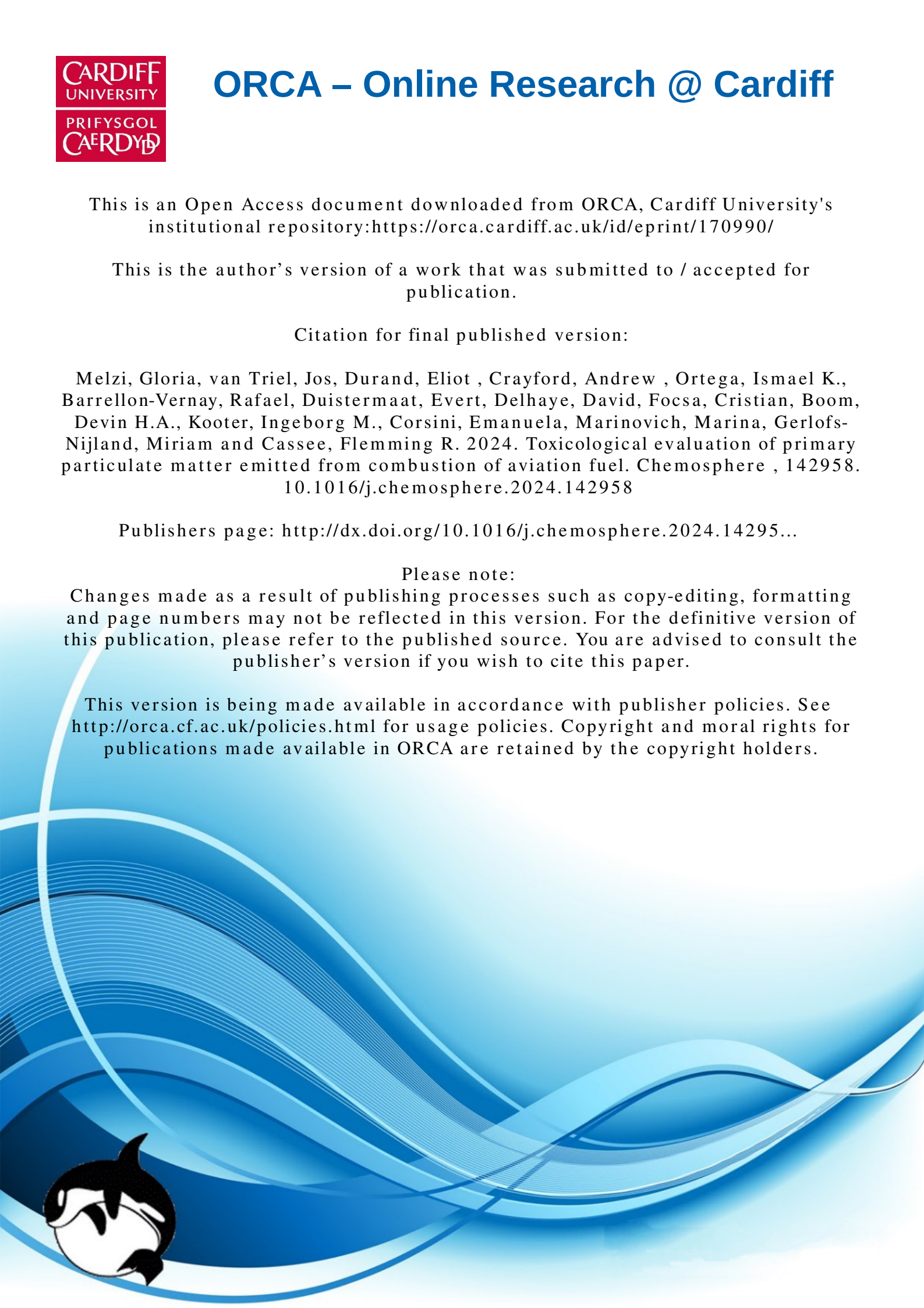

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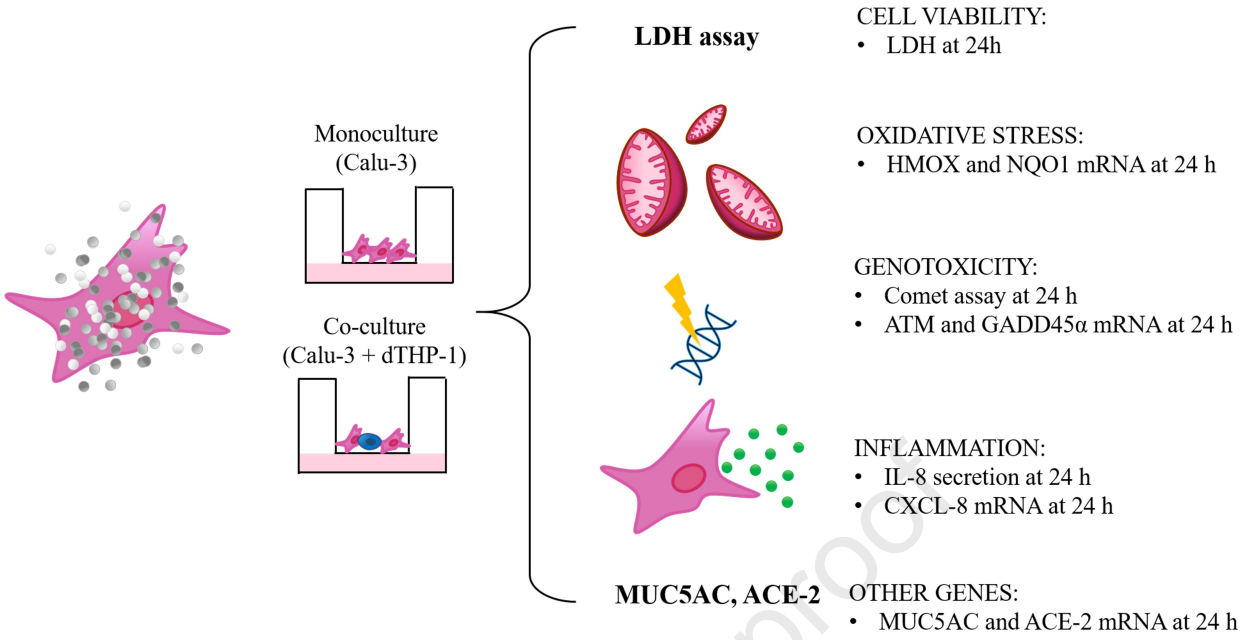

Toxicological evaluation of primary particulate matter emitted from combustion of aviation fuel

Authors: Gloria Melzi [1], Jos van Triel [2], Eliot Durand [3], Andrew Crayford [3], Ismael K. Ortega [4], Rafael Barrellon-Vernay [4,5], Evert Duistermaat [2], David Delhaye [4], Cristian Focsa [5], Devin H.A. Boom [6], Ingeborg M. Kooter [6, 7], Emanuela Corsini [1], Marina Marinovich [1], Miriam Gerlofs-Nijland [2], Flemming R. Cassee [2,8]

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ABSTRACT

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

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

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

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

INTRODUCTION

Increasing levels of air pollution are detrimental to human health, correlating with enhanced cardio-pulmonary mortality and lung cancer (Pope III et al., 2002). Particulate Matter (PM) is one of the most abundant pollutants, associated with combustion, and considered to impact human health significantly. PM is composed of a complex and heterogeneous mixture of solid and liquid particles suspended in the atmosphere which evolves as a result of cooling and chemical reactions occurring between pollutants from other natural and anthropogenic sources. The source of emission influences the size, shape, and composition of the particles (Perrone et al., 2013). Inorganic elements (i.e., metals) and ions (e.g., ammonium, sulphate, and nitrate), mineral dust, elemental and organic carbon, and aromatic compounds are the main components of PM (Rönkkö et al., 2018). Guidelines and standards exist to limit the emission of several airborne pollutants such as nitrogen oxides (NO_X), carbon oxide (CO), unburned hydrocarbons (UHC), ozone, and PM. In 2021, the WHO recommended lowering the PM2.5 air quality annual guideline level from 10 to 5 $\mu g/m^3$ to reflect new evidence of detrimental impacts at low levels of exposure (World Health Organization, 2021).

