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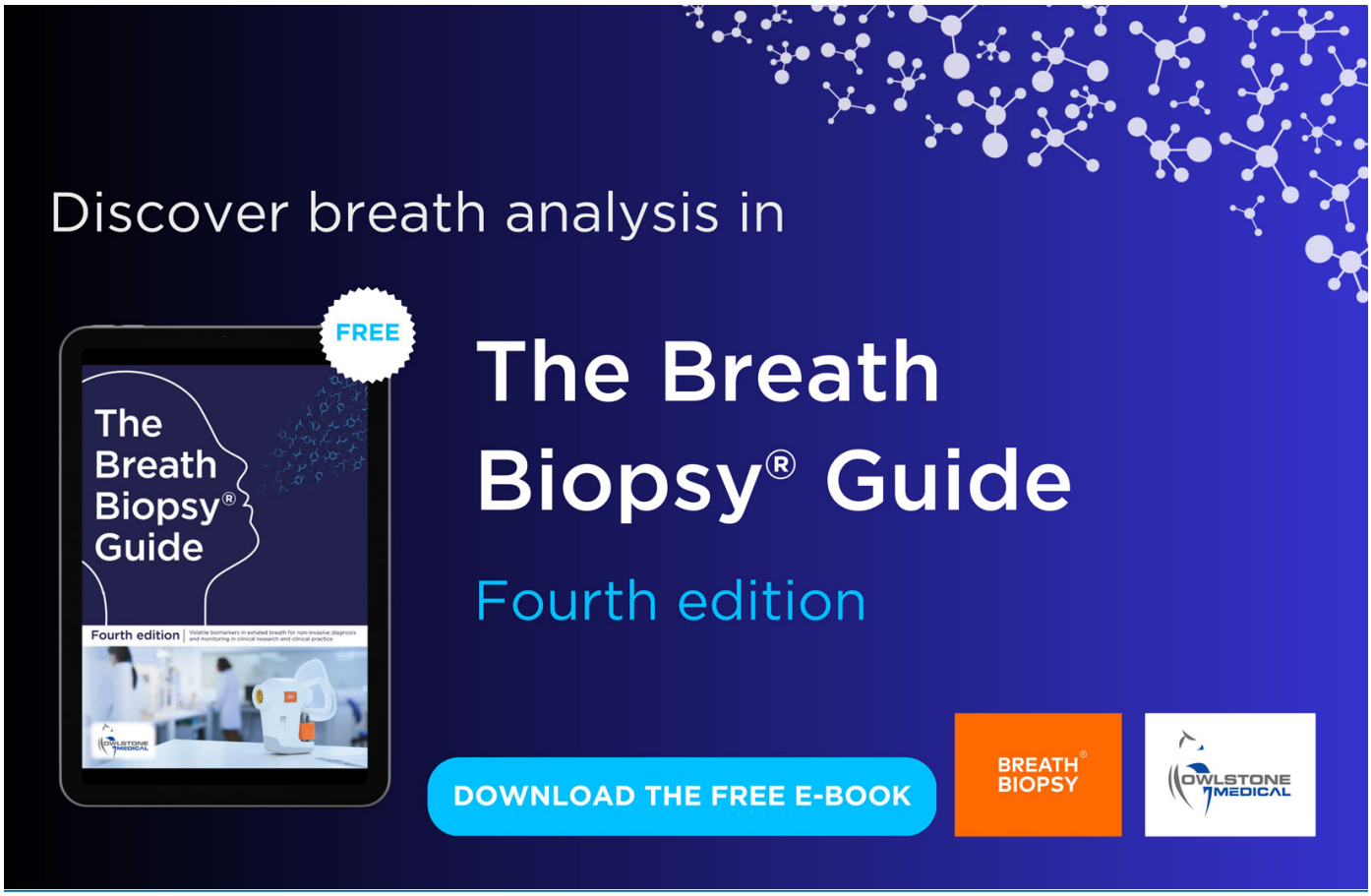

