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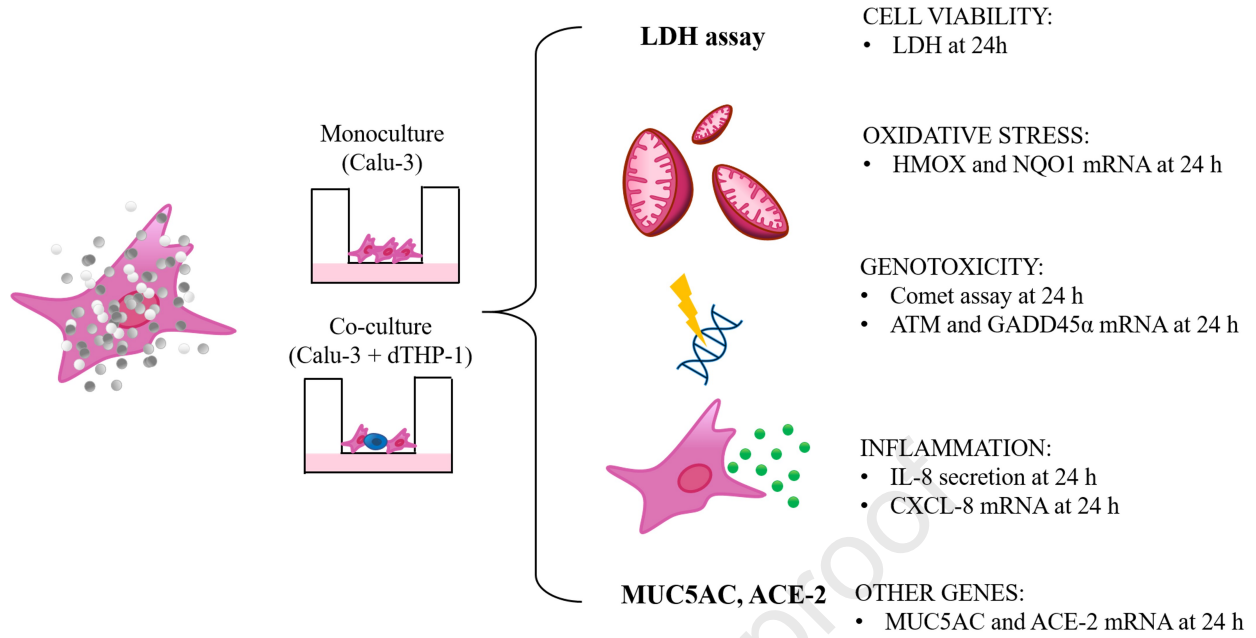
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1 **Toxicological evaluation of primary particulate matter emitted from combustion** 2 **of aviation fuel**

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22 **ABSTRACT**

23 Recently, Sustainable Aviation Fuel (SAF) blends and novel combustion technologies have been introduced
24 to reduce aircraft engine emissions. However, there is limited knowledge about the impact of combustion
25 technology and fuel composition on toxicity of primary Particulate Matter (PM) emissions, comparable to
26 regulated non-volatile PM (nvPM).

27 In this study, primary PM was collected on filters using a standardised approach, from both a Rich-Quench-
28 Lean (RQL) combustion rig and a bespoke liquid fuelled Combustion Aerosol Standard (CAST) Generator
29 burning 12 aviation fuels including conventional Jet-A, SAFs, and blends thereof. The fuels varied in aromatics
30 (0-25.2%), sulphur (0-3000 ppm) and hydrogen (13.43-15.31%) contents. Toxicity of the collected primary
31 PM was studied *in vitro* utilising Air-Liquid Interface (ALI) exposure of lung epithelial cells (Calu-3) in
32 monoculture and co-culture with macrophages (differentiated THP-1 cells). Cells were exposed to PM
33 extracted from filters and nebulised from suspensions using a cloud-based ALI exposure system. Toxicity
34 readout parameters were analysed 24h after exposure.

35 Results showed presence of genotoxicity and changes in gene expression at dose levels which did not induce
36 cytotoxicity. DNA damage was detected through Comet assay in cells exposed to CAST generated samples.
37 Real-Time PCR performed to investigate the expression profile of genes involved in oxidative stress and DNA
38 repair pathways showed different behaviours after exposure to the various PM samples. No differences were
39 found in pro-inflammatory interleukin-8 secretion. This study indicates that primary PM toxicity is driven by
40 wider factors than fuel composition, highlighting that further work is needed to substantiate the full toxicity of
41 aircraft exhaust PM inclusive of secondary PM emanating from numerous engine technologies across the
42 power range burning conventional Jet-A and SAF.

43

44 **Key words:** air-liquid interface, *in vitro*, DNA damage, inflammation, toxicity, aircraft PM emission, aviation
45 fuel.

46

47 INTRODUCTION

48 Increasing levels of air pollution are detrimental to human health, correlating with enhanced cardio-pulmonary
49 mortality and lung cancer (Pope III et al., 2002). Particulate Matter (PM) is one of the most abundant pollutants,
50 associated with combustion, and considered to impact human health significantly. PM is composed of a
51 complex and heterogeneous mixture of solid and liquid particles suspended in the atmosphere which evolves
52 as a result of cooling and chemical reactions occurring between pollutants from other natural and
53 anthropogenic sources. The source of emission influences the size, shape, and composition of the particles
54 (Perrone et al., 2013). Inorganic elements (i.e., metals) and ions (e.g., ammonium, sulphate, and nitrate),
55 mineral dust, elemental and organic carbon, and aromatic compounds are the main components of PM (Rönkkö
56 et al., 2018). Guidelines and standards exist to limit the emission of several airborne pollutants such as nitrogen
57 oxides (NO_x), carbon oxide (CO), unburned hydrocarbons (UHC), ozone, and PM. In 2021, the WHO
58 recommended lowering the PM_{2.5} air quality annual guideline level from 10 to 5 µg/m³ to reflect new evidence
59 of detrimental impacts at low levels of exposure (World Health Organization, 2021).

60 In the proximity of the emission source, PM is more concentrated, hence people can be easily exposed to high
61 levels of toxicants. Anthropogenic sources, including automotive combustion sources, generate the majority
62 of ultrafine particles in urbanized areas and they may on a mass basis be more toxic for human health compared
63 to fine particles (Cassee et al., 2013; Ghio et al., 2012; Schilirò et al., 2015). In urban areas, PM from
64 automotive traffic comprising both exhaust and non-exhaust emissions is highly hazardous (Corsini et al.,
65 2019, 2017; Domingues et al., 2018). In recent years, emissions from the aviation industry and their associated
66 health effects are getting more and more attention given its continued growth, and include pollutants coming
67 from aircraft and ground traffic operations (He et al., 2018; Masiol and Harrison, 2014). These pollutants are
68 not only generated by the aircraft engines, but are also derived from the wear of tires, brakes, and asphalt along
69 with the re-suspension of particles due to aircraft movements, and to a lesser extent from maintenance work,
70 heating facilities, vapours from refuelling operations, and restaurants in the air terminals (Masiol and Harrison,
71 2014; Pirhadi et al., 2020). Since the combustion of fossil fuels remains the main source of harmful pollutants,
72 nowadays the study and development of more sustainable and less damaging fuels is a priority. In terms of
73 civil aviation the two main operational standard fuels are Jet-A (mostly used in US in civil aviation) and Jet-

74 A1 (used elsewhere in the world), which contain a kerosene distillation fraction of crude oil, a complex mixture
75 of more than 1000 chemical compounds (Kallio et al., 2014). New fuel standards have been developed to
76 mitigate emissions, for example lowering aromatic and sulphur compounds in Jet-A1 and permitting the use
77 of gas-to-liquid (GTL) kerosene fuel, catalytic hydrothermal conversion jet (CHCJ) fuel, hydroprocessed esters
78 and fatty acids (HEFA) fuels, and others (Christie et al., 2012; Kallio et al., 2014; Luning Prak et al., 2017;
79 Onorato et al., 2022) in recent ASTM revisions.

