response (Rösler and Herold, 2016), was released after exposure to ULSD exhaust and three B20 exhaust types (soy B20, tallow B20 and cottonseed B20).

The three most toxic exhaust exposures in terms of mediator release (palm B20, soy B20 and tallow B20) also resulted in increased release of IL-8 and IL-9. These mediators stimulate the innate neutrophil and adaptive allergic airway responses respectively (Abe et al., 2000; Little et al., 2001; Sokol and Luster, 2015; Zhou et al., 2001). IL-8 has previously been shown to be important in diesel exhaust exposure studies (Dai et al., 2018; Swanson et al., 2009). Conversely, to the best of our knowledge, IL-9 has not been measured in this context outside our group. In addition, IL-6 was released after exposure to both soy B20 and tallow B20 exhaust and is associated with the innate acute inflammatory response (Holdsworth and Gan, 2015), palm B20 and ULSD exhaust induced MIP-1β release, which is associated with the innate neutrophilic and natural killer cell response (Garofalo and Haeberle, 2000; Sokol and Luster, 2015) and soy B20 and palm B20 induced RANTES release, which is associated with the adaptive recruitment and activation of T-cells (Garofalo and Haeberle, 2000; Olszewska-Pazdrak et al., 1998; Sokol and Luster, 2015). All three of these mediators have previously been found to be dysregulated in exhaust exposure studies (Behndig et al., 2011; Dai et al., 2018; Matsumoto et al., 2006; Swanson et al., 2009). ULSD, canola B20 and WCO B20 did not induce the release of mediators that alter adaptive immunity, impacting the release of innate mediators only.

It is difficult to compare the results of our data to those of previous studies for a number of reasons. Firstly, we used whole exhaust in our exposure so the toxic effects of both the gaseous components and a more realistic particle spectrum are included in our study when they would not be with many previous studies that only used particles collected on filters as their exposure method (André et al., 2015; Morin et al., 2008; Larcombe et al., 2015; Surawski et al., 2011). Secondly, we used the exhaust from an engine equipped with exhaust after-treatment devices (including a diesel particulate filter and oxidative catalyst), which are known to greatly impact exhaust output (Khalek et al., 2011; Magnusson et al., 2017). Most previous studies in this field use older engines without these devices, and hence those exhausts contain significantly more particles, higher particle mass and higher CO (Larcombe et al., 2015; Valand et al., 2018). Finally, the findings for blended biodiesel fuel toxicological studies are inconsistent (Larcombe et al., 2015; Møller et al., 2020). This is in part due to the fact that, while B20 is arguably one of the most common blend types in scientific studies, blends of B30 and B50 are also commonly studied (Betha et al., 2012; Gerlofs-Nijland et al., 2013; Libalova et al., 2016). This, combined with the exhaust profile and toxic exposure consequences of steadily increasing biodiesel amounts in blended fuels not being a linear trend between ULSD and pure B100, makes it difficult to draw overarching conclusions. For example, previous studies have found blends to be more toxic in terms of oxidative potential and DNA damage compared with both diesel and B100, and to contain more PM and NO_x (Ackland et al., 2007; Adenuga et al., 2016; Graver et al., 2016).

That said, our results indicate that the toxic results of exposure to B20 exhaust were slightly more inflammatory than exposure to B100 in three of our six biodiesel fuels (Landwehr et al., 2021b). This is not the first study to find blended biodiesel fuels can be more toxic than B100, with previous studies finding blends of all ranges between B20-B80 to have more oxidative potential and more DNA damage causing capability than B100 (Ackland et al., 2007; Adenuga et al., 2016; Krahl et al., 2008). Unfortunately, these studies do not always state the type of feedstock used to create the biodiesel, with only blended rapeseed (canola) biodiesel known to be more toxic than its B100 counterpart (Krahl et al., 2008). We also found that in the remaining three fuels, toxic consequences of exhaust exposure were similar to that of matched B100 fuels. Again, this has previously been reported with equal levels of DNA damage and gene dysregulation in cell exposure studies and comparable inflammatory responses in mice (Brito et al., 2010; Cervena et al., 2017; Libalova et al., 2016).

The results of our study raise the question of whether the toxicological results of B20 exhaust exposures are so inconsistent in the literature because different feedstocks have been used by different studies. Soy and canola are the most common biodiesel types used in blended fuel studies (Larcombe et al., 2015; Møller et al., 2020) and we have found soy B20 to be amongst the most toxic, more than that of ULSD, whereas canola B20 was amongst the least toxic, less than that of ULSD. There are also several studies that use less common feedstock types such as animal fat or corn (Hemmingsen et al., 2011; Yanamala et al., 2013) or don't report the type of feedstock used to create the biodiesel (Ackland et al., 2007; Magnusson et al., 2019), which makes comparisons difficult. This could explain the inconsistencies in toxicological findings, with attempts being made to define the differences between diesel, B20 and B100 while also correlating exposure endpoints of biodiesels made from varyingly toxic feedstock types. Unfortunately, methodological differences make comparisons between different feedstocks unadvisable unless those comparisons are performed within the same study and so previous attempts to review literature and attribute particular toxic effects, such as inflammation or DNA damage, to a particular biodiesel feedstock have been inconclusive (Møller et al., 2020).

5. Conclusion

The feedstock used to create biodiesel has a significant effect on the resulting exhaust toxicity, even when only blended 20% within the fuel. The future of biodiesel research needs to become more standardised so that comparisons between different studies can be accurately made and the widest range of biodiesel types compared. At the very least, engine type, drive cycle type or constant speed and load settings, after-treatment devices, sulfur levels in diesel fuel and the exact type of feedstock, preferably down to the FAME profile, need to be reported consistently before any sort of comparison between studies can be accurately performed. As biodiesel can be made from almost any fat or oil, this will be an undertaking and our study is just a small part of what will be required to find the least toxic feedstock for biodiesel creation.