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NOTE

Chloroform release from ageing cells and *Drosophila DJ-1β* mutants

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Abstract

Volatile organic compounds (VOCs) offer potential for non-invasive diagnosis as biomarkers of disease and metabolism. In complex biological matrices, such as breath however, identifying useful biomarkers from hundreds, or even thousands of VOCs can be challenging. Models of disease, such as cellular or animal models, offer a means to elucidate VOC metabolisms, for accurate targeted studies in patient samples. Neurodegenerative conditions, such as Parkinson's have been associated with changed VOCs, offering a potential for early diagnostics and interventions improving treatments and outcomes for patients. Here, three separate models including; human HEK-293t cells, isolated primary rat glial cells, and *Drosophila* fruit flies (wild type and a mutant of the Parkinson's associated gene, *DJ-1β*) were grown for an extended period and levels of the VOC chloroform investigated using custom static headspace sampling chambers. Samples were analysed using targeted gas chromatography mass spectroscopy with selected ion monitoring mode, measuring chloroform at masses 83/85. Chloroform levels were shown to dramatically increase in all models over time. HEK-293t cells revealed a 60-fold increase after 10 weeks, glial cells revealed a 10-fold increase after 3–4 weeks and *DJ-1β* mutant flies revealed significant increases compared to control flies at 4 weeks. These results, taken together, suggest that chloroform release is related to ageing in these models and may provide a target for neurodegenerative studies. We present here the first evidence of chloroform being actively produced by human and rat cells and the first observation of volatile metabolisms in *Drosophila*. Recent clinical studies have also identified increased chloroform flux in the breath of patients, supporting the translational potential of our findings.

1. Introduction

Volatile organic compounds (VOCs) diffuse readily through cellular membranes and liquids and are released and consumed through cellular metabolism, offering potential non-invasive diagnosis [1, 2]. VOCs define odour and examples of smell being used for the diagnosis of neurodegenerative conditions have encouraged efforts for non-invasive diagnostic technologies [3, 4]. Despite significant advances in understanding of VOC mechanisms [5, 6] and technologies for diagnosis via VOCs [7], applications within the clinic are still limited [2, 3].

Neurodegenerative diseases, such as Parkinson's, are defined by a change in function of the neurological system. There are many causes of neurodegenerative disease [8] but early intervention can improve outcomes and therapeutic options for patients [8]. Because the neurological system is highly metabolically active [9], VOCs, as gaseous metabolites, offer an opportunity for non-invasive diagnosis.

A critical need to accelerate translation of this tool to the clinic is identification of biomarkers, linked to neurodegenerative conditions, which would allow targeted methodologies to simplify analysis of complex biological matrices, such as breath or blood.

VOCs from cells in culture have been shown to distinguish between tissue of origin and pathology [10], cell status [11], or response to stress [12, 13]. Recent breath flux studies have demonstrated that chloroform can be detected and measured in human breath, providing potential translational applications for biomarkers identified in model systems [14]. Cellular models of disease have been pivotal in medical research and animal models are powerful tools for modelling disease and key in translational medicine [15–17]. Taken together, cellular and animal models of disease are therefore useful tools through which we may identify target biomarkers for human studies, framing the purpose of this work.

In this study we investigated the release of chloroform ($CHCl_3$) in relation to ageing cells and flies as a result of a chance observation when profiling VOCs from HEK293t cells which were passaged (grown) for an extended period of time. We hypothesised that cells accumulate cellular products over time and proceeded to test if chloroform release in ageing cells was observed in terminally-differentiated cell cultures sourced from rat brain. Furthermore, we aimed to establish if this could be observed in a model of neurological dysfunction, such as Parkinson's, using *Drosophila melanogaster*. In Parkinson's disease, and a range of neurological conditions the clearing of cellular metabolic waste products is inhibited [18] which may be a source of biomarkers. Protein degylcase (*DJ-1β*) also known as PARK7 has been used as a model of neurological dysfunction in *Drosophila* as it is altered in Parkinson's disease [19]. *DJ-1β* protects against accumulation of reactive oxygen species (ROS), *DJ-1β* mutant flies have increased levels of ROS [20], and here we hypothesise that *DJ-1β* may present with elevated levels of chloroform over time in comparison to controls.

2. Methods

2.1. Cell culture and cellular isolation

HEK-293t were grown in Dulbecco's Modified Eagle Medium (DMEM, Thermo Scientific, Waltham, MA, USA), 25 mM glucose, supplemented with L-glutamine (4 mM) and 5% foetal bovine serum (Thermo Scientific, Waltham, MA, USA). Cells were passaged twice a week.