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

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

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

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

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

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

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

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

MATERIAL AND METHODS

Filter collection

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

Filter samples and fuel properties

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

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

Samples	**Aromatic content (%wt)**	**Napththalene (di-aromatics) (%wt)**	**Sulphur content (ppm)**	**Hydrogen content (%wt)**
Measurement method	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%*

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

PM extraction from filters

The PM samples on the different filters were individually extracted in methanol (Haleyur et al., 2016; Happo 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.

Cell culture

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.

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

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

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

Air-liquid interface exposure

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

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

Table 2. Deposited dose expressed in ng/cm^2 for each nebulization of the PM samples (data are expressed as mean ± SEM). The expected deposition is around 450 ng/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

Lactate Dehydrogenase assay

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

Comet assay modified with enzymes (ENDO III and FPG)

The analysis of the DNA damage was performed after 24 h from the exposure to the nebulised PM samples. Cells were washed with 0.05% of PBS-EDTA and detached with 0.05% trypsin-EDTA (Gibco) for 5 min. Cells were collected in medium and centrifuged at g-force of 150 for 5 min.

Pellets were resuspended in 100 µL of MEM. The assay was performed following the Trevigen kit instructions. Cells were mixed with low melting agarose (1 : 3 – cells : agarose), seeded on microscope slides and allowed to solidify at 4 °C. Three slides were prepared for each sample and treated with the different enzymes (Endonuclease III – ENDO III, and Formamidopyrimidine DNA Glycosylase – FPG). Slides were submerged with lysis buffer (Trevigen) for 30 min at 4 °C and then incubated with the enzymes ENDOIII and FPG for 45 min at 37 °C. Slides were submerged in unwinding buffer for 30 min at 4 °C. Electrophoresis was then performed for 20 min at 300 mA constant and 25 V. At the end, slides were dehydrated through incubation with cold ethanol for 5 min. Once dry, slides were stained with SYBR Gold (Thermo Fisher Scientific Inc.) solution and stored at 4 °C in the dark. Slides were analysed with Comet IV, magnification 10X, reading and analysing 100 cells for each sample to obtain the value of tail moment (TM) used for the quantification of DNA damage.

Gene expression

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

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

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

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

Inflammatory response

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

Statistical analysis

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

RESULTS

Transepithelial electrical resistance (TEER)

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

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

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

Lactate Dehydrogenase assay

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

Comet assay

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

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

Gene expression

Gene expression analysis was performed in samples collected after 24 h from the exposure. Genes involved in oxidative stress response (HMOX and NQO1), DNA repair (ATM and GADD45α), inflammation (CXCL-8), and protection mechanisms (ACE-2 and MUC5AC) were evaluated. Results are shown in **Figure 3**.

There is a trend of upregulation for the genes ATM, GADD45α, and MUC5AC following the treatment with the FCE PM samples, and of downregulation with the other genes. Statistically significant differences in ATM expression were detected in the monoculture following the treatment with CAST J-LAS, CAST J-HA2, CAST J-HS, CAST J-HAS, RQL B-GTL75, and with the Diesel soot. The increase of the expression of GADD45α was detected in all the samples in a statistically significant manner, both in the monoculture and the co-culture, with the only exception of the samples treated with CAST J-HS and RQL A-GTL100. Also, for MUC5AC the statistical analysis showed significant increase of expression following the treatment with CAST J-HA2, CAST B-REF, RQL J-REF2, RQL J-HA, and RQL A-HA for the monoculture, and for and CAST J-REF also in the co-culture. Statistically different behaviour of the monoculture and the co-culture was detected for ATM following the treatment with CAST J-HS and CAST J-HAS; for GADD45α following RQL A-HA and Diesel soot; for ACE-2 following CAST J-HA2, CAST J-REF, RQL J-REF2, RQL A-HA, RQL B-GTL75; for MUC5AC following CAST J-HA2 and CAST B-REF; and for NQO1 following RQL J-REF2, RQL A-HA, and diesel soot treatments.