Declarations:

Availability of data and materials: Supplementary information is available at_____. All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Authors' contributions:

Katherine Landwehr; Conceptualization, Methodology, Verification, Formal Analysis, Investigation, Writing - Original Draft, Writing - Review & Editing. **Jessica Hillas**; Methodology, Resources. **Ryan Mead-Hunter**; Methodology, Resources, Writing - Review & Editing. **Andrew King**; Resources. **Rebecca O'Leary**; Methodology, Formal Analysis, Writing - Review & Editing. **Anthony Kicic**; Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision. **Benjamin Mullins**; Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision. **Alexander Larcombe**; Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition

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6. References

Abe, S., Takizawa, H., Sugawara, I., Kudoh, S., 2000. Diesel Exhaust (DE)–Induced Cytokine Expression in Human Bronchial Epithelial Cells. American Journal of Respiratory Cell and Molecular Biology 22, 296-303.

ACCC, Australian Competition & Consumer Commission, 2021. Biofuels.

Ackland, M.L., Zou, L., Freestone, D., Van De Waasenburg, S., Michalczyk, A.A., 2007. Diesel exhaust particulate matter induces multinucleate cells and zinc transporter-dependent apoptosis in human airway cells. Immunology and cell biology 85, 617-622.

Adenuga, A.A., Wright, M.E., Atkinson, D.B., 2016. Evaluation of the reactivity of exhaust from various biodiesel blends as a measure of possible oxidative effects: A concern for human exposure. Chemosphere 147, 396-403.

Amanatidis, S., Ntziachristos, L., Giechaskiel, B., Bergmann, A., Samaras, Z., 2014. Impact of Selective Catalytic Reduction on Exhaust Particle Formation over Excess Ammonia Events. Environmental Science & Technology 48, 11527-11534.

André, V., Barraud, C., Capron, D., Preterre, D., Keravec, V., Vendeville, C., Cazier, F., Pottier, D., Morin, J.P., Sichel, F., 2015. Comparative mutagenicity and genotoxicity of particles and aerosols emitted by the combustion of standard vs. rapeseed methyl ester supplemented bio-diesel fuels: Impact of after treatment devices: Oxidation catalyst and particulate filter. Mutation Research-Genetic Toxicology and Environmental Mutagenesis 777, 33-42.

ASTM, ASTM International, 2020a. ASTM D975-20c, Standard Specification for Diesel Fuel,, West Conshohocken, PA.

ASTM, ASTM International, 2020b. ASTM D7467-20a, Standard Specification for Diesel Fuel Oil, Biodiesel Blend (B6 to B20), West Conshohocken, PA.

Bamgboye, A., Hansen, A.C., 2008. Prediction of cetane number of biodiesel fuel from the fatty acid methyl ester (FAME) composition. International Agrophysics 22, 21.
Barros, S., 2020. Biofuels Annual- Brazil.
Behndig, A.F., Larsson, N., Brown, J.L., Stenfors, N., Helleday, R., Duggan, S.T., Dove, R.E., Wilson, S.J., Sandstrom, T., Kelly, F.J., Mudway, I.S., Blomberg, A., 2011. Proinflammatory doses of diesel exhaust in healthy subjects fail to elicit equivalent or augmented airway inflammation in subjects with asthma. Thorax 66, 12-19.
Betha, R., Pavagadhi, S., Sethu, S., Hande, M.P., Balasubramanian, R., 2012. Comparative in vitro cytotoxicity assessment of airborne particulate matter emitted from stationary engine fuelled with diesel and waste cooking oil-derived biodiesel. Atmospheric Environment 61, 23-29.
Brito, J.M., Belotti, L., Toledo, A.C., Antonangelo, L., Silva, F.S., Alvim, D.S., Andre, P.A., Saldiva, P.H.N., Rivero, D.H.R.F., 2010. Acute Cardiovascular and Inflammatory Toxicity Induced by Inhalation of Diesel and Biodiesel Exhaust Particles. Toxicological Sciences 116, 67-78.
Bünger, J., Müller, M.M., Krahl, J., Baum, K., Weigel, A., Hallier, E., Schulz, T.G., 2000. Mutagenicity of diesel exhaust particles from two fossil and two plant oil fuels. Mutagenesis 15, 391-397.
Bünger, J., Krahl, J., Munack, A., Ruschel, Y., Schröder, O., Emmert, B., Westphal, G., Müller, M., Hallier, E., Brüning, T., 2007. Strong mutagenic effects of diesel engine emissions using vegetable oil as fuel. Archives of Toxicology 81, 599-603.
Cardone, M., Prati, M.V., Rocco, V., Seggiani, M., Senatore, A., Vitolo, S., 2002. *Brassica carinata* as an Alternative Oil Crop for the Production of Biodiesel in Italy: Engine Performance and Regulated and Unregulated Exhaust Emissions. Environmental Science & Technology 36, 4656-4662.
Cervena, T., Rossnerova, A., Sikorova, J., Beranek, V., Vojtisek-Lom, M., Ciganek, M., Topinka, J., Rossner, P., 2017. DNA Damage Potential of Engine Emissions Measured In Vitro by Micronucleus Test in Human Bronchial Epithelial Cells. Basic & Clinical Pharmacology & Toxicology 121, 102-108.
Cox, G., Gauldie, J., Jordana, M., 1992. Bronchial epithelial cell-derived cytokines (G-CSF and GM-CSF) promote the survival of peripheral blood neutrophils in vitro. Am J Respir Cell Mol Biol 7, 507-513.
Dai, Y., Ren, D., Bassig, B.A., Vermeulen, R., Hu, W., Niu, Y., Duan, H., Ye, M., Meng, T., Xu, J., Bin, P., Shen, M., Yang, J., Fu, W., Meliefste, K., Silverman, D., Rothman, N., Lan, Q., Zheng, Y., 2018. Occupational exposure to diesel engine exhaust and serum cytokine levels. Environmental and molecular mutagenesis 59, 144-150.
Dayer, J.-M., Oliviero, F., Punzi, L., 2017. A Brief History of IL-1 and IL-1 Ra in Rheumatology. Frontiers in Pharmacology 8.
Duffy, A.M., Bouchier-Hayes, D.J., Harmey, J.H., 2013. Vascular endothelial growth factor (VEGF) and its role in non-endothelial cells: autocrine signalling by VEGF, Madame Curie Bioscience Database [Internet]. Landes Bioscience.
Eea, E., 2013. Bioenergy potential from a resource-efficiency perspective. European Environment Agency. EEA Report, 2013.
EERE, Energy Efficiency & Renewable Energy, 2020. Alternate Fuels Data Centre, in: US Department of Energy Efficiency & Renewable Energy (Ed.).
EIA, US Energy and Information Administration, 2020. International energy statistics, in: EIA (Ed.). United States Energy and Information Administration.
Elmore, S., 2007. Apoptosis: a review of programmed cell death. Toxicologic pathology 35, 495-516.
EU, European Union, 2009. Directive 2009/28/EC of the European Parliament and of the Council of 23 April 2009 on the promotion of the use of energy from renewable sources and amending and subsequently repealing Directives 2001/77/EC and 2003/30/EC. Official Journal of the European Union 5, 2009.
EU, European Union, 2016. Directive (EU) 2016/802 of the European Parliament and of the Council codification of 11 May 2016.