To initiate the volatile collection for HEK293t, cells were trypsinised and ∼500 000 cells were seeded into 8 mL complete media. Cells were then allowed to attach for 3 h, washed twice with warm PBS and an 8 mL treatment medium was applied. Volatile headspace sampling was performed 24 h later as previously described [10, 12].

2.2. Glial cell isolation and maintenance

Timed-mated female Wistar rats (Charles River UK) (RRID:RGD_737929) were maintained in accordance with the UK Animals (Scientific Procedures) Act (1986). Cortices were dissected from postnatal day 1 (P1) mixed sex rat pups. Animals were euthanized using pentobarbital injection followed by cervical dislocation, according to Home Office guidelines. Cortical cell suspensions were obtained as previously described [21] and cells were maintained in DMEM supplemented with 10% foetal bovine serum [22]. Media was refreshed once per week and glial identity was confirmed through monitoring the establishment of an astrocyte monolayer and the absence of neurons.

2.3. Fly strains and husbandry

Wild-type and transgenic strains were reared and maintained on standard yeast–agar–cornmeal medium [23] and kept at 25 °C for 12 h light/dark cycles. Wild-type, white-eyed flies (w^{-118}) and *DJ-1β*$^{\Delta 93}$ mutants [24] were maintained for the indicated times prior to analysis, with the flies flipped onto fresh food 1–2 times per week.

2.4. Static volatile headspace sampling

The custom static headspace sampling chambers used in this study have been described in detail with schematics and images in our previous publications [10, 12]. Briefly, these chambers consist of transparent acrylic tanks (25 l volume) with silicone gaskets and butyl seals. Prior to sampling, all chambers were thoroughly cleaned and tested for background chloroform levels.

For cell culture samples, 8 ml fresh medium was applied to cells in 10 cm petri dishes 24 h before sampling. Upon sampling initiation, 5 ml medium and petri dish lid was removed and dishes were placed in static chambers. Both flies and cell cultures in headspace chambers were placed on a rocker on the lowest setting (5 rpm) with lab air flowing through the chamber for 10 min at 750 ml per min. Chambers were then sealed and time point 0 samples taken.

T1 samples were then taken 2 h later for cells in culture and 4 h for flies. The 2 h sampling point for flies was insufficient for observation of adequate signal, possibly due to passive respiration. All samples were collected through pressure differential into pre-evacuated, electropolished, stainless steel canisters (500 ml volume, LabCommerce, San Jose, CA, United States). All canisters underwent a standardized cleaning procedure involving repeated evacuation and flushing with ultra-pure nitrogen prior to use, and blank analyses were performed to ensure no carryover between samples.

Media-only controls were analysed alongside all cell samples to account for any background chloroform present in culture media. These control measurements were subtracted from the cell sample measurements to isolate the cellular contribution to chloroform flux. Similarly, for fly experiments, empty chamber measurements were subtracted from chambers containing flies to determine fly-specific volatile flux. This subtraction approach ensures that the

reported flux values represent biological production or consumption rather than background volatiles from media or environmental sources.

2.5. Gas chromatography (GC)-mass spectroscopy (MS), calibration and peak analysis

Collected canister samples were transferred to a liquid nitrogen trap through pressure differential. The liquid nitrogen trap consisted of a stainless steel loop immersed in liquid nitrogen (−196 °C), capable of condensing volatile compounds with boiling points above approximately −190 °C. Pressure change between beginning and end of 'injection' was measured, allowing calculation of the moles of canister collected air injected. Sample in the trap was then transferred, via heated helium flow (250 °C for 3 min), to an Aglient/HP 5972 MSD system (Santa Clara, CA, United States) equipped with a PoraBond Q column (25 m × 0.32 mm × 0.5 μm film thickness; Restek©, Bellefonte, PN, United States).

Targeted samples were analysed in selected ion monitoring (SIM) mode with a dwell time of 50 ms per mass. The mass spectrometer was operated in electron impact ionisation mode with 70 eV ionisation energy, and transfer line, ion source, and quadrupole temperatures of 250, 280, and 280 °C, respectively. A known standard was used with masses 83/85 for identification of chloroform. The oven programme for SIM analysis was as follows: 35 °C for 2 min, 10 °C min^{-1} to 155 °C, 1 °C min^{-1} to 131 °C, and 25 °C min^{-1} to 250 °C with a 5 min 30 s hold.