Pro-inflammatory cytokines

The secretion of two cytokines was used as an indicator for a pro-inflammatory response: IL-8 and TNF-α. Data for TNF-α are not reported here because all results were below the limit of detection (15.6 pg/mL). The 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.

DISCUSSION

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

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

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

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

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

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

GADD45α is also involved in the response to environmental genotoxicant-induced stress (Higashi et al., 2006; Rossner et al., 2015) and the results presented in this research showed that it is even more susceptible than ATM in Calu-3 cells (mono and co-cultures) exposed to primary PM. The high level of aromatic compounds contained in the fuels could be also responsible for the activation of the gene MUC5AC, which is one of the major lung mucus component (Juarez-Facio et al., 2021; Leclercq et al., 2016; Sotty et al., 2019). Following the exposure to primary PM, the gene was generally upregulated in the samples obtained from fuels with high level of aromatic compounds with the CAST generator. The upregulation of this gene would probably provide a defence of the cells exposed to the particles since it is well known that the mucus clearance system is the dominant mechanical host defence system of the human lung (Hill et al., 2022). Additionally, previous studies identified differences in gene expression in oxidative stress related genes (HMOX and NQO1) following the treatment with Jet-A and HEFA fuels (Gualtieri et al., 2022; Jonsdottir et al., 2019). However, in the present study no modulation of these genes was observed, and this is consistent with the results observed in the comet assay modified with enzymes, which did not show increased oxidation at of DNA bases. Although the analysis of IL-8 secretion was not conclusive, exposure to PM collected from aviation FCE can induce the secretion of IL-8 in most of the samples, and for this reason further investigation on the secretion of other inflammatory mediators should be more considered.

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

CONCLUSION

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

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

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

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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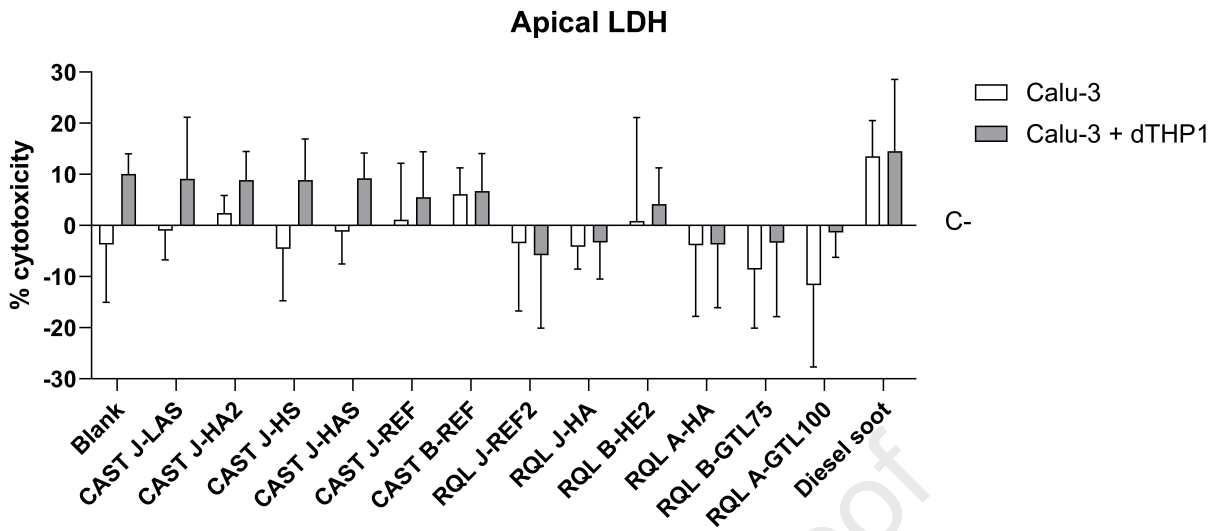
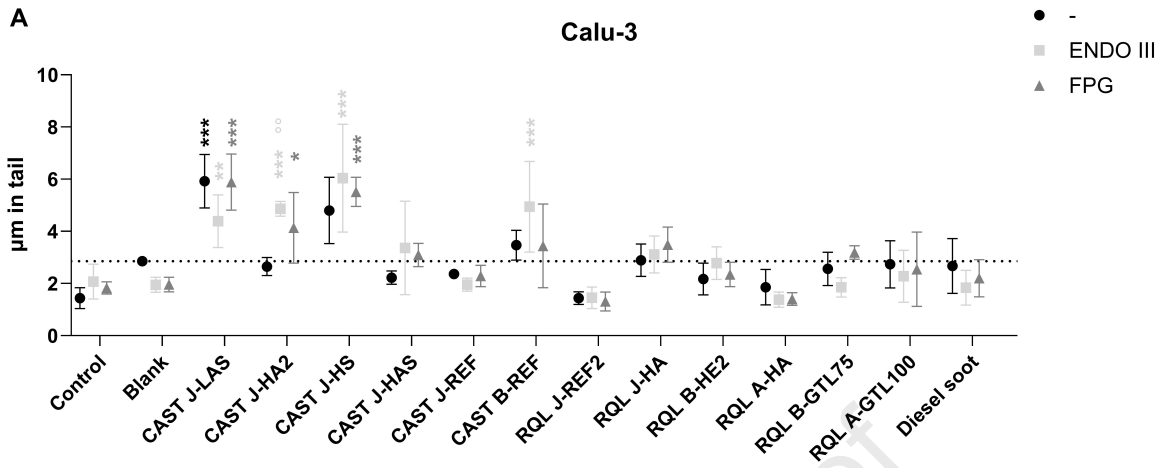
Figure 1. LDH release in Calu-3 monoculture and Calu-3 + dTHP-1 co-culture after 24 h of exposure to the PM resulting from combustion of different fuels. Supernatants were collected from the apical and basal compartments of the transwell inserts; only data from the apical compartment are shown, since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