Filograna, R., Civiero, L., Ferrari, V., Codolo, G., Greggio, E., Bubacco, L., Beltramini, M., Bisaglia, M., 2015. Analysis of the Catecholaminergic Phenotype in Human SH-SY5Y and BE(2)-M17 Neuroblastoma Cell Lines upon Differentiation. PLoS ONE 10, e0136769.
Fontaras, G., Karavalakis, G., Kousoulidou, M., Tzamkiozis, T., Ntziachristos, L., Bakeas, E., Stournas, S., Samaras, Z., 2009. Effects of biodiesel on passenger car fuel consumption, regulated and non-regulated pollutant emissions over legislated and real-world driving cycles. Fuel 88, 1608-1617.
Garofalo, R.P., Haeberle, H., 2000. Epithelial Regulation of Innate Immunity to Respiratory Syncytial Virus. American Journal of Respiratory Cell and Molecular Biology 23, 581-585.
Gerlofs-Nijland, M.E., Totlandsdal, A.I., Tzamkiozis, T., Leseman, D.L.A.C., Samaras, Z., Låg, M., Schwarze, P., Ntziachristos, L., Cassee, F.R., 2013. Cell Toxicity and Oxidative Potential of Engine Exhaust Particles: Impact of Using Particulate Filter or Biodiesel Fuel Blend. Environmental Science & Technology 47, 5931-5938.
Graboski, M.S., McCormick, R.L., Alleman, T.L., Herring, A.M., 2003. The Effect of Biodiesel Composition on Engine Emissions from a DDC Series 60 Diesel Engine, Colorado Institute for Fuels and Engine Research, Colorado School of Mines, Golden, CO.
Graver, B.M., Frey, H.C., Hu, J., 2016. Effect of Biodiesel Fuels on Real-World Emissions of Passenger Locomotives. Environmental Science & Technology 50, 12030-12039.
Hemmingsen, J.G., Møller, P., Nøjgaard, J.K., Roursgaard, M., Loft, S., 2011. Oxidative Stress, Genotoxicity, And Vascular Cell Adhesion Molecule Expression in Cells Exposed to Particulate Matter from Combustion of Conventional Diesel and Methyl Ester Biodiesel Blends. Environmental Science & Technology 45, 8545-8551.
Holdsworth, S.R., Gan, P.-Y., 2015. Cytokines: Names and Numbers You Should Care About. Clinical journal of the American Society of Nephrology : CJASN 10, 2243-2254.
Jalava, P.I., Aakko-Saksa, P., Murtonen, T., Happo, M.S., Markkanen, A., Yli-Pirilä, P., Hakulinen, P., Hillamo, R., Mäki-Paakkanen, J., Salonen, R.O., Jokiniemi, J., Hirvonen, M.-R., 2012. Toxicological properties of emission particles from heavy duty engines powered by conventional and bio-based diesel fuels and compressed natural gas. Particle and Fibre Toxicology 9, 37.
Karavalakis, G., Bakeas, E., Fontaras, G., Stournas, S., 2011. Effect of biodiesel origin on regulated and particle-bound PAH (polycyclic aromatic hydrocarbon) emissions from a Euro 4 passenger car. Energy 36, 5328-5337.
Karthikeyan, S., Thomson, E.M., Kumarathasan, P., Guénette, J., Rosenblatt, D., Chan, T., Rideout, G., Vincent, R., 2013. Nitrogen Dioxide and Ultrafine Particles Dominate the Biological Effects of Inhaled Diesel Exhaust Treated by a Catalyzed Diesel Particulate Filter. Toxicological Sciences 135, 437-450.
Khalek, I.A., Bougher, T.L., Merritt, P.M., Zielinska, B., 2011. Regulated and Unregulated Emissions from Highway Heavy-Duty Diesel Engines Complying with U.S. Environmental Protection Agency 2007 Emissions Standards. Journal Of The Air & Waste Management Association 61, 427-442.
Kicic, A., Sutanto, E.N., Stevens, P.T., Knight, D.A., Stick, S.M., 2006. Intrinsic Biochemical and Functional Differences in Bronchial Epithelial Cells of Children with Asthma. American Journal of Respiratory and Critical Care Medicine 174, 1110-1118.
Knothe, G., de Castro, M.E.G., Razon, L.F., 2015. Methyl Esters (Biodiesel) from and Fatty Acid Profile of Gliricidia sepium Seed Oil. Journal of the American Oil Chemists' Society 92, 769-775.
Knothe, G., Steidley, K.R., 2005. Kinematic viscosity of biodiesel fuel components and related compounds. Influence of compound structure and comparison to petrodiesel fuel components. Fuel 84, 1059-1065.
Krahl, J., Munack, A., Ruschel, Y., Schröder, O., Bünger, J., 2008. Exhaust Gas Emissions and Mutagenic Effects of Diesel Fuel, Biodiesel and Biodiesel Blends. SAE International.
Landwehr, K.R., Hillas, J., Mead-Hunter, R., Brooks, P., King, A., O'Leary, R.A., Kicic, A., Mullins, B.J., Larcombe, A.N., 2021a. In Vitro primary human airway epithelial whole exhaust exposure. MethodsX 8, 101561.