Calibration was performed using standard gases (BOC Specialty Gases) at known concentrations for chloroform. Linear regression of calibration curves confirmed strong, positive linear relationships between observed compound peak areas and moles of gas injected ($r^2 > 0.9$). The method limit of detection for chloroform was 5 pptv, with a limit of quantification of 15 pptv, and method reproducibility of 5%–8% RSD.

All samples were analysed within 6 d of collection to prevent sample degradation. Flux values (pg/hr/μg) were calculated by normalizing to protein content for cellular samples or to fly numbers for Drosophila samples. All quantification was performed with reference to CFC-11 as an internal standard (240 pptv) to correct for any analytical variations.

2.6. Calculation of VOC flux

Volatile organic compound flux was calculated as previously described [10, 12]. Briefly, the difference in chloroform concentration between time points (T0 and T1) was determined for each sample. Media-only controls were analysed in parallel and the average flux values from media-only samples were subtracted from the cell-containing samples to account for any non-cellular contributions. For Drosophila experiments, empty chamber measurements were subtracted from chambers containing flies.

Concentrations were calculated using calibration curves relating peak areas to moles of compound. Sample VOC concentrations were normalized to CFC-11 concentrations (240 pptv) as an internal standard. To account for differences in cellular growth rates, results from GC-MS analysis were normalized to protein content determined by BCA assay for cellular samples, or to fly numbers for Drosophila samples.

Final flux values were expressed as pg/hr/μg protein for cellular samples and converted to comparable units for fly samples. Positive flux values indicate net production of chloroform, while negative values represent net consumption.

2.7. Western blotting

Thirty *Drosophila* heads were ground on dry ice and resuspended in 75 μl RIPA buffer with PMSF (0.5% v/v) and protease inhibitor (Sigma, P8340). Lysates were incubated on ice for 30 min and debris removed via centrifugation for 20 min at 13 000 RPM and 4 °C. Protein concentration was normalised to 2 μg^{-1} μl^{-1} using a Pierce BCA assay (Thermo ScientificTM, 23 225). 2X SDS Loading buffer (30% v/v glycerol, 4% w/v SDS, 160 mM Tris-HCl pH 6.8, 0.1% w/v Bromophenol Blue, 10 mM DTT) was added and samples were heated to 80 °C for 10 min. 15 μg of protein was loaded into pre-cast gels (NuPAGE, 4%–12% Bis-Tris Gels, Thermo) and samples were run in MOPS buffer (20X Bolt, Invitrogen) at 150 V for 80 min.

Wet transfer to PVDF membranes (Immobilon-P, Merck Millipore) was performed in a transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol, 0.005% w/v SDS) at 30 V and 4 °C overnight. Blots were blocked in 5% w/v skimmed milk powder in TBS-T (TBS with 0.1% Tween-20) for 2 h, followed by overnight incubation in primary antibodies. Blots were incubated at room temperature for 2 h with secondary HRP-conjugated antibodies. The concentrations and details of the primary and secondary antibodies used are shown in table S1. Methods for the generation of *DJ-1β* antibodies are detailed in supplementary material. Chemiluminescent HRP substrate (Immobilon, Merck Millipore) was added and chemiluminescence was detected using an iBright (Thermo ScientificTM).

2.8. Statistics

Statistical analysis and figure arrangement was performed in Graphpad Prism (v10). Student's t-test was performed for figures 1(A) and (B), one way ANOVA with Bonferroni post hoc analysis was performed for figure 1(D).

3. Results

HEK-293 t cells passaged over 20 times (10–15 weeks in culture) produced chloroform at significantly higher levels than cells below this age (figure 1(A),