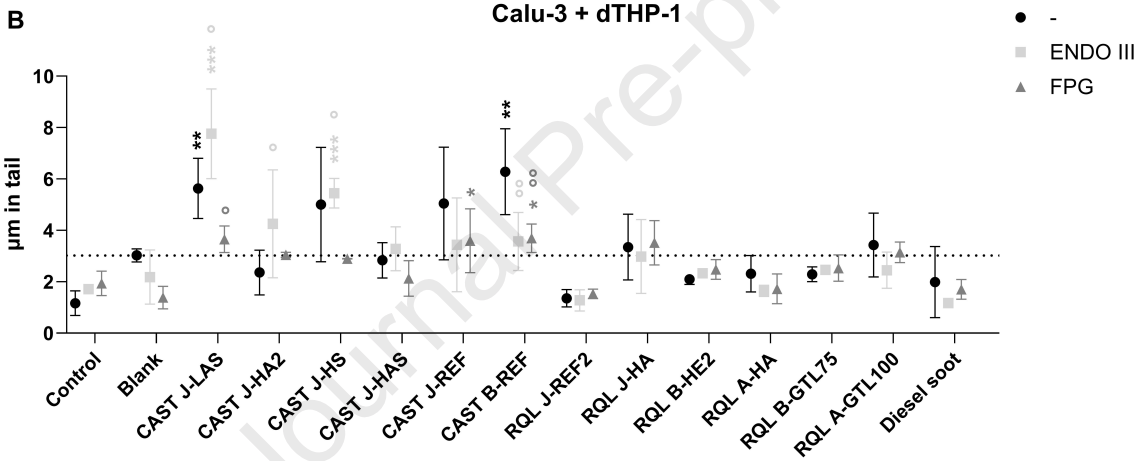
Figure 2. Evaluation of DNA damage after ALI exposure as assessed by comet assay modified with enzymes. Cells were collected after 24 h from the exposure. Data are expressed as μm tail length of DNA detected in tails of the nuclei. Treatments were compared to the blank (represented by the dashed line – 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 ± 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), °p<0.05, °°p<0.01 *vs.* same sample without enzyme (-).