80 Another approach that can be used to reduce aircraft engine emissions is the development of new combustor
81 technologies. Unfortunately gaining access to full-scale aircraft engine exhaust is expensive and technically
82 challenging, with fuel flow rates often prohibitive to acquiring sufficient volumes of non-conventional fuels.
83 As such surrogate sources of combustion emissions, representative of an engine burning Jet fuels have been
84 investigated. One example is a Combustion Aerosol Standard (CAST) generator designed by Jing Ltd, which
85 is specifically designed to burn liquid fuel and is able to work with liquid aeronautic fuel (Jing-CAST
86 Technology GmbH, 2003). The concept is to mimic a practical combustion process via quenching of a
87 diffusion flame. As in a real engine, fuels are atomised to produce fine droplets which are pyrolyzed to
88 particles, then mixed with a quenching gas to prevent combustion, stabilise the soot particles, and inhibit
89 condensation (Jing-CAST Technology GmbH, 2003; Mueller et al., 2015). This generator is based on the
90 design of mini-CAST, a well-known standard source of soot (Moore et al., 2014). The main advantage of the
91 CAST generator is the low fuel consumption (a few mL/h).

92 Similarly, combustor rigs are often used to develop and understand low-emission combustor technologies
93 inclusive of Rich-Quench-Lean (RQL), which were traditionally designed to control NO_x production by
94 controlling localised combustor zone temperatures whilst affording high global efficiency and reliability
95 (Harper et al., 2022).

96 Aircraft emissions can impact travellers, the local demographic of the airports, and airport workers who are
97 exposed for prolonged periods (Westerdahl et al., 2008), with the workers continuously exposed on a daily
98 basis to airport pollutants for the duration of their careers. For humans, the main route of exposure to airborne
99 pollutant is through respiration. The regional deposition of particles in the airways is influenced by several

100 factors including particle size, lung morphology and physiology, fluid dynamics of the inhaled airflow, and
101 particle features (Nozza et al., 2021; Sznitman, 2022). It is known that larger particles (with aerodynamic
102 diameter higher than 2.5 μm) can affect the upper respiratory tract, while small particles penetrate deeply in
103 lungs reaching bronchioles and alveoli (Salma et al., 2002; Valavanidis et al., 2008).

104 Aircraft engine emissions are composed of gases (CO_2 , NO_x , CO, UHCs, SO_x , etc.), volatiles (sulphates,
105 nitrates, oil, unburnt fuels, etc.) and non-volatile PM (soot) typically consisting of solid carbon and formed
106 from polycyclic aromatic hydrocarbons (PAHs) (Bendtsen et al., 2019; Gualtieri et al., 2022; Miake-Lye et al.,
107 1998). In terms of number concentration, non-volatile PM (nvPM) derived from aircraft engines is typically
108 between 10 and 100 nm in mobility size (Boies et al., 2015; Durand et al., 2021; Durdina et al., 2014; Harper
109 et al., 2022), which is particularly prone to reach the lower part of the respiratory tract (Durdina et al., 2014;
110 Lighty et al., 2000; Stacey et al., 2020). Despite the presence of protection mechanisms in the lungs, PM can
111 cause toxicity, e.g., oxidative stress induction, generation of inflammatory mediators, DNA oxidative damage
112 and breaks (Cavallo et al., 2006; Corsini et al., 2019; Marabini et al., 2017; Møller et al., 2020).
113 Epidemiological studies have demonstrated that proximity to running aircraft engines or to airports is
114 associated with increased exposure to PM and risk of disease, hospital admission, and lung dysfunctions
115 (Bendtsen et al., 2021; Habre et al., 2018; Lin et al., 2008).

116 Different methods can be found in the literature for assessing the toxicity of combustion emissions, ranging
117 from collection-based methods (Karavalakis et al., 2017; McCaffery et al., 2022) to direct deposition onto cells
118 (Jonsdottir et al., 2019). In the present study, the toxicity of primary PM generated by combustion of different
119 aviation fuels was evaluated in a model of Calu-3 human lungs epithelial cells. Raw exhaust PM was collected
120 onto filters, extracted and then used to expose the test cells by Air-Liquid Interface (ALI) exposure. The ALI
121 exposure was conducted using a "radial *in vitro* aerosol exposure system" (RIVAES; developed at RIVM based
122 on the VITROCELL® Cloud system) in which PM suspensions are nebulized above the cells grown on
123 transwell inserts. Cytotoxicity, oxidative stress, genotoxicity, and the production of pro-inflammatory
124 cytokines were investigated to ascertain the possible toxicity induced and the differences in potential health
125 outcomes resulting from the different fuels.

126 MATERIAL AND METHODS

127 Filter collection

128 Raw PM emissions were collected on PTFE filters during measurement campaigns performed as part of the
129 RAPTOR (Research of Aviation PM Technologies, mOdelling and Regulation) project at Cardiff University's
130 Gas Turbine Research Centre (GTRC) and UNREAL (Unveiling nucleation mechanism in aircraft engine
131 exhaust and its link with fuel composition) project with French aerospace lab ONERA CAST burner. Aircraft
132 engine emission-like PM was produced using a small-scale (<250 kW) non-proprietary Rich-Quench-Lean
133 (RQL) combustion rig at pressures ranging from 1.0 to 2.4 bar (Harper et al., 2022), and a bespoke liquid
134 fuelled Combustion Aerosol STandard (CAST) generator (Jing-CAST Technology GmbH, 2003). All filters
135 were collected using unheated filter holders supplied with 160 °C raw exhaust. This setup, derived from
136 regulatory raw emissions measurements (ICAO, 2023) and reproducible across different combustion
137 technologies, was used to suppress volatile (e.g., unburnt fuel, oil) and water condensation in the filter
138 collection sampling system. Dilution was not utilised as is the case in regulatory nvPM measurements to
139 achieve sufficient PM loadings within practical sampling times. For the RQL testing, filters were collected
140 simultaneously in parallel using three nominally identical 47 mm aluminium filter holders connected to a
141 heated 2 m long 3/8" internal diameter carbon loaded PTFE sampling line (160 °C) sampling from the water-
142 cooled emissions probe (160 °C) at flow rates of approximately 40 L/min per filter (120 L/min in sample line).
143 Sample times of up to 1 h were used to achieve minimum filter loadings of 1 mg per filter, with the exception
144 of the RQL AGTL-100, where approximately 0.2 mg was collected on each filter due to limited PM mass
145 concentration and available sampling time. PTFE Membrane Filter, ZeFluor, 2 µm pore size (Pall Corporation)
146 were used for *in vitro* toxicity testing. After sampling, filters were individually stored in plastic filter holders,
147 wrapped, as sets of 3, in aluminium foil and stored in a freezer, before being shipped (for *in vitro* toxicity
148 testing) in a thermal case kept cold using ice blocks, with a blank filter which was loaded into and out of the
149 filter holder and stored in an identical manner. For the CAST testing, raw emissions were also sampled on
150 PTFE filters at 5 L/min for 10 seconds, resulting in significantly lower PM mass on the filters (i.e., ~10-100
151 µg). Filters in this experiment were stored in Petri dishes in the dark below 7 °C, before shipping.

152 Filter samples and fuel properties

153 Aviation relevant emissions were generated with the CAST or RQL burning twelve different fuels, from
 154 standard Jet-A to 100% GTL fuel allowing for a large variation in emitted nvPM morphology representative
 155 of the current aircraft fleet (Harper et al., 2022), with details of the varying aromatic, sulphur, and hydrogen
 156 contents provided in Table 1. The fuels included seven conventional aviation Jet-A fuels (J-LAS, J-HA, J-
 157 HA2, J-HS, J-HAS, J-REF and J-REF2) covering the permissible range of fuel aromatic and sulphur contents
 158 specified by ASTM D1655 for aviation fuels, two SAFs (A-HA and A-GTL), and three blends (B-REF, B-
 159 HE2 and B-GTL). B-REF is a mixture of 70% J-REF with 30% A-LA, B-HE2 consists of 70% J-LA with 30%
 160 HEFA, and the GTL blend is made of 75% A-GTL and 25% of J-REF2. In line with terminology proposed by
 161 Harper et al., 2022, 'J-' refers to Jet fuels, 'B-' refers to Blend fuels, and 'A-' refers to Alternative fuels. The
 162 suffixes '-H', '-A', '-S', and '-HE' correspond to High, Aromatic, Sulphur, and HEFA, respectively. A blank
 163 filter (Blank) was left overnight in the filter holder but otherwise handled identically as the PM samples to
 164 have a control for the experiments, to assess the filter handling and shipping procedures.

165 **Table 1.** Tested Fuel Combustion Emissions (FCE) and composition expressed as content of aromatic
 166 compound (%wt), Naphthalene (di-aromatics) (%wt), sulphur (ppm), and hydrogen content (%wt).

Samples	Aromatic content (%wt)	Naphthalene (di-aromatics) (%wt)	Sulphur content (ppm)	Hydrogen content (%wt)
<i>Measurement method</i>	GC x GC	GC x GC	ASTM D2622	ASTM D7171 or GCxGC*
Blank	-	-	-	-
CAST J-LAS	16%	0.5%	4 ppm	14.02%
CAST J-HA2	23%	3%	4 ppm	13.55%
CAST J-HS	16%	0.5%	3000 ppm	14.02%
CAST J-HAS	23%	3%	3000 ppm	13.55%
CAST J-REF	20.2%	1.8%	200 ppm	14.02%
CAST B-REF	14.2%	1.3%	140 ppm	14.41%
RQL J-REF2	20.8%	0.8%	36 ppm	13.43%*
RQL J-HA	22.8%	2.2%	105 ppm	13.65%
RQL B-HE2	12.8%	0.1%	4 ppm	14.51%
RQL A-HA	25.2%	0.3%	0 ppm	13.51%
RQL B-GTL75	5.3%	0.3%	25 ppm	14.90%*
RQL A-GTL100	0.1%	<0.1%	0 ppm	15.47%*

167 **Legend.** CAST: Combustion Aerosol Standard generator; RQL: Rich-Quench-Lean; J-: Jet fuel; B-: Blend
 168 fuel; A-: Alternative fuel; -L: low; -H: High; -A: Aromatic compounds; -S: Sulphur compounds; -HE: HEFA;
 169 -REF: Reference; GTL75 or GTL100: Gas-To-Liquid (75 and 100 = % blended); * hydrogen content derived
 170 from GCxGC analysis.
 171

172 PM extraction from filters

173 The PM samples on the different filters were individually extracted in methanol (Haleyur et al., 2016; Happonen et al., 2008; Pennanen et al., 2007). The filters were transferred to a clean Petri dish with 2 mL of HPLC-grade methanol (BioSolve BV). Petri dishes were held at two millimetres in the ultrasonic bath above the central point of the sonic burst for 30 seconds with water contamination avoided. Afterwards the Petri dish was tilted, and the supernatant transferred into a pre-weighed and labelled cryovial. Filters were flipped over with a clean plastic tweezer and the extraction step was repeated with fresh methanol. The methanol suspension was transferred into the same cryovial. Particles extraction efficiency was generally >90%. The extracted solution was dried overnight at 25 °C in an incubator under a constant flow of nitrogen to prevent further PM degradation or oxidation. The cryovials with the extracts were reweighed to calculate the extraction yield. PM was resuspended in ultrapure water to obtain a solution concentrate of 1 mg/mL and the vials were stored at -20 °C until further analysis. The Blank was kept in the Petri dishes and extracted in the same manner as the samples.

185 Cell culture

186 Calu-3 (cod. HTB-55, American Tissue Culture Collection - ATCC, Rockville, US) is a lung epithelial cell line obtained from a patient with a lung adenocarcinoma that is commercially available and can be used in cancer and toxicology research (Zhu et al., 2010). Cells were cultured in Minimum Essential Medium (MEM 1X) + GlutaMAX™-I [+] Earle's Salts [+] 25 mM HEPES (Gibco) supplemented with 10% of Foetal Bovine Serum (FBS) Heat Inactivated (HI) (Gibco), 1% of Minimum Essential Medium Non-Essential Amino Acid (MEM NEAA 100X – Gibco), and 1% of Penicillin Streptomycin (P/S – [+] 10000 Units/mL Penicillin [+] 10000 µg/mL Streptomycin – Gibco). THP-1 cells were cultured in RPMI Medium 1640 (1X) [+] L-Glutamine (Gibco), supplemented with 10% FBS-HI and 1% P/S.

194 At day 0, Calu-3 cells were seeded at a cell density of 2×10^5 cells/well on the apical part of 12 mm diameter Transwell® Polyester Membrane Polystyrene inserts with 0.4 µm pore (Costar Corning). The apical compartment was filled with 500 µL of complete medium and the basal compartment with 1 mL. Culture medium was changed every other day, until day 14 when the medium was removed from the apical layer of the transwell and kept only on the basal, to start the Air-Liquid Interface (ALI) condition. Calu-3 cells were

199 kept in ALI condition for 7 days meaning a cell preparation period of 21 days. During this preparation phase,
200 differentiation of THP-1 cells (2.0×10^5 cell/mL) was performed, on day 19, using phorbol 12-myristate 13-
201 acetate (PMA, Sigma Aldrich) 10 ng/mL (Park et al., 2007). The differentiation lasted 48 h. At day 21, the
202 differentiated THP-1 cells were detached using a cell scraper. 2×10^5 differentiated THP-1 (dTHP-1) cells were
203 seeded in the apical compartment of half of the inserts to start the co-culture, while the other half were used as
204 monoculture. After the preparation period, on day 22, the cells were exposed to the extracted PM.

205 As an indicator of barrier integrity, transepithelial electrical resistance (TEER) was measured using an Evom2
206 Voltohmmeter equipped with 4 mm chopstick electrodes (World Precision Instruments Inc.). TEER was
207 measured before setting ALI condition (Day 14) and before starting the co-culture (Day 21). The different
208 TEER measurements were used to determine if it was appropriate or not to set ALI condition. Values around
209 $800\text{-}1000 \Omega/\text{cm}^2$ were considered acceptable to set the ALI condition. To measure TEER at the ALI, 500 μL
210 of corresponding medium was added onto the apical side of the inserts. All TEER values were corrected for
211 the resistance of cell-free insert ($\approx 100 \Omega/\text{cm}^2$).

212 **Air-liquid interface exposure**

213 The PM solutions were at a starting concentration of 1 mg/mL in ultrapure water. Before cells exposure, they
214 were diluted and subsequently nebulised onto the apical side of mono- and co-culture models in inserts using
215 a "radial *in vitro* aerosol exposure system" (RIVAES; designed by RIVM, inspired by the design of the
216 VITROCELL® Cloud exposure system (VitroCell, Waldkirch, Germany)) on Day 22. A photo of RIVAES is
217 provided in the supplementary materials (**Figure S1**). In this exposure system, the transwell inserts are placed
218 radially to minimize variation in deposition. The system has a slightly smaller surface area than the
219 VITROCELL® Cloud system, resulting in a slightly higher deposition. It is equipped with a refined
220 temperature control system resulting in a stable temperature at the transwell inserts. The nebulizer used for the
221 exposure of the cells is the Aeroneb® nebulizer, 4.0 – 6.0 μm volume median diameter (VMD) (Aerogen Ltd.,
222 Galway Ireland). The injection volume of the PM samples (diluted in saline solution – 0.9 mg of NaCl in 1
223 mL of sterile water – at a PM concentration of 250 $\mu\text{g}/\text{mL}$) for nebulization was 200 μL and the deposited dose
224 in each insert is presented in **Table 2**. Deposition was measured using a quartz crystal microbalance (QCM).
225 The expected deposition was around $450 \text{ ng}/\text{cm}^2$, which was calculated based on the concentration of the PM

226 suspensions including solutes (NaCl), the total surface area of the RIVAES and assuming a deposition
 227 efficiency of 80% (which is typically observed upon nebulization of PM suspensions in the RIVAES). Some
 228 differences were observed between the samples' deposition doses may be due to several factors (e.g.
 229 differences in physicochemical properties). Controls were treated with 0.9% saline solution. NIST2975 Diesel
 230 Particulate Matter (Industrial Forklift – Diesel soot) was used as positive control. After exposure, 1% FBS
 231 medium was added in the wells, and apical (500 μ L added 30 min before collecting) and basolateral medium
 232 were collected separately after 24 h.

233 **Table 2.** Deposited dose expressed in ng/cm^2 for each nebulization of the PM samples (data are expressed as
 234 mean \pm SEM). The expected deposition is around $450 \text{ ng}/\text{cm}^2$.

Samples	Deposition dose ng/cm^2
Blank	0 ± 0
CAST J-LAS	580 ± 24
CAST J-HA2	670 ± 68
CAST J-HS	565 ± 6
CAST J-HAS	610 ± 65
CAST J-REF	478 ± 84
CAST B-REF	491 ± 33
RQL J-REF2	541 ± 102
RQL J-HA	650 ± 14
RQL B-HE2	700 ± 70
RQL A-HA	535 ± 20
RQL B-GTL75	240 ± 32
RQL A-GTL100	351 ± 15
Diesel soot	456 ± 32

235 Lactate Dehydrogenase assay

236 The Lactate Dehydrogenase (LDH) release was quantified to evaluate the cytotoxicity. To measure the
 237 maximum LDH release, cells were incubated with 2% Triton X-100 (Thermo Fisher Scientific Inc.) for 30
 238 min. The medium was collected from the apical and basal layer of the transwell insert, after 24 h from the time
 239 of nebulization and immediately analysed for LDH measurement; the test was performed following
 240 manufacturer's instructions (Roche Diagnostics). Briefly, 50 or 100 μ L of medium (apical and basal
 241 respectively) and 100 μ L reaction reagent were added into a 96-well flat-bottomed plates and incubated in the
 242 dark for 30 min at room temperature. The absorbance was measured at the wavelength of 490 nm with a
 243 microplate reader (Molecular Devices). All LDH values were normalised for the maximum LDH release per
 244 cell type or for the controls.

245 Comet assay modified with enzymes (ENDO III and FPG)

246 The analysis of the DNA damage was performed after 24 h from the exposure to the nebulised PM samples.
247 Cells were washed with 0.05% of PBS-EDTA and detached with 0.05% trypsin-EDTA (Gibco) for 5 min.
248 Cells were collected in medium and centrifuged at g-force of 150 for 5 min.
249 Pellets were resuspended in 100 μ L of MEM. The assay was performed following the Trevigen kit instructions.
250 Cells were mixed with low melting agarose (1 : 3 – cells : agarose), seeded on microscope slides and allowed
251 to solidify at 4 °C. Three slides were prepared for each sample and treated with the different enzymes
252 (Endonuclease III – ENDO III, and Formamidopyrimidine DNA Glycosylase – FPG). Slides were submerged
253 with lysis buffer (Trevigen) for 30 min at 4 °C and then incubated with the enzymes ENDOIII and FPG for 45
254 min at 37 °C. Slides were submerged in unwinding buffer for 30 min at 4 °C. Electrophoresis was then
255 performed for 20 min at 300 mA constant and 25 V. At the end, slides were dehydrated through incubation
256 with cold ethanol for 5 min. Once dry, slides were stained with SYBR Gold (Thermo Fisher Scientific Inc.)
257 solution and stored at 4 °C in the dark. Slides were analysed with Comet IV, magnification 10X, reading and
258 analysing 100 cells for each sample to obtain the value of tail moment (TM) used for the quantification of
259 DNA damage.

260 Gene expression

261 Cells were lysed with 300 μ L of TRIzol® reagent (Life Technologies) and stored at -80 °C until analysis. RNA
262 was extracted using Direct-zol™ RNA MiniPrep Kit following the manufacturer's instructions (Zymo
263 Research). RNA concentration and purity were evaluated by spectrophotometer (NanoVue Plus, NanoDrop
264 Technologies, Biochrom™, Cambridge, UK) calculating the 260/230 and 260/280 absorbance ratios. 300
265 nanograms of total RNA were retro-transcribed with random primers (Promega, Milan, Italy) and M-MLV
266 Reverse Transcriptase Kit (Promega, Milan, Italy), according to manufacturer's instructions. Analysis of gene
267 expression was carried out with 2 μ L of cDNA using Luna® Universal qPCR Master Mix (New England
268 BioLabs, Ipswich, USA) and analysed on an CFX Connect Real-Time PCR Detection System (BIO-RAD,
269 Hercules, USA). All reactions were run in duplicate, and the relative abundance of the specific mRNA levels
270 were calculated by normalizing to GAPDH expression using the $2^{-\Delta\Delta ct}$ method. The complete list of genes and

271 primer sequences is reported in **Table 3**. All the sequences were obtained using Primer designing tool – NCBI
 272 and NIH (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

273 **Table 3.** Primer sequences (Primer designing tool – NCBI and NIH).

Gene	Oligonucleotide sequence	
	Forward (5' – 3')	Reverse (5' – 3')
<i>ACE-2</i>	TCCATTGGTCTTCTGTCACCCG	AGACCATCCACCTCCACTTCTC
<i>ATM</i>	GTTTATTGTCACCCTGCTGCC	ACTTCTTTCTTTCGTTCTGTAGCTC
<i>CYP1A1</i>	GCAGATCAACCATGACCAGAAG	TCACCGATACACTTCCGCTT
<i>CXCL-8</i>	GAAGTTTTTGAAGAGGGCTGAGA	CACTGGCATCTTCACTGATTCT
<i>GADD45α</i>	GAGAGCAGAAGACCGAAAGGA	CACAACACCACGTTATCGGG
<i>GAPDH</i>	TCGGAGTCAACGGATTTGGT	TGAAGGGGTCATTGATGGCA
<i>HMOX</i>	CAACAAAGTGCAAGATTCTGCC	TGGCATAAAGCCCTACAGCA
<i>MUC5AC</i>	TCTGAGCGTGGAGAATGAGAAG	CTCACAGTTGCAGGTGTCAA
<i>NQO1</i>	AGTATCCACAATAGCTGACG	TTTGTGGGTCTGTAGAAATG

274 **Inflammatory response**

275 Supernatants were collected from the apical and basal layer of the transwell and stored at -20 °C until analysis.
 276 Interleukin (IL)-8 and Tumour Necrosis Factor (TNF)- α secretion was evaluated by commercially available
 277 ELISA kits (ImmunoTools for IL-8 and R&D DuoSet® ELISA for TNF- α). Samples were diluted 1:50 for IL-
 278 8 and undiluted for TNF- α .

279 **Statistical analysis**

280 Every PM sample was tested using $n=6$ inserts, exposed using one nebulization. Three inserts were dedicated
 281 to Comet assay while the other three to gene expression analysis; all the inserts were used for the other assays.
 282 Results were analysed using ANOVA to assess statistical significance, two-way ANOVA analysis followed
 283 by post-hoc Dunnett's tests for multiple comparisons. Results were considered statistically significant at
 284 $p<0.05$. Statistical analysis was carried out using the software package GraphPad Prism version 9.0 (GraphPad
 285 Software). Results are expressed as means \pm standard deviation (SD).

286 **RESULTS**287 **Transepithelial electrical resistance (TEER)**

288 TEER is a measurement of electrical resistance across a cellular monolayer which is used to verify the integrity
 289 of a monolayer (Srinivasan et al., 2015). TEER was measured immediately before and 24 h after the ALI
 290 exposure. Results are shown in **Table 4** as ratio post/pre-exposure. No statistically significant differences were
 291 detected among the samples and the controls.

292 **Table 4.** TEER ratio post-exposure/pre-exposure of Calu-3 cells in mono- and in co-culture with differentiated
 293 THP-1 (dTHP-1) cells. Data are expressed as mean \pm SD.

	Calu-3	Calu-3 + dTHP-1
	Mean \pm SD	Mean \pm SD
Control	0.9682 \pm 0.1395	1.0305 \pm 0.0954
Blank	1.1617 \pm 0.0645	1.1410 \pm 0.0311
CAST J-LAS	1.0984 \pm 0.0301	1.0656 \pm 0.0450
CAST J-HA2	1.0663 \pm 0.0698	0.9918 \pm 0.0376
CAST J-HS	1.0676 \pm 0.1083	1.0648 \pm 0.0283
CAST J-HAS	1.1994 \pm 0.1352	1.1203 \pm 0.0961
CAST J-REF	0.8877 \pm 0.0332	0.8764 \pm 0.0332
CAST B-REF	0.9264 \pm 0.0259	0.9420 \pm 0.0456
RQL J-REF2	0.7420 \pm 0.0377	0.7411 \pm 0.0284
RQL J-HA	1.0321 \pm 0.0651	1.0396 \pm 0.0789
RQL B-HE2	0.9315 \pm 0.0313	0.9411 \pm 0.0391
RQL A-HA	1.0143 \pm 0.1065	1.0007 \pm 0.0664
RQL B-GTL75	0.9057 \pm 0.0603	0.8859 \pm 0.0888
RQL A-GTL100	1.1429 \pm 0.0634	1.2027 \pm 0.0593
Diesel soot	1.0881 \pm 0.0724	1.1365 \pm 0.1087

294 **Lactate Dehydrogenase assay**

295 Twenty-four hours after the exposure, supernatants were collected from the apical and the basal compartments
 296 of the transwell inserts. Leakage of LDH was measured immediately after supernatant collection, and results
 297 are shown in **Figure 1**. In general, slightly higher levels of LDH were detected in the apical supernatants
 298 (Figure 1) compared to the basal supernatants (data not shown), but no significant cytotoxicity was observed
 299 after exposure to any of the primary PM samples.

300 **Comet assay**

301 Cytotoxicity was considered acceptable to perform genotoxicity assay as was always below 30% (Tice et al.,
 302 2000). The comet assay was performed to detect the amount of DNA damage. In addition, ENDOIII and FPG
 303 were used for the identification of oxidative type of DNA damage in the samples. The results are shown in

304 **Figure 2.** Statistically significant increase in DNA damage was detected mainly in the samples treated with
305 the PM obtained from CAST-generated filters (CAST J-LAS, CAST J-HA2, CAST J-HS, CAST B-REF), both
306 in the monoculture and the co-culture. Increase of DNA damage was detected also following ENDOIII and
307 FPG treatment, with comparable results between samples treated with the enzymes and not, indicating that the
308 induced DNA damage was not related to oxidation of DNA bases. No statistically significant differences were
309 detected between the monoculture and the co-culture.

310 **Gene expression**

311 Gene expression analysis was performed in samples collected after 24 h from the exposure. Genes involved in
312 oxidative stress response (HMOX and NQO1), DNA repair (ATM and GADD45 α), inflammation (CXCL-8),
313 and protection mechanisms (ACE-2 and MUC5AC) were evaluated. Results are shown in **Figure 3.**

314 There is a trend of upregulation for the genes ATM, GADD45 α , and MUC5AC following the treatment with
315 the FCE PM samples, and of downregulation with the other genes. Statistically significant differences in ATM
316 expression were detected in the monoculture following the treatment with CAST J-LAS, CAST J-HA2, CAST
317 J-HS, CAST J-HAS, RQL B-GTL75, and with the Diesel soot. The increase of the expression of GADD45 α
318 was detected in all the samples in a statistically significant manner, both in the monoculture and the co-culture,
319 with the only exception of the samples treated with CAST J-HS and RQL A-GTL100. Also, for MUC5AC the
320 statistical analysis showed significant increase of expression following the treatment with CAST J-HA2, CAST
321 B-REF, RQL J-REF2, RQL J-HA, and RQL A-HA for the monoculture, and for and CAST J-REF also in the
322 co-culture. Statistically different behaviour of the monoculture and the co-culture was detected for ATM
323 following the treatment with CAST J-HS and CAST J-HAS; for GADD45 α following RQL A-HA and Diesel
324 soot; for ACE-2 following CAST J-HA2, CAST J-REF, RQL J-REF2, RQL A-HA, RQL B-GTL75; for
325 MUC5AC following CAST J-HA2 and CAST B-REF; and for NQO1 following RQL J-REF2, RQL A-HA,
326 and diesel soot treatments.

327 **Pro-inflammatory cytokines**

328 The secretion of two cytokines was used as an indicator for a pro-inflammatory response: IL-8 and TNF- α .
329 Data for TNF- α are not reported here because all results were below the limit of detection (15.6 pg/mL). The
330 results related to the production of the pro-inflammatory mediator IL-8 assay are shown in **Figure 4.** The

331 secretion of the IL-8 protein resulted increase in statistically significant manner for most of the samples in
332 different manner in the apical and basolateral compartment. The secretion parameter evaluated in this study
333 does not show consistent results when compared with findings in the scientific literature.

Journal Pre-proof

334 **DISCUSSION**

335 The majority of aviation fuels used nowadays are jet fuels, the kerosene distillation fraction of crude oil (Masiol
336 and Harrison, 2014). However, with the drive to net-zero there will be an inevitable shift towards different
337 SAFs (Kallio et al., 2014; Liu et al., 2013). Previous studies have demonstrated that the most abundant species
338 of particle bound-PAHs in airport emissions are naphthalene, phenanthrene, fluoranthene, acenaphthene, and
339 pyrene, with total concentrations between 0.152 - 0.189 $\mu\text{g}/\text{m}^3$ depending on the ambient conditions (Lai et al.,
340 2013). Adoption of SAFs is expected to reduce nvPM emissions in terms of mass and number concentrations
341 and alter their composition, as alternative fuels typically contain lower concentrations of harmful materials
342 such as aromatic compounds (PAHs) (Christie et al., 2012; Masiol and Harrison, 2014). Currently, information
343 examining the potential adverse health effects of emissions from these new SAFs is sparse, especially
344 concerning genotoxicity and carcinogenicity (Gualtieri et al., 2022; Møller et al., 2020).

345 As with other combustion sources, aircraft engines produce high concentrations of PM containing black carbon
346 (BC) and Organic Carbon (OC). Incomplete combustion of fuels, including kerosene, results in the formation
347 of carbon-rich aromatic by-products and condensates (Bendtsen et al., 2021). In the development of SAF, the
348 presence of aromatic compounds gained attention due to their toxicological properties (Sterner et al., 2020).
349 The toxicity of PAHs is highly dependent on their chemical structure Arias-Pérez et al. (2020) and McCaffery
350 et al. (2022), and different isomers are classified from toxic to extremely toxic, carcinogenic, mutagenic, and
351 teratogenic (Working Group on PAH, 2001). This underscores the importance of detailed PAH analysis, as
352 these substances can induce DNA detriment and micronuclei formation in different cells of the respiratory
353 system, as well as triggering inflammation (Michael et al., 2013; Oh et al., 2011).

354 The aim of this study was to assess the effects of primary PM emitted from aviation representative combustion
355 sources burning a range of aviation fuels, namely an RQL combustion rig and a CAST on cell viability,
356 genotoxicity, oxidative stress, and generation of pro-inflammatory cytokines in Calu-3 epithelial cells
357 (monocultured and co-cultured with macrophages differentiated from THP-1 cells) exposed in ALI conditions.
358 Primary PM emissions were collected on filters using a standardised approach, then were extracted and
359 nebulized on the cells using an air-liquid interface exposure system to mimic the real-life exposure of airway
360 epithelium. It is noted that the sampling approach employed in this study did not replicate full condensation of

361 OC, of which PAH are a subset, as would be the case in real-world conditions. Instead, it was only concerned
362 with primary PM, which is the current regulatory metric. This also meant that although the fuel sulphur content
363 was varied, it was not expected to impact the results of this study given that sulphur-derived PM occurs in the
364 secondary aerosol.

365 No differences were found in the release of the pro-inflammatory IL-8 among the samples. The fuel impact on
366 genotoxicity of primary PM appeared limited, however it is noted that lower fuel aromatic content results in
367 lower concentrations of nvPM (i.e., primary PM) (Durand et al., 2021; Durdina et al., 2021), therefore
368 suggesting that strategies lowering aromatic content, conducive to increased SAF adoption, may result in less
369 toxic primary PM emission (e.g. due to the lower PM emission). However, further analysis as to the secondary
370 PM emissions would be needed to confirm real-world toxicity of aircraft engines burning SAF. Results also
371 showed absence of cytotoxicity and no significant changes in TEER measurement following exposure to the
372 tested samples. Although the sampling approach used in this study was designed to maximise the amount of
373 primary PM material collected onto the filters (0.2 – 2.6 mg/filter), the limited sample availability was
374 considered a significant limitation for toxicological analysis. Similarly, previous studies showed absence of
375 cytotoxicity following 1-4 h exposure to PM obtained from the Jet-A1 and HEFA fuels emissions, as well as
376 a little decrease in TEER after 24 h (Grant et al., 2001; Gualtieri et al., 2022; He et al., 2020). In future studies,
377 on-site *in vitro* ALI toxicity testing could be considered to address this limitation, thereby eliminating the need
378 for extensive PM collection and extraction procedures.

379 Nevertheless, the absence of significant cytotoxicity did not imply the absence of genotoxicity, or changes in
380 gene expression, with most of the CAST-generated primary PM samples seen to increase DNA damage and
381 modulate gene expression, notably ATM. Indeed, ATM is a key protein in the signal transduction pathways
382 that detects DNA damage and controls several cellular responses, like recruiting DNA repair machinery
383 (Tanaka et al., 2007). In the samples showing higher level of DNA damage, genes involved in DNA repair are
384 strongly upregulated (more than 10-fold increase in some treatments). Until now, there has been limited
385 literature focusing on DNA repair gene expression following PM exposure, and to the authors' knowledge,
386 none have investigated the impact of PM generated from different aviation fuels. In this study, the expression
387 of genes involved in DNA repair was found highly upregulated after 24 h following the nebulisation with PM.

388 GADD45 α is also involved in the response to environmental genotoxicant-induced stress (Higashi et al., 2006;
389 Rossner et al., 2015) and the results presented in this research showed that it is even more susceptible than
390 ATM in Calu-3 cells (mono and co-cultures) exposed to primary PM. The high level of aromatic compounds
391 contained in the fuels could be also responsible for the activation of the gene MUC5AC, which is one of the
392 major lung mucus component (Juarez-Facio et al., 2021; Leclercq et al., 2016; Sotty et al., 2019). Following
393 the exposure to primary PM, the gene was generally upregulated in the samples obtained from fuels with high
394 level of aromatic compounds with the CAST generator. The upregulation of this gene would probably provide
395 a defence of the cells exposed to the particles since it is well known that the mucus clearance system is the
396 dominant mechanical host defence system of the human lung (Hill et al., 2022). Additionally, previous studies
397 identified differences in gene expression in oxidative stress related genes (HMOX and NQO1) following the
398 treatment with Jet-A and HEFA fuels (Gualtieri et al., 2022; Jonsdottir et al., 2019). However, in the present
399 study no modulation of these genes was observed, and this is consistent with the results observed in the comet
400 assay modified with enzymes, which did not show increased oxidation at of DNA bases. Although the analysis
401 of IL-8 secretion was not conclusive, exposure to PM collected from aviation FCE can induce the secretion of
402 IL-8 in most of the samples, and for this reason further investigation on the secretion of other inflammatory
403 mediators should be more considered.

404 Generally, our study found that toxicity did not appear to correlate with the different cell deposition dose of
405 the tested primary PM or with any varied fuel properties. Instead, the toxicity indicators investigated in this
406 study were more generally exacerbated by the larger, more fractal primary PM with higher organic carbon
407 content for CAST compared with RQL and/or aircraft engine (Crayford, 2022).

408 **CONCLUSION**

409 This study assessed the toxicity of primary PM, comparable to regulatory nvPM, from two combustion
410 technologies and twelve aviation fuels using a standardised filter-collection approach. ALI exposure of Calu-
411 3 lung epithelial cells in monoculture and in co-culture with macrophages generally indicated lower toxicity
412 after exposure to primary PM samples generated from a RQL rig when compared to a CAST generator. Indeed,
413 most of the primary PM samples generated from the CAST generator specifically burning jet fuels with higher

414 aromatic content displayed enhanced genotoxicity even at the relatively low deposition doses achieved in this
415 study. This effect is attributed to the larger, more fractal primary PM with higher organic carbon content from
416 the CAST compared to combustor rigs and full gas turbine engines. It is noted that assessing toxicity from
417 indirect exposure of cells to primary PM requires extensive extraction and processing steps, which can alter
418 the physicochemical properties of the test material before toxicity assessment.

419 Overall, this study suggests that the development of strategies that result in lower fuel aromatic content, as
420 generally witnessed in SAF, may also lead to primary PM emissions that are not more toxic than conventional
421 jet fuel. It also indicates that primary PM toxicity is driven by wider factors than fuel composition. As such,
422 further work is required to substantiate the real-world toxicity of aircraft exhaust PM inclusive of secondary
423 PM emanating from numerous engine technologies across the power range burning conventional Jet-A and
424 SAF.

425 **CONFLICT OF INTEREST**

426 Authors declare no conflict of interest.

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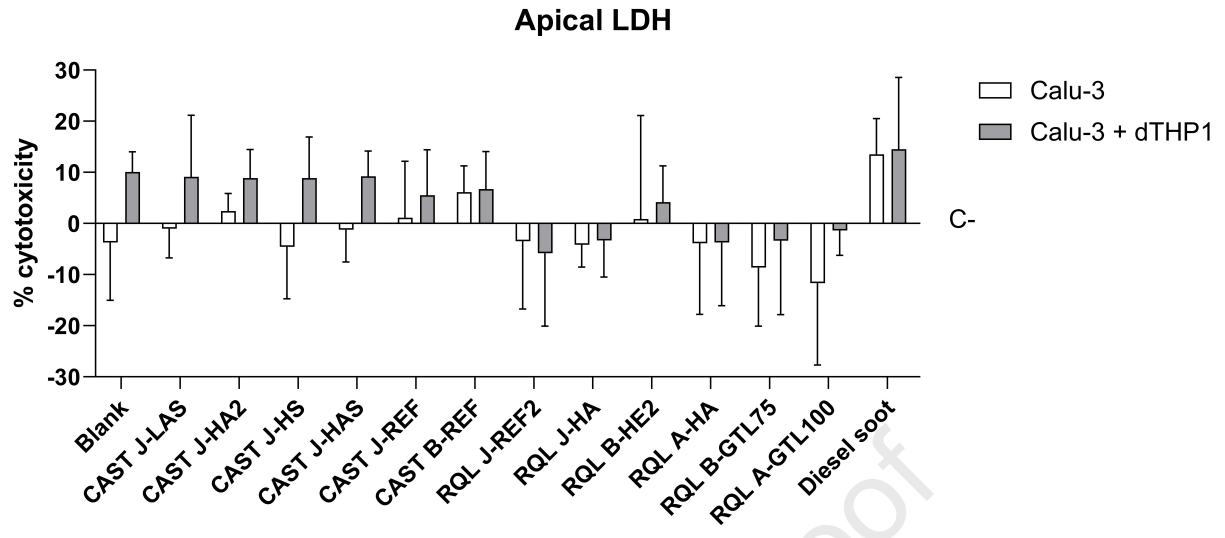
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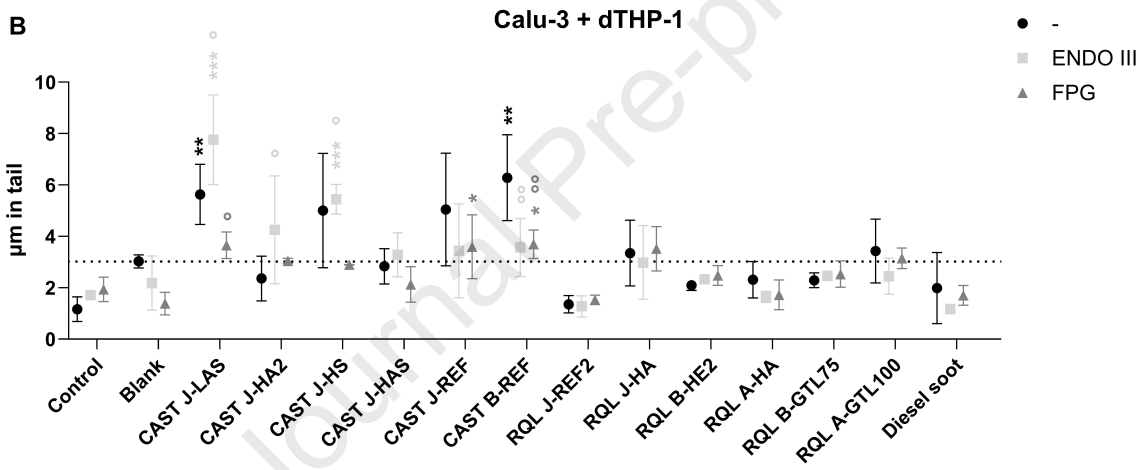
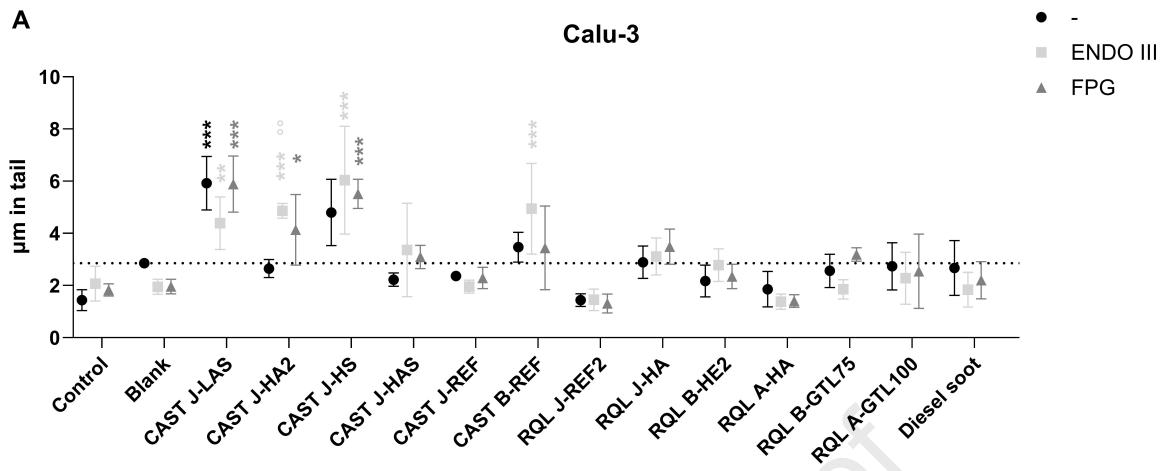
Figure 1. LDH release in Calu-3 monoculture and Calu-3 + dTHP-1 co-culture after 24 h of exposure to the PM resulting from combustion of different fuels. Supernatants were collected from the apical and basal compartments of the transwell inserts; only data from the apical compartment are shown, since analysis of the basal medium shown similar, albeit slightly lower LDH levels. Control is set at 0%. Data are expressed as mean \pm SD. Statistical analysis: Two-way ANOVA.

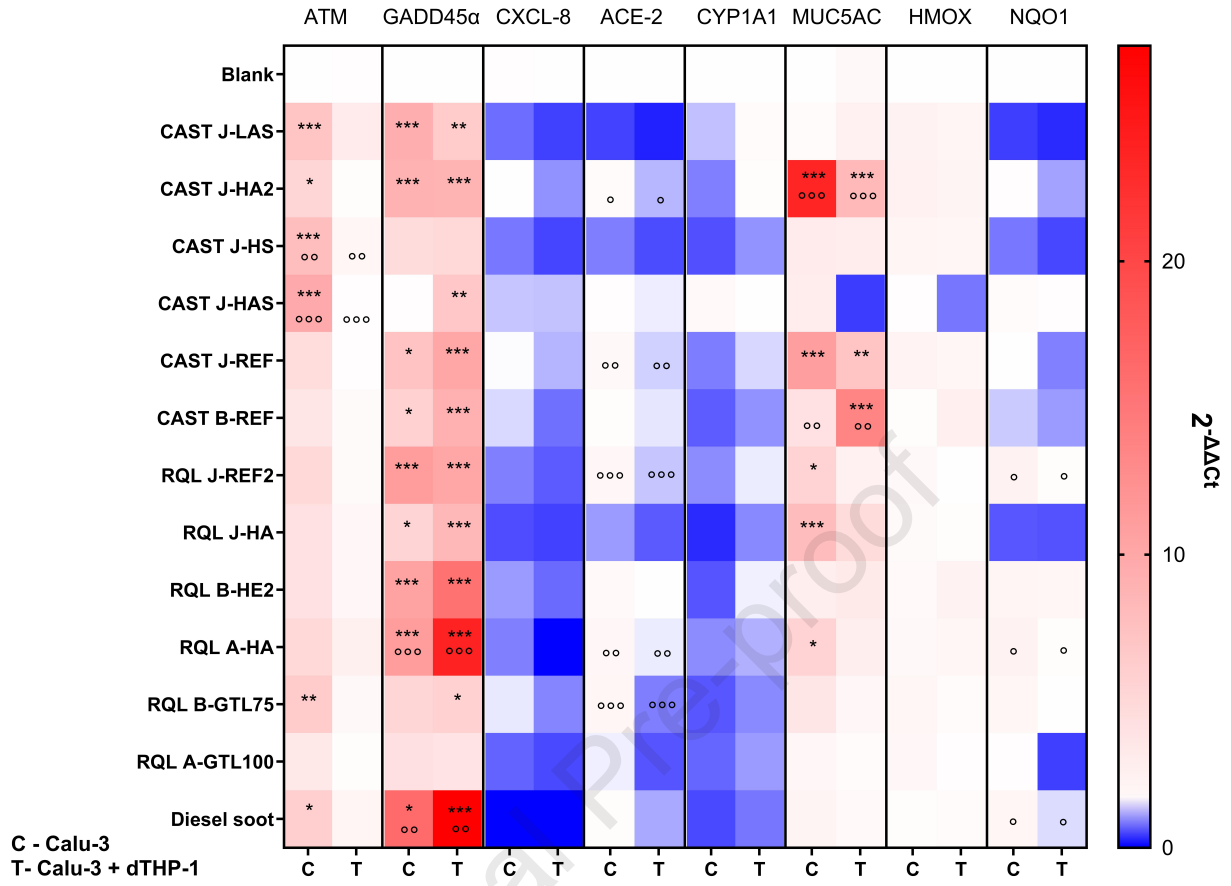
Figure 2. Evaluation of DNA damage after ALI exposure as assessed by comet assay modified with enzymes. Cells were collected after 24 h from the exposure. Data are expressed as μ m tail length of DNA detected in tails of the nuclei. Treatments were compared to the blank (represented by the dashed line – no enzymes). Results of the monoculture are shown in the graph **A** and of the co-culture in graph **B**. Data are expressed as mean \pm SD. Statistical analysis: Two-way ANOVA, followed by Dunnett's multiple comparison test. * p <0.05, ** p <0.01, *** p <0.001 vs. Blank (no enzymes); ** p <0.01, *** p <0.001 vs. Blank (ENDOIII); * p <0.05, ** p <0.01, *** p <0.001 vs. Blank (FPG), $^{\circ}$ p <0.05, $^{\circ\circ}$ p <0.01 vs. same sample without enzyme (-).

Figure 3. Heat map analysis of the gene expression analysis of Calu-3 monoculture and Calu-3+dTHP-1 co-culture treated with PM from FCE. Cells were collected after 24 h from the exposure. Data are expressed as $2^{-\Delta\Delta C_t}$. The monoculture is reported as C (Calu-3), while the co-culture as T (Calu-3 + dTHP-1) on the x-axis. Fuels' treatments reported in the y-axis were compared to the blank (first row). On the x-axis are reported the genes evaluated (on the top of the heat map). The colour gradient indicates the expression of the gene. Upregulated genes in red, downregulated genes in blue. The statistical analysis was performed between the samples and the blank and to compare the sample of the monoculture and the samples of the co-culture. Statistical analysis: Two-way ANOVA, followed by Dunnett's multiple comparison test (fuel sample vs. blank) and Šidák's multiple comparison test (monoculture vs. co-culture and vice versa). * p <0.05, ** p <0.01, *** p <0.001 vs. blank; $^{\circ}$ p <0.05, $^{\circ\circ}$ p <0.01, $^{\circ\circ\circ}$ p <0.001 monoculture (C) vs. co-culture (T) and *vice versa*.

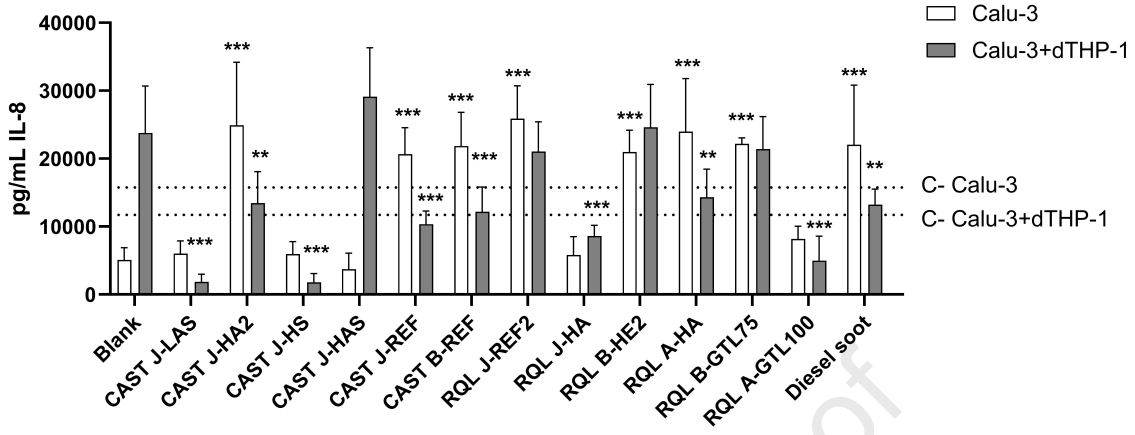
Figure 4. Interleukin-8 secretion of Calu-3 and Calu-3 + dTHP-1 co-culture after 24 h exposure to the different FCE. Medium was collected from the apical and basal compartments of the transwell, and the results are reported in graph **A** and **B**, respectively. Dashed lines are representing the basal control, cell not exposed to FCE or blank. Data are expressed as pg/mL and reported as mean \pm SD. Statistical analysis: Two-way ANOVA, Dunnett's Multiple Comparison test. * p <0.05, ** p <0.01, *** p <0.001 vs. Blank (Calu-3 or Calu-3 + dTHP-1 respectively).



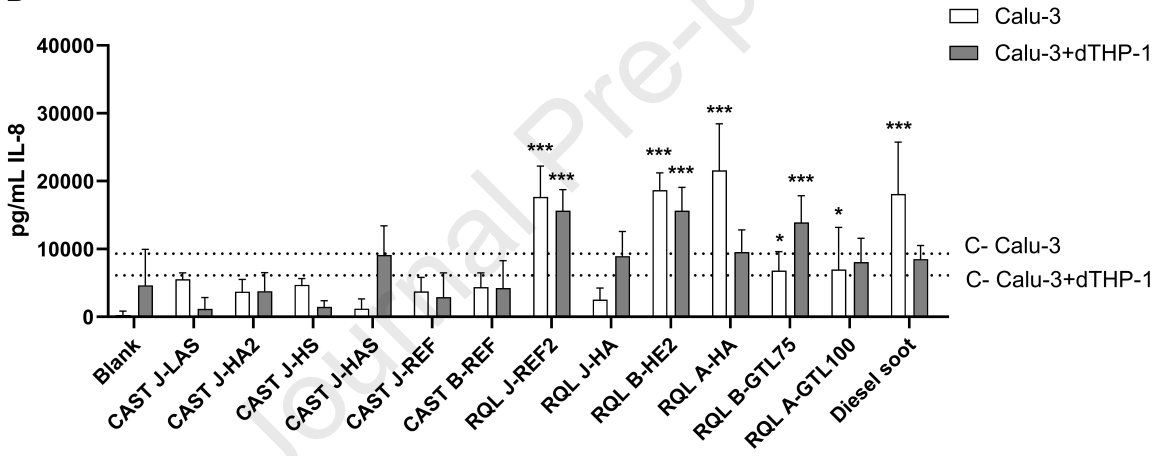




A



B



Human lung cells were exposed to primary PM from combustion of aviation fuels.

Toxicity depended on combustion technology but did not correlate with fuel properties.

Genotoxicity induced even at the relatively low PM deposition doses.

Strategies of lowering aromatic content may result in less harmful PM emissions.

Journal Pre-proof

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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