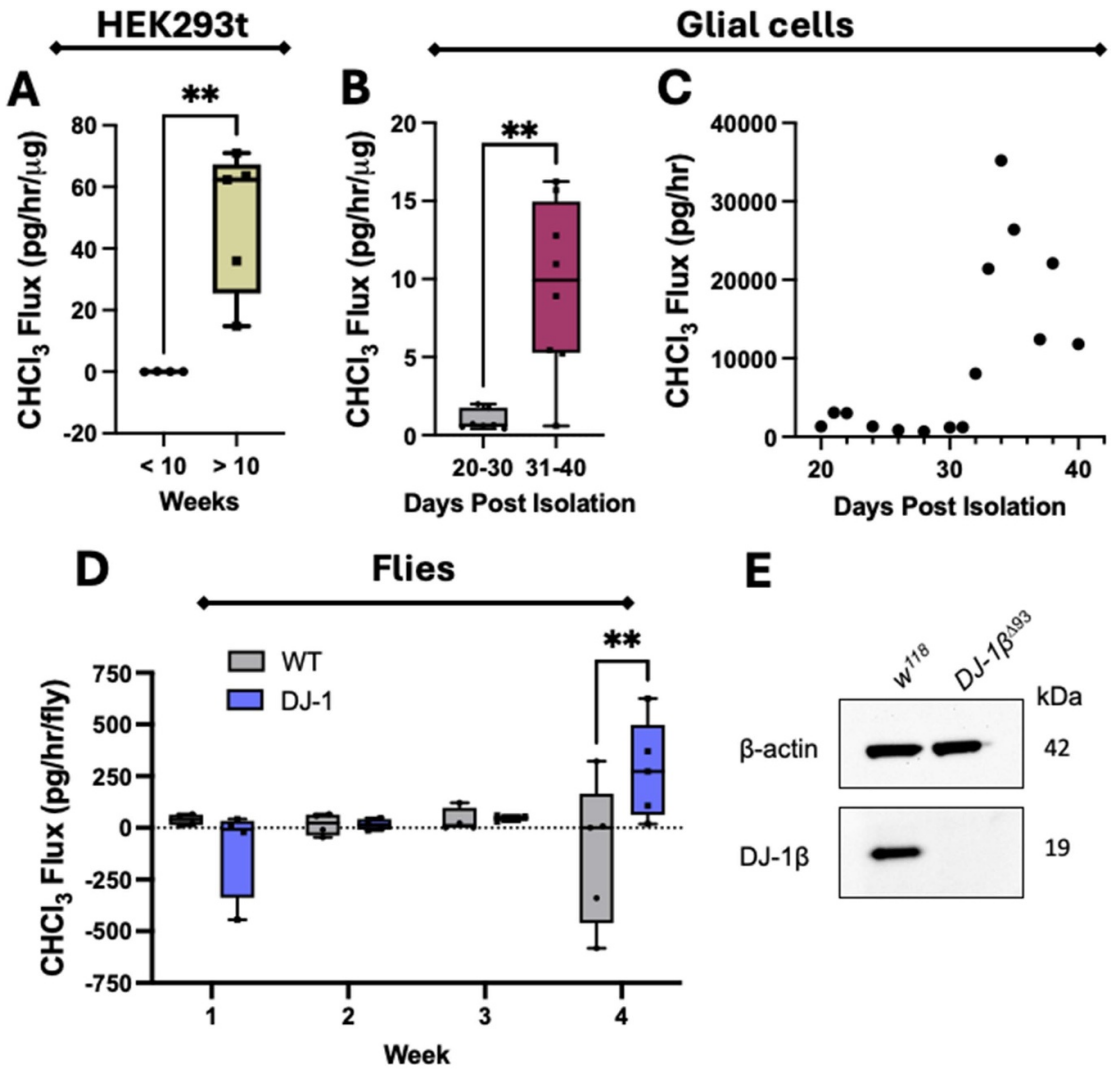

Figure 1. Chloroform (CHCl3) flux in aging HEK293t cells, rat glial cells and wild-type (WT) or DJ-1 mutant Drosophila melanogaster flies. (A) CHCl3 flux (pg CHCl3/hour/μg total protein), with media subtracted for human HEK-293 t cells below (<10, $n = 4$) or above (>10, $n = 5$) 10 weeks of growth (passages on average twice a week). (B) CHCl3 flux (pg CHCl3/hour/μg total protein), media subtracted for primary rat glial cells for days post isolation(20–30, $n = 7$; 31–40, $n = 8$). (C) CHCl3 flux (pg CHCl3/hour) for glial cells days post isolation. (D) CHCl3 flux (pg CHCl3/hour/per fly) of WT or *DJ-1β* mutant flies at 1, 2, 3 ($n = 4$) and 4 ($n = 5$) weeks of age. (E) Western blot of fly head lysates probed for *DJ-1β* and *β*-actin in wild type (w118) or *DJ-1β*Δ93 mutants. Students *t*-test performed for A and B, two-way ANOVA with Bonferroni post-hoc analysis performed for D; $^{**}p = 0.001$.

0.05 ± 0.046 pg hr^{-1} μg^{-1} for <10 weeks and 49.5 ± 23.5 pg hr^{-1} μg^{-1} for >10 weeks in culture).

Rat glial cell cultures were confirmed following isolation at day 20. Glial cells released chloroform with cells over 30 d in culture releasing significantly more chloroform than those between 20 and 30 d (figure 1(B), 0.95 ± 0.64 pg hr^{-1} μg^{-1} for 20–30 d and 9.47 ± 5.49 pg hr^{-1} μg^{-1} for 30–40 d). Release of chloroform in glial cells occurred at 33 d with slight increases occurring in cells between 30 and 32 d (figure 1(C)).

Over 4 weeks, control flies showed no significant alterations in chloroform flux whereas the *DJ-1β* mutants revealed significant production of chloroform at week 4 compared to wild type flies (figure 1(D), −118.22 ± 349.12 pg hr^{-1} μg^{-1} vs 278.72 ± 237.39 pg hr^{-1} μg^{-1}). Knockdown of *DJ-1β* was confirmed by western blot (figure 1(E)).

4. Discussion

We present here the first example of chloroform release from human cells, rat glial cells and the first documented example of volatile metabolisms from *Drosophila melanogaster*. We have previously investigated chloroform flux in HEK-293t and other cells in cultures, as well as the response of these cells to hypoxia and chemotherapeutic stress [10, 12]. Modulation of chloroform metabolism was not observed from between cell lines or under conditions of stress/starvation as well as

no active metabolism observed in the breath of mice [10].

The strikingly large amount of chloroform released by both HEK-293t and glial cells as they age (10–50 times greater flux) could be a result of accumulating chloride-containing compounds, potentially as cell waste, however this requires further investigation.

We observed release of chloroform from *DJ-1β* mutant flies, compared with control flies at 4 weeks. There was considerable loss of viability in *DJ-1β* flies into week 5 and we were unable to measure past 4 weeks (over 28 d) which would have allowed clearer comparison with glial cells. We did not identify this as a confounding factor as all flies showed normal behaviour upon measurement.

Few sources observe metabolic production of chloroform but it has been observed in fungi [25] and is persistent in the environment and drinking water [26], however this is likely driven by human pollution [27, 28]. All water and culture media used in this study were tested for background chloroform levels, which were found to be minimal and stable, confirming that the observed increases were due to biological production rather than environmental contamination. Cellular accumulation of chloride or chloride-containing compounds in culture could be a source of chloroform. In culture, glial cells, which are highly metabolically active, accumulate metabolic waste which would normally be cleared by macrophages and immune cells [26]. Furthermore, poor waste management is a hallmark of neurodegenerative conditions [26].

Carbon tetrachloride (CCl_4) has been demonstrated to be a source of chloroform through cytochrome P450-dependent metabolism of CCl_4 in hepatic microsomes [29], degradation in mouse liver [30] and aerobic transformation by poplar cells [31]. Although CCl_4 was not detected in our samples and would not have been seen using our SIM mode targeting chloroform, based on existing literature, accumulation of CCl_4 over time in our models could explain the observation of increased chloroform flux as neurons and glial cells are able to metabolise CCl_4 [32]. However, presence of CCl $\sim$ 4$\sim$ in our models requires further investigation and other chloride-containing molecules could be sources. Alternatively, there may be some other, as yet, undetermined source.

In conclusion, we have demonstrated the *positive* release of chloroform in ageing cells and *DJ-1β* mutant flies. Recent studies have also shown increased chloroform flux in the breath of patients [14], suggesting potential clinical relevance of our findings. Chloroform therefore presents as a potential biomarker to target in diseases associated with ageing, such as Parkinson's disease.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

Acknowledgment

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Ethical statement

This study was performed in accordance with the Declaration of Helsinki. Animal primary cell lines included in this study were approved as part of this study protocol. This animal study was approved by University of York Ethics Committee. Timed-mated female Wistar rats (Charles River UK) (RRID:RGD_737929) were maintained in accordance with the UK Animals (Scientific Procedures) Act (1986).

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