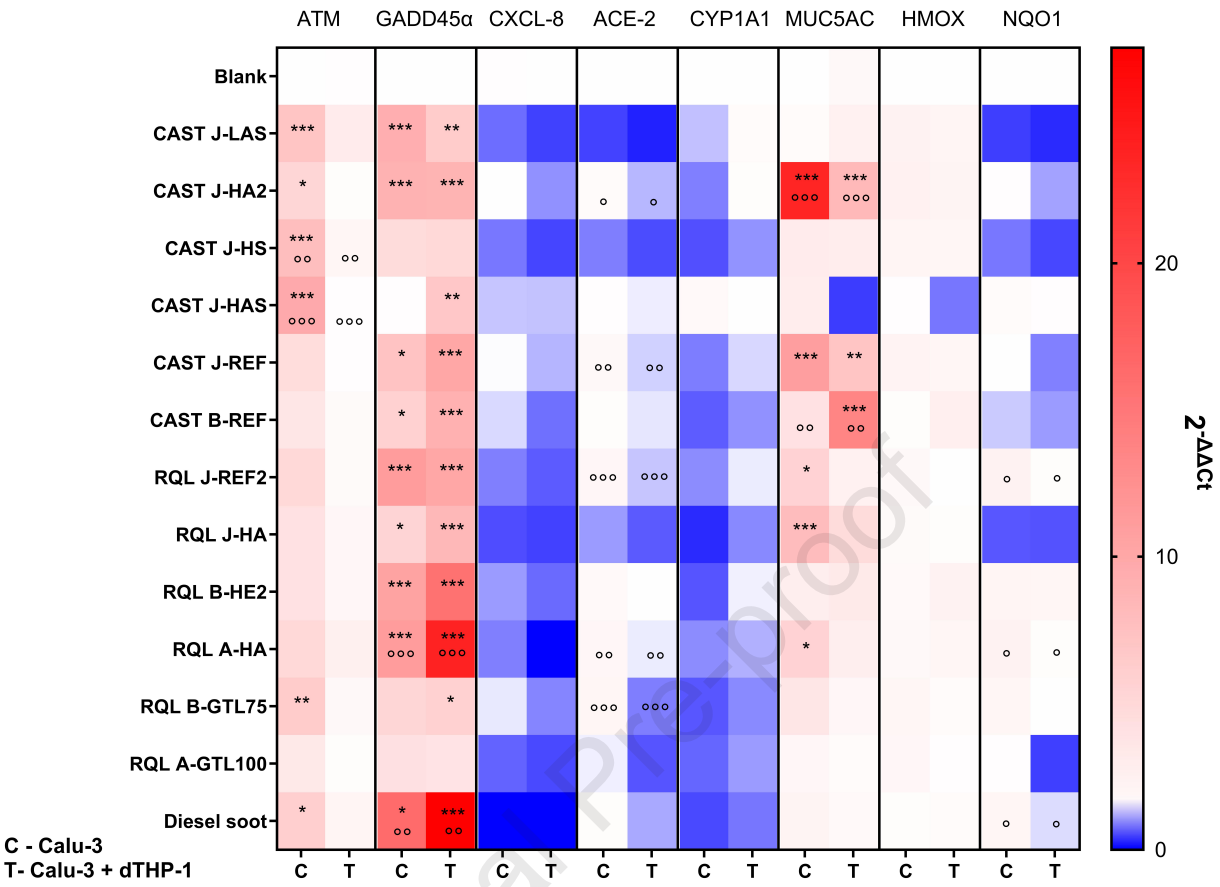
Figure 3. Heat map analysis of the gene expression analysis of Calu-3 monoculture and Calu-3+dTHP-1 co-culture treated with PM from FCE. Cells were collected after 24 h from the exposure. Data are expressed as $2^{-\Delta\Delta Ct}$. The monoculture is reported as C (Calu-3), while the co-culture as 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; °p<0.05, °°p<0.01, °°°p<0.001 monoculture (C) *vs.* co-culture (T) and *vice versa*.