623 Landwehr, K.R., Hillas, J., Mead-Hunter, R., Brooks, P., King, A., O'Leary, R.A., Kicic, A., Mullins, B.J.,
624 Larcombe, A.N., 2021b. Fuel feedstock determines biodiesel exhaust toxicity in a human airway
625 epithelial cell exposure model. Journal of Hazardous Materials 420, 126637.
626 Landwehr, K.R., Hillas, J., Mead-Hunter, R., O'Leary, R.A., Kicic, A., Mullins, B.J., Larcombe, A.N.,
627 2019. Soy Biodiesel Exhaust is More Toxic than Mineral Diesel Exhaust in Primary Human Airway
628 Epithelial Cells. Environmental Science & Technology 53, 11437-11446.
629 Lane, C., Burgess, S., Kicic, A., Knight, D., Stick, S., 2005. The use of non-bronchoscopic brushings to
630 study the paediatric airway. Respiratory Research 6, 53-53.
631 Lankoff, A., Brzoska, K., Czarnocka, J., Kowalska, M., Lisowska, H., Mruk, R., Øvrevik, J., Wegierek-
632 Ciuk, A., Zuberek, M., Kruszewski, M., 2017. A comparative analysis of in vitro toxicity of diesel
633 exhaust particles from combustion of 1st- and 2nd-generation biodiesel fuels in relation to their
634 physicochemical properties—the FuelHealth project. Environmental Science and Pollution Research
635 24, 19357-19374.
636 Larcombe, A.N., Kicic, A., Mullins, B.J., Knothe, G., 2015. Biodiesel exhaust: The need for a systematic
637 approach to health effects research. Respirology 20, 1034-1045.
638 Li, F., Liu, Z., Ni, Z., Wang, H., 2019. Effect of biodiesel components on its lubrication performance.
639 Journal of Materials Research and Technology 8, 3681-3687.
640 Libalova, H., Rossner, P., Vrbova, K., Brzicova, T., Sikorova, J., Vojtisek-Lom, M., Beranek, V., Klema,
641 J., Ciganek, M., Neca, J., Pencikova, K., Machala, M., Topinka, J., 2016. Comparative Analysis of Toxic
642 Responses of Organic Extracts from Diesel and Selected Alternative Fuels Engine Emissions in Human
643 Lung BEAS-2B Cells. International Journal of Molecular Sciences 17, 1833.
644 Little, F.F., Cruikshank, W.W., Center, D.M., 2001. Il-9 stimulates release of chemotactic factors from
645 human bronchial epithelial cells. Am J Respir Cell Mol Biol 25, 347-352.
646 Lloyd, C., 2002. Chemokines in allergic lung inflammation. Immunology 105, 144-154.
647 Magnusson, P., Dziendzikowska, K., Oczkowski, M., Øvrevik, J., Eide, D.M., Brunborg, G., Gutzkow,
648 K.B., Instanes, C., Gajewska, M., Wilczak, J., Sapierzynski, R., Kamola, D., Królikowski, T., Kruszewski,
649 M., Lankoff, A., Mruk, R., Duale, N., Gromadzka-Ostrowska, J., Myhre, O., 2019. Lung effects of 7-
650 and 28-day inhalation exposure of rats to emissions from 1st and 2nd generation biodiesel fuels with
651 and without particle filter – The FuelHealth project. Environmental Toxicology and Pharmacology 67,
652 8-20.
653 Magnusson, P., Oczkowski, M., Øvrevik, J., Gajewska, M., Wilczak, J., Biedrzycki, J., Dziendzikowska,
654 K., Kamola, D., Królikowski, T., Kruszewski, M., Lankoff, A., Mruk, R., Brunborg, G., Instanes, C.,
655 Gromadzka-Ostrowska, J., Myhre, O., 2017. No adverse lung effects of 7- and 28-day inhalation
656 exposure of rats to emissions from petrodiesel fuel containing 20% rapeseed methyl esters (B20)
657 with and without particulate filter – the FuelHealth project. Inhalation Toxicology 29, 206-218.
658 Martinovich, K.M., Iosifidis, T., Buckley, A.G., Looi, K., Ling, K.-M., Sutanto, E.N., Kicic-Starcevich, E.,
659 Garratt, L.W., Shaw, N.C., Montgomery, S., Lannigan, F.J., Knight, D.A., Kicic, A., Stick, S.M., 2017.
660 Conditionally reprogrammed primary airway epithelial cells maintain morphology, lineage and
661 disease specific functional characteristics. Scientific Reports 7, 17971.
662 Matsumoto, A., Hiramatsu, K., Li, Y., Azuma, A., Kudoh, S., Takizawa, H., Sugawara, I., 2006.
663 Repeated exposure to low-dose diesel exhaust after allergen challenge exaggerates asthmatic
664 responses in mice. Clinical Immunology 121, 227-235.
665 Mazzon, E., Cuzzocrea, S., 2007. Role of TNF-α in lung tight junction alteration in mouse model of
666 acute lung inflammation. Respiratory Research 8, 75.
667 McCormick, R.L., Graboski, M.S., Alleman, T.L., Herring, A.M., Tyson, K.S., 2001. Impact of Biodiesel
668 Source Material and Chemical Structure on Emissions of Criteria Pollutants from a Heavy-Duty
669 Engine. Environmental Science & Technology 35, 1742-1747.
670 Miller, J.A., Bowman, C.T., 1989. Mechanism and modeling of nitrogen chemistry in combustion.
671 Progress in Energy and Combustion Science 15, 287-338.

672 Møller, P., Scholten, R.H., Roursgaard, M., Krais, A.M., 2020. Inflammation, oxidative stress and
673 genotoxicity responses to biodiesel emissions in cultured mammalian cells and animals. Critical
674 Reviews in Toxicology, 1-19.
675 Morin, J.-P., Hasson, V., Fall, M., Papaioanou, E., Preterre, D., Gouriou, F., Keravec, V.,
676 Konstandopoulos, A., Dionnet, F., 2008. Prevalidation of in vitro continuous flow exposure systems
677 as alternatives to in vivo inhalation safety evaluation experimentations: Outcome from MAAPHRI-
678 PCRD5 research program. Experimental and Toxicologic Pathology 60, 195-205.
679 Mullins, B.J., Kicic, A., Ling, K.-M., Mead-Hunter, R., Larcombe, A.N., 2016. Biodiesel Exhaust–Induced
680 Cytotoxicity and Proinflammatory Mediator Production in Human Airway Epithelial Cells.
681 Environmental Toxicology 31, 44-57.
682 OECD/FAO, 2020. OECD-FAO Agricultural Outlook 2020-2029, Organisation for Economic Co-
683 operation and Development/ Food and Agriculture Organization of the United Nations.
684 Olfert, J.S., Symonds, J.P.R., Collings, N., 2007. The effective density and fractal dimension of
685 particles emitted from a light-duty diesel vehicle with a diesel oxidation catalyst. Journal of Aerosol
686 Science 38, 69-82.
687 Olszewska-Pazdrak, B., Casola, A., Saito, T., Alam, R., Crowe, S.E., Mei, F., Ogra, P.L., Garofalo, R.P.,
688 1998. Cell-specific expression of RANTES, MCP-1, and MIP-1alpha by lower airway epithelial cells and
689 eosinophils infected with respiratory syncytial virus. Journal of virology 72, 4756-4764.
690 Peng, D.-X., 2017. Biodiesel Improves Lubricity of Low-Sulfur Petro-Diesels. Chemistry and
691 Technology of Fuels and Oils 52, 699-703.
692 Price, M., 2019. Fuel Quality Standards (Automotive Diesel) Determination 2019, in: Government, A.
693 (Ed.), F2019L00456, Federal Register of Legislation.
694 R Team, 2021. R: A language and environment for statistical computing. , R: Foundation for
695 Statistical Computing, Vienna, Austria.
696 Ragauskas, A.J., Williams, C.K., Davison, B.H., Britovsek, G., Cairney, J., Eckert, C.A., Frederick, W.J.,
697 Hallett, J.P., Leak, D.J., Liotta, C.L., Mielenz, J.R., Murphy, R., Templer, R., Tschaplinski, T., 2006. The
698 Path Forward for Biofuels and Biomaterials. Science 311, 484-489.
699 Rösler, B., Herold, S., 2016. Lung epithelial GM-CSF improves host defense function and epithelial
700 repair in influenza virus pneumonia—a new therapeutic strategy? Molecular and Cellular Pediatrics
701 3, 29.
702 Rynning, I., Arlt, V.M., Vrbova, K., Neča, J., Rossner, P., Jr., Klema, J., Ulvestad, B., Petersen, E., Skare,
703 Ø., Haugen, A., Phillips, D.H., Machala, M., Topinka, J., Mollerup, S., 2019. Bulky DNA adducts,
704 microRNA profiles, and lipid biomarkers in Norwegian tunnel finishing workers occupationally
705 exposed to diesel exhaust. Occupational and Environmental Medicine 76, 10-16.
706 SHELL, Australia, 2018. Shell diesel extra, automotive diesel fuel (SDS).
707 Sokol, C.L., Luster, A.D., 2015. The chemokine system in innate immunity. Cold Spring Harbor
708 perspectives in biology 7, a016303.
709 Steiner, S., Czerwinski, J., Comte, P., Popovicheva, O., Kireeva, E., Müller, L., Heeb, N., Mayer, A.,
710 Fink, A., Rothen-Rutishauser, B., 2013. Comparison of the toxicity of diesel exhaust produced by bio-
711 and fossil diesel combustion in human lung cells in vitro. Atmospheric Environment 81, 380-388.
712 Surawski, N.C., Miljevic, B., Ayoko, G.A., Elbagir, S., Stevanovic, S., Fairfull-Smith, K.E., Bottle, S.E.,
713 Ristovski, Z.D., 2011. Physicochemical Characterization of Particulate Emissions from a Compression
714 Ignition Engine: The Influence of Biodiesel Feedstock. Environmental Science & Technology 45,
715 10337-10343.
716 Swanson, K.J., Kado, N.Y., Funk, W.E., Pleil, J.D., Madden, M.C., Ghio, A.J., 2009. Release of the pro-
717 inflammatory markers by BEAS-2B cells following in vitro exposure to biodiesel extracts. Open
718 Toxicology Journal 3, 8-15.
719 Tong, H., Rappold, A.G., Caughey, M., Hinderliter, A.L., Graff, D.W., Berntsen, J.H., Cascio, W.E.,
720 Devlin, R.B., Samet, J.M., 2014. Cardiovascular effects caused by increasing concentrations of diesel
721 exhaust in middle-aged healthy GSTM1 null human volunteers. Inhalation Toxicology 26, 319-326.

Valand, R., Magnusson, P., Dziendzikowska, K., Gajewska, M., Wilczak, J., Oczkowski, M., Kamola, D., Królikowski, T., Kruszewski, M., Lankoff, A., Mruk, R., Marcus Eide, D., Sapierzyński, R., Gromadzka-Ostrowska, J., Duale, N., Øvrevik, J., Myhre, O., 2018. Gene expression changes in rat brain regions after 7- and 28 days inhalation exposure to exhaust emissions from 1st and 2nd generation biodiesel fuels - The FuelHealth project. Inhalation Toxicology 30, 299-312.

Vogel, C.F.A., Kado, S.Y., Kobayashi, R., Liu, X., Wong, P., Na, K., Durbin, T., Okamoto, R.A., Kado, N.Y., 2019. Inflammatory marker and aryl hydrocarbon receptor-dependent responses in human macrophages exposed to emissions from biodiesel fuels. Chemosphere 220, 993-1002.

Wang, J.-S., Tseng, C.-Y., Chao, M.-W., 2017. Diesel Exhaust Particles Contribute to Endothelia Apoptosis via Autophagy Pathway. Toxicological Sciences 156, 72-83.

Yanamala, N., Hatfield, M.K., Farcas, M.T., Schwegler-Berry, D., Hummer, J.A., Shurin, M.R., Birch, M.E., Gutkin, D.W., Kisin, E., Kagan, V.E., Bugarski, A.D., Shvedova, A.A., 2013. Biodiesel versus diesel exposure: Enhanced pulmonary inflammation, oxidative stress, and differential morphological changes in the mouse lung. Toxicology and applied pharmacology 272, 373-383.

Yang, P.-M., Wang, C.-C., Lin, Y.-C., Jhang, S.-R., Lin, L.-J., Lin, Y.-C., 2017. Development of novel alternative biodiesel fuels for reducing PM emissions and PM-related genotoxicity. Environmental Research 156, 512-518.

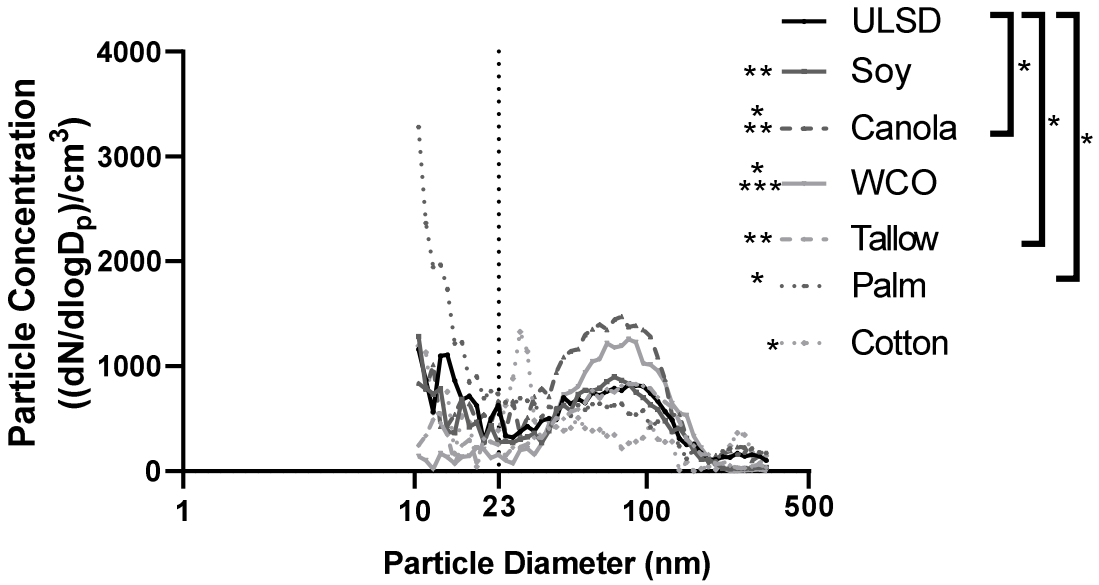
Zhang, J.J., McCreanor, J.E., Cullinan, P., Chung, K.F., Ohman-Strickland, P., Han, I.K., Jarup, L., Nieuwenhuijsen, M.J., 2009. Health effects of real-world exposure to diesel exhaust in persons with asthma. Res Rep Health Eff Inst, 5-109; discussion 111-123.

Zhou, Y., McLane, M., Levitt, R.C., 2001. Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. Respiratory Research 2, 80-84.

Table 1: Mean (standard deviation) gas measurements for all fuels. All significances displayed are compared to ULSD. Measurements are shown as the mean concentration for the entire exposure, with the exception of CO which is shown as the peak measurement.

Fuel	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cotton B20
O_2 (%)	20.4 (0.2)	20.4 (0.1)	20.5 (0.1)	20.3 (0.1) *	20.4 (0.1)	20.4 (0.2)	20.5 (0.1) **
CO (ppm)	3.5 (2.4)	2.2 (1.7)	5.1 (5.8)	1.4 (0.5)	1.2 (0.7)	2.6 (2.5)	1.3 (0.5)
CO_2 (%)	0.3 (0.1)	0.4 (0.1) *	0.3 (0.1)	0.4 (0.1)	0.4 (0.1) **	0.4 (0.1)	0.3 (0.1)
NO_x (ppm)	21.9 (6.1)	20.3 (4.5)	19.4 (2.1) **	22.4 (4.2)	22.2 (5.1)	22.1 (5.6)	17.7 (4.0) ***
NO (ppm)	15.1 (3.9)	13.7 (3.0) *	12.8 (3.1) ***	16.3 (3.6)	14.8 (3.3)	15.3 (3.7)	11.9 (2.5) ***
NO_2 (ppm)	6.7 (2.2)	6.6 (1.4)	6.5 (1.8)	7.1 (1.8) +	7.4 (1.8) *	6.8 (1.9)	6.0 (1.1)
SO_2 (ppm)	1.5 (0.3)	1.4 (0.2)	1.4 (0.4)	1.3 (0.3)	1.1 (0.4)	1.4 (0.6)	1.2 (0.3)

* Significantly different to ULSD (*=p <0.05, **=p <0.01, ***=p <0.001)



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773 **Figure 1:** Particle size spectra for all fuels averaged over the 60 minute exposure. Data were
774 analysed using total particle number concentration between the size of 10 and 340 nm for
775 each fuel (*=p value<0.05, **=p value<0.01, ***=p value<0.001). The dotted line indicates
776 the particle size spectra of 23nm. Bars linking fuel types in the figure key indicate significant
777 difference in particle number between the different fuels. Significance indicators to the left of
778 the figure key indicate significant differences between the B100 and B20 of the same fuel
779 type.

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795 **Table 2:** Mean (standard deviation) particle characteristics between the sizes of 10-340 nm
796 for all fuels.

Particle Characteristic	Fuel						
	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cottonseed B20
Particle Mass Concentration ($\mu g/m^3$)	12.9 (8.6)	7.0 (1.7) [0.5]	16.4 (2.7) [1.3]	8.6 (0.7) [0.7]	8.6 (3.2) [0.7]	13.7 (5.8) [1.1]	11.7 (9.3) [0.9]
Median Particle Size (nm)	44	51 [1.2]	58 [1.3]	72 [1.6]	63 [1.4]	21 [0.5]	31 [0.7]
Total Particle Number ($particles/cm^3$)	25993 (12596)	22335 (7896) [0.7]	34804 (6682) [1.3] *	21409 (2253) [0.8]	18891 (4630) [0.7] *	33809 (7894) [1.5] *	19165 (8415) [1.6]
Particle Concentration Between 80-100 nm ($particles/cm^3$)	3187 (2470)	2919 (727) [0.9]	5392 (935) [1.7] *	4526 (529) [1.4] *	3267 (1053) [1.0]	2084 (1185) [0.7]	1318 (1079) [0.4]
Particle Concentration Between 20-35 nm ($particles/cm^3$)	3453 (1211)	2525 (2313) [0.7]	4524 (1354) [1.3]	1293 (912) [0.4] *	2023 (1067) [0.6]	5302 (613) [1.5] *	5558 (3964) [1.6] *
Particle Number >23 nm ($particles/cm^3$)	16900 (10326) 65.0%	15442 (2357) 69.1%	27299 (4752) 78.4%	19776 (1702) 92.3%	15484 (4412) 82.0%	16125 (4433) 47.7%	12711 (6563) 66.3%
Particle Number <23 nm ($particles/cm^3$)	9093 (3472) 35.0%	6893 (6250) 30.9%	7505 (2452) 21.6%	1633 (1819) 7.6%	3407 (2163) 18.0%	17684 (5791) 52.3%	6454 (3517) 33.7%

797 a Values in square brackets [ ] represent proportional changes in comparison to ULSD.
798 b Percentage values in the last two rows represent the percentage of the total particle number
799 concentration within each fuel.
800 * Significantly different to ULSD (*=p<0.05, **=p<0.01, ***=p<0.001).

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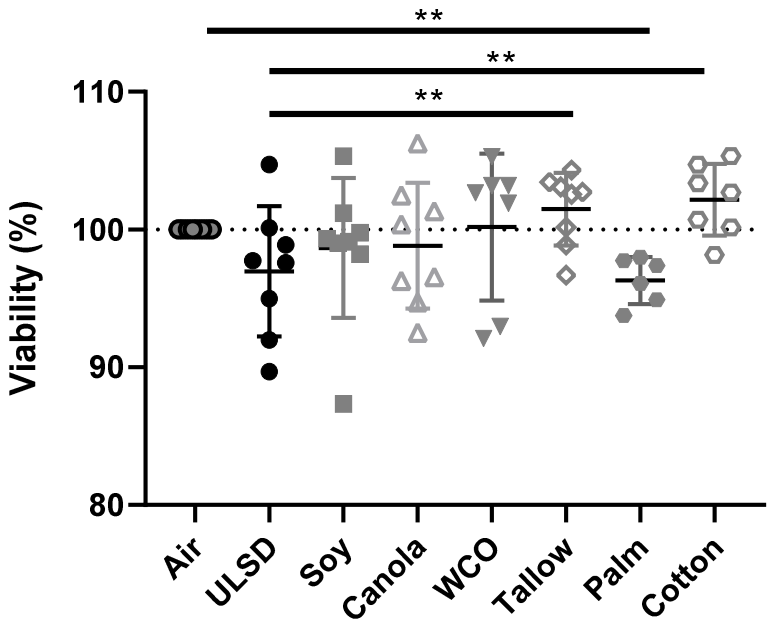


Figure 2: Viability measurements normalised to air controls 24 hours after exposure. Mean viability measurements were: 97. 0 ± 4.7%, 98.6 ± 5.1%, 98.8 ± 4.6%, 100.2 ± 5.3%, 101.5 ± 2.6%, 96.3 ± 1.7% and 102.2 ± 2.6% for ULSD, soy B20, canola B20, WCO B20, tallow B20, palm B20 and cottonseed B20 exposures respectively. Linking bars on the top of the graph indicate significant differences to air or ULSD controls compared to the linked fuel (**=$p<0.01$).

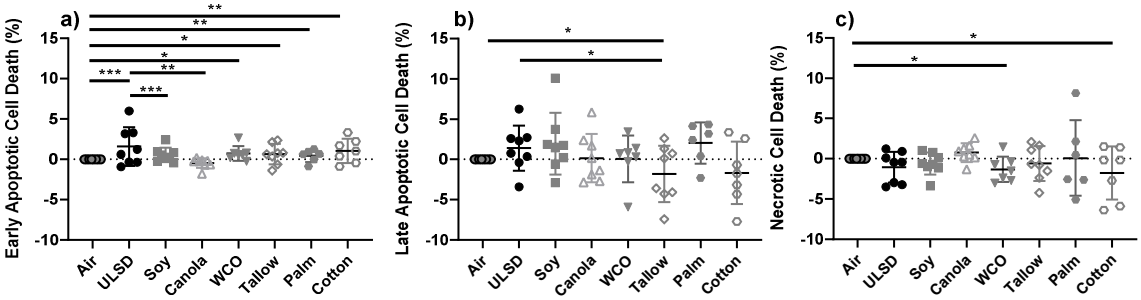


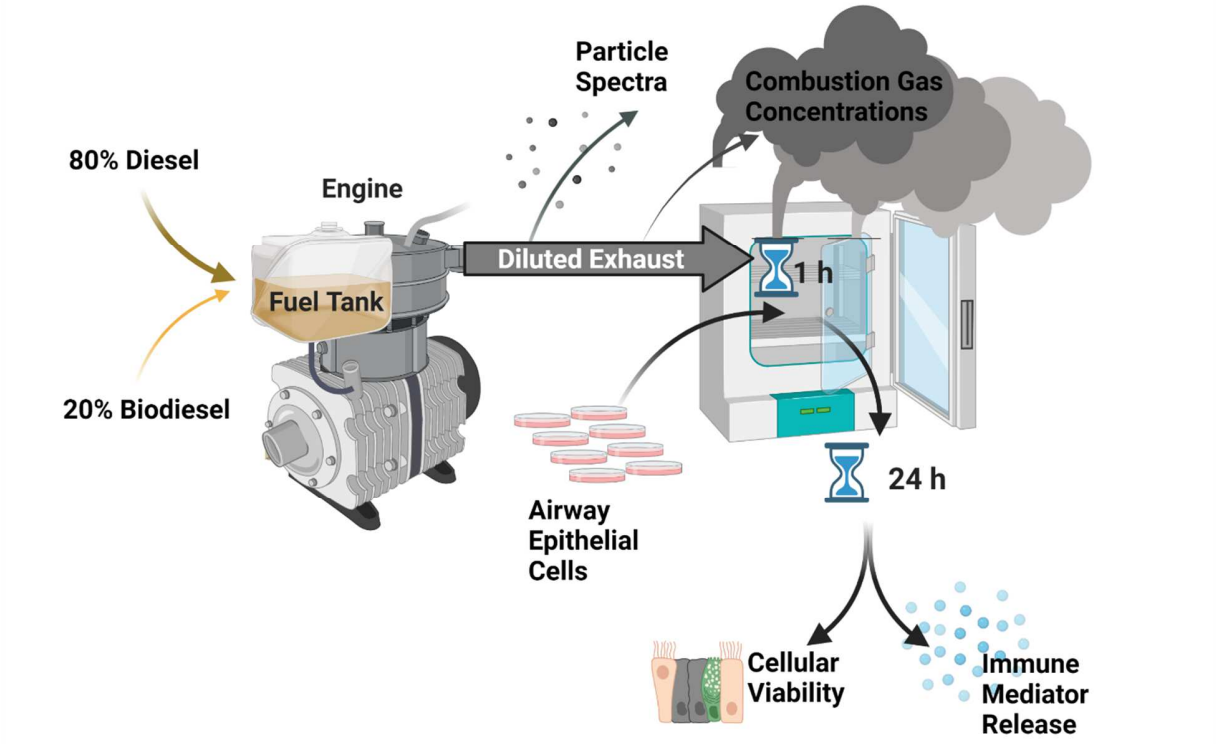
Figure 3: a) Percentage change in early apoptotic cell death 24 hours after exposure. b) Percentage change in late apoptotic cell death 24 hours after exposure. c) Percentage change in necrotic cell death 24 hours after exposure. All cell death mechanisms were normalised by subtracting air controls. Linking bars on the top of the graph indicate significant differences to air or ULSD controls compared to the linked fuel (*=p<0.05, **=p<0.01, ***=p<0.001).

Table 3: Mean (standard deviation) mediator release for the 14 cytokines released above the limits of detection 24 hours after exposure. All values have been normalised by subtracting air controls for each individual participant so as to minimise variability between samples from different volunteers. See supplementary Table S3 for significant differences between biodiesel fuels.

Mediator Concentration (pg/mL)	Fuel						
	ULSD	Soy B20	Canola B20	WCO B20	Tallow B20	Palm B20	Cottonseed B20
IL-1β	0.2 (0.7) *	0.2 (0.6) **	0.2 (0.3)	0.1 (0.1)	-0.0 (0.1) #	0.1 (0.2)	0.1 (0.4)
IL-1RA	10.1 (42.5)	9.3 (297.0)	-26.7 (87.3)	102.8 (213.8)	-13.3 (121.2)	35.1 (206.6)	143.0 (223.8)
IL-6	-70.8 (117.1)	121.4 (238.4) *** ##	-21.8 (110.3)	19.8 (147.7)	217.1 (297.6) ** ####	27.4 (269.2)	-0.1 (259.4)
IL-8	255.5 (705.6)	2180.7 (3774.0) **** ####	-29.9 (151.5)	153.6 (430.8)	3003.1 (5481.2) **** ####	920.3 (1307.3) *** #	-4.2 (513.9)
IL-9	2.0 (4.0)	4.4 (9.5) ***	1.7 (4.1)	2.5 (6.4)	3.7 (6.2) **	5.4 (6.6) *	2.3 (7.2)
G-CSF	28.8 (62.4) **	59.2 (113.8) **** ##	56.2 (69.3) ***	34.8 (45.7) *	124.6 (196.7) **** ####	49.9 (46.8) *	23.9 (64.5) #
GM-CSF	8.4 (14.4) *	12.7 (19.5) **	7.2 (9.6)	5.8 (8.2)	19.9 (24.0) ** #	19.1 (23.6)	17.0 (22.6) *
IFN-γ	-2.0 (5.6)	1.8 (8.8)	1.3 (4.2)	8.5 (13.4)	1.9 (7.1)	6.7 (11.7) *	16.1 (14.2) ** ##
IP-10	-8.8 (200.8)	143.6 (204.3) #	27.2 (157.2)	-22.1 (95.0)	32.4 (112.2)	18.3 (203.8)	68.6 (227.4)
MCP-1	24.8 (56.0) *	61.7 (105..0) *	3.2 (3.3)	7.0 (12.0)	53.5 (126.8) *	57.5 (132.7) *	41.8 (108.5) *
MIP-1β	1.2 (1.6) **	0.4 (1.4)	-0.2 (1.2) ##	0.2 (2.0) ##	0.3 (1.1)	2.4 (1.1) ***	0.2 (1.7) ##
RANTES	-0.3 (1.0)	3.1 (4.4) *** ##	-0.1 (1.0)	0.9 (3.2)	0.3 (0.4)	6.3 (5.3) **** ###	1.2 (0.8)
TNF-α	5.2 (4.3) **	7.1 (7.1) ***	5.1 (2.8) **	8.0 (10.4) ***	4.7 (5.7) **	12.4 (16.7) **** #	15.6 (12.0) **** ###
VEGF	93.6 (167.3)	-39.3 (166.7)	-1.8 (127.1)	16.5 (59.7)	10.1 (237.8)	81.9 (201.2)	-92.8 (197.1) * #

* 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)


Particle
Spectra
Combustion Gas
Concentrations
80% Diesel
Engine
Diluted Exhaust
1 h
Fuel Tank
20% Biodiesel
24 h
Airway
Epithelial
Cells
Cellular
Viability
Immune
Mediator
Release