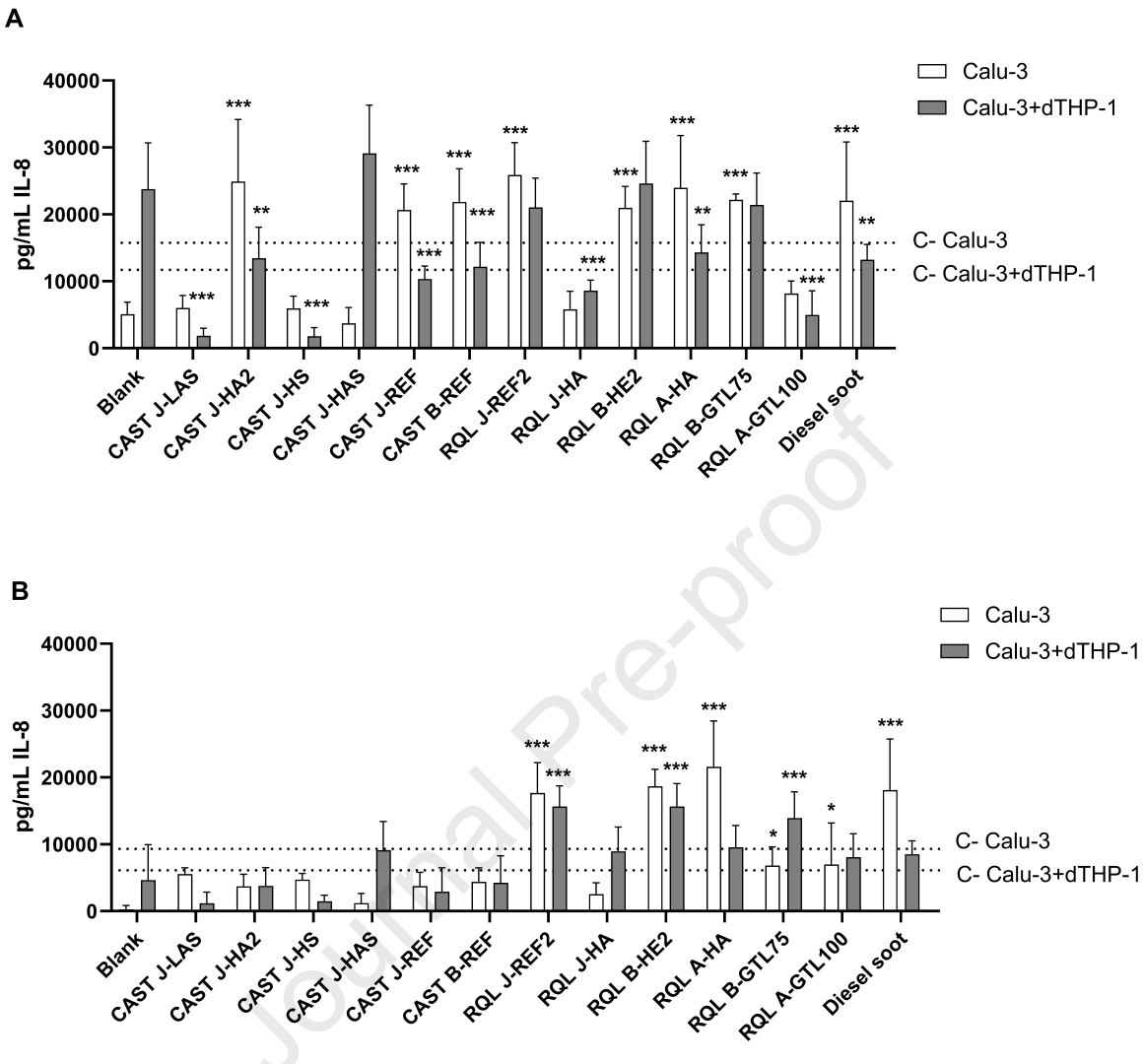
Figure 4. Interleukin-8 secretion of Calu-3 and Calu-3 + dTHP-1 co-culture after 24 h exposure to the different FCE. Medium was collected from the apical and basal compartments of the transwell, and the results are reported in graph **A** and **B,** respectively. Dashed lines are representing the basal control, cell not exposed to FCE or blank. Data are expressed as pg/mL and reported as mean ± 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).











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.

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: