



Published in final edited form as:

J Breath Res. ; 13(3): 036014. doi:10.1088/1752-7163/ab1fc4.

Modeling cellular metabolomic effects of oxidative stress impacts from hydrogen peroxide and cigarette smoke on human lung epithelial cells

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Abstract

The respiratory system is continuously exposed to variety of biological and chemical irritants that contain reactive oxygen species, and these are well known to cause oxidative stress responses in lung epithelial cells. There is a clinical need to identify biomarkers of oxidative stress which could potentially support early indicators of disease and health management. To identify volatile biomarkers of oxidative stress, we analyzed the headspace above human bronchial epithelial cell cultures (HBE1) before and after hydrogen peroxide (H₂O₂) and cigarette smoke extract (CSE) exposure. Using stir bar and headspace sorptive extraction-gas chromatography-mass spectrometry, we searched for volatile organic compounds (VOC) of these oxidative measures. In the H₂O₂ cell peroxidation experiments, four different H₂O₂ concentrations (0.1, 0.5, 10, 50 mM) were applied to the HBE1 cells, and VOCs were collected every 12 h over the time course of 48 h. In the cigarette smoke extract cell peroxidation experiments, four different smoke extract concentrations (0, 10, 30, 60 %) were applied to the cells, and VOCs were collected every 12 h over the time course of 48 h. We used partial-least squares (PLS) analysis to identify putative compounds from the mass spectrometry results that highly correlated with the known applied oxidative stress. We observed chemical emissions from the cells that related to both the intensity of the oxidative stress and followed distinct time courses. Additionally, some of these chemicals are aldehydes, which are thought to be non-invasive indicators of oxidative stress in exhaled human breath. Together, these results illustrate a powerful *in situ* cell culture model of oxidative

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Conflict of interest disclosure

The authors have no conflicts to declare.

stress that can be used to explore the putative biological genesis of exhaled breath biomarkers that are often observed in human clinical studies.

Keywords

breath analysis; aldehydes; oxidative stress; peroxidation; mass spectrometry

Introduction

The lungs are continuously exposed to a variety of environmental chemicals and potential insults, ranging from particulate matter to potentially oxidizing compounds [1]. This includes subsets of reactive oxygen species (ROS) such as superoxide anions ($O_2^{\cdot -}$), alkoxy (RO^{\cdot}), alkylperoxy (ROO^{\cdot}) hydroxyl ($^{\cdot}OH$) free radicals and hydrogen peroxide (H_2O_2) [2]. Hydrogen peroxide is one of the most stable of the reactive oxygen species and tightly regulated under physiological conditions along with other ROSs. These endogenously produced ROSs play important roles in various biological processes under low concentration, such as intracellular signaling [3], respiration [4], immune cell activation and vascular remodeling in mammals [5, 6]. However, exogenously produced H_2O_2 exposes cells to high concentrations of ROS, and is implicated as an important mediator of cellular toxicity [7]. Previous studies have shown that oxidants like H_2O_2 cause oxidative stress in airway epithelial cells [2, 7, 8]. Oxidative stress, which refers to a state of elevated levels of ROS, occurs from a variety of conditions that produce unbalanced ROS production or a decline in antioxidant defenses [9]. These oxidants are reported to damage cell components including proteins, lipids, and nucleic acids [10–12]. Moreover, proteins can be modified by aldehydes and ketones produced during reactions of ROS with lipids [13–15].

One critically important source of exogenous H_2O_2 into the lung is cigarette smoke [2, 8, 16], a well-studied contributor that initiates development of many smoking-related diseases. It disrupts many biological processes partially by exposing the lung to ROS, including H_2O_2 in a complex smoke matrix [2, 8, 16–18]. Prior research has looked at many different cellular changes and damage that occurs due to cigarette smoke exposure [19–23], but very little is known about the cellular level volatile organic compound (VOC) metabolite emissions that result from this exposure.

Oxidative stress is implicated with host immune-inflammatory processes and infectious diseases as well [24, 25]. In earlier work, we cultured human B-cells *in situ* and monitored VOCs that were released before and after infection with various influenza virus strains [26, 27]. We observed several biomarkers (alcohols, ketones, aldehydes, and other aromatic compounds) that were produced after virus infection, and some of these were specific to the infecting strain of virus. Some of the VOCs were emitted over specific time courses, suggesting that viral infection stimulated certain cell pathways as a mechanism of action. A key finding was oxidative stress markers that was present in all virus infections, and the abundance was correlated to the multiplicity of infection (MOI) used to infect the cells. Similar generic oxidative stress biomarkers were also observed by Phillips, *et al.* after influenza vaccines were administered to normal healthy subjects [28]. In this latter

case, a live attenuated vaccine was used to stimulate the immune response, which may have contributed to minor variations in the specific oxidative stress biomarker signals measured. Ahmed *et al.* observed significant upregulation of heptanol, propanal and benzeneacetaldehyde active in Crohn's disease patients [29], and those VOCs may have been produced during the inflammatory processes as a result of lipid peroxidation and oxidative stress [30]. However, the detailed pathway and response mechanism that produced these exact VOC biomarkers at the cellular level is still unclear. Other researchers have replicated some of these findings from *in situ* cell culture studies [31, 32] and *in vivo* animal models [33]. We seek to further define a cell-based assay to elucidate some of these mechanisms that translate between cell damage and VOC release.

In the field of breath analysis, we exploit both volatile and non-volatile exhaled biomarkers when they associate with disease or health states. These breath biomarkers are thought to be endogenously produced and partitioned into the breath through two potential processes: diffusion equilibrium between blood and exhaled respiratory gasses in the capillary bed compartment of the lung (with the origin of the biomarker systemically elsewhere in the body) and generation originating directly from the airway epithelial cell surfaces/ in the lung itself. We believe both pathways are probably active, and our present work aims to reveal the potential oxidative stress markers that can be generated directly from lung cells themselves. Given that the airway epithelium is the first line of defense in the lung against agents such as cigarette smoke, this paper investigates the effects of H₂O₂ and cigarette smoke directly on airway epithelial cells.

Material and Methods

We previously published an initial protocol paper with details of our experimental procedure for growing an immortalized line of human bronchial epithelial cells and the process of monitoring emitted volatile organic compounds [34]. These are briefly described again below, along with new details on the protocols we developed for the H₂O₂ and smoke extract oxidative stress assays. Research Cigarettes (3R4F) were purchased from the Tobacco Research Institute (University of Kentucky, Kentucky, USA).

Cell Culture Conditions

Immortalized human bronchial epithelial HBE1 cells [35] were plated on Transwell (Corning Costar, Corning, NY) 12 mm chambers at $1-2 \times 10^4$ cells/cm² in LHC Basal Medium (Life Technologies, Carlsbad, CA) supplemented with: insulin (5 µg/mL), transferrin (10 µg/mL), epidermal growth factor (24 ng/mL), hydrocortisone (0.1 µM), triiodothyronine (0.01 µM), bovine hypothalamus extract (10 µg/mL), bovine serum albumin (0.5 mg/mL), epinephrine (0.6 µg/mL), phosphorylethanolamine (0.5 µM), ethanolamine (0.5 µM), zinc sulfate (3 µM), ferrous sulfate (1.5 µM), magnesium chloride (0.6 mM), calcium chloride (0.11 mM) and trace elements (selenium, manganese, silicone, molybdenum, vanadium, nickel sulfate and tin) [36]. All additives were purchased from Sigma Aldrich (St. Louis, MO). Confluent HBE1 cultures were transferred to ALI culture conditions in Pneumacult ALI medium (StemCell Technologies, Vancouver, BC) for 5–7 days before experiments began.

Cell metabolic activity was quantified using an alamarBlue[®] cell viability reagent per manufacturer recommendations (ThermoFisher) to ensure cells were alive and viable for measurements.

VOC Extraction Protocol

We utilized the same VOC extraction protocol previously published [34], and is briefly described here. Confluent cells were placed into sterile borosilicate glass jars and capped, with the apical and basolateral surfaces bathed in their appropriate media, Opti-MEM (Life Technologies, Carlsbad, CA). The internal standard dactone-22 was dissolved at 1 μ L of standard solution per 5 mL of cell media. Twisters[®] (Part 011222-001-00, Gerstel, Inc., Linthicum, MD) were conditioned and sterilized in 70% aqueous ethanol prior to introduction to cultures, and sampling was achieved using the magnetized surface of the jar lids at 37 °C. Experiments were started at $t=0$ h. Twelve hours extractions were removed at $t=12$ h, etc. Cells were confluent at $t=0$ but were also undergoing normal senescence over the duration of each experiment. During the sampling period, the lids were left ajar for 3 min every 12 h to allow for normal cellular respiration.

Hydrogen Peroxide Oxidative Stress Assay

Concentrated H₂O₂ stock solution (30%, EMD Millipore) was diluted into Opti-MEM medium to concentrations of 0.1, 0.5, 10, and 50 mM H₂O₂. Monolayer cultures in transwells were exposed to the various H₂O₂ concentrations to measure VOC dose responses over timepoints of 12, 24, 36 and 48 hours at 37°C. (Figure 1A). Cell VOCs were collected with Twisters[®] every 12 h until 48 h elapsed (Figure 1B). Two medium-only controls were used: Opti-MEM medium and Opti-medium with H₂O₂. Two cell culture treatments were also used: cell cultures with Opti-medium only and cell culture with Opti-medium with H₂O₂. Jar blank samples were also collected at each time point to provide background controls. Each treatment included two jar biological replicates and two Twister[®] technical replicates. The cell viability assay was conducted after 24 h in each concentration of H₂O₂ exposure, and was measured three times among three transwells.

Cigarette Smoke Extract (CSE) Oxidative Stress Assay

Researchers have previously reported methods to generate cigarette smoke extract (CSE) medium that can be used to expose cell cultures [37, 38], and we adopted our method from these prior studies. Cigarette smoke extract (CSE) was prepared daily in a custom designed smoking apparatus. Briefly, a cigarette was attached to a 3-way stopcock attached to a 60 mL syringe containing 6 mL of Opti-medium, once the cigarette was lit, mainstream smoke was withdrawn from the cigarette under negative pressure from the syringe and passed through the 6 mL of Opti-medium within the syringe. The cigarette was smoked to within 3 mm of the cigarette filter, with an average burn time of 7 min. After the cigarette was removed, the medium solution containing the extra was incubated at 37 °C for 30 min, and the solution was filtered (0.22 μ m) and used immediately. The CSE absorbance was measured (OD 1.1, 320 nm) using a spectrophotometer, and this resulting extract was designated as 100 % CSE. Dilutions were performed in Opti-medium to 10, 30, 60 % CSE (Figure 1A). Cell VOCs were collected with Twister[®] for every 12 h until 48 h (Figure 1B). The medium control was defined as 0 % CSE (100 % Opti-medium). Two cell culture

treatments were also applied to the experiment; cell culture exposed to Opti-medium alone and cell culture exposed to CSE at various concentrations. Each treatment had two jar biological replicates and two Twister[®] technical replicates. The cell viability assay was conducted after 48 h in each concentration of CSE exposure, and was measured three times among three transwells.

GC-MS Analysis

After VOC extractions, Twisters[®] were loaded into thermal desorption tubes. A 1.0 μL aliquot of a 10 ppm naphthalene-d8 solution in 100% ethanol was added to each tube as a second internal standard.

Cellular VOCs are desorbed from Twisters[®] using a thermal desorption unit (TDU) and cooled injection system (Gerstel, Inc.) and are analyzed using a GC-MS system as previously noted [34]. Briefly, the TDU heats a Twister[®] to 300 °C and holds for 5 min. As VOCs desorb from the Twister[®], they are lead via a flow of helium to the CIS, which is held at -80 °C. After desorption, the CIS quickly heats to 300 °C, splitlessly injecting the VOCs onto the GC column.

Analytes are separated on an Agilent 7890B GC (Agilent Technologies Inc., Santa Clara, CA) with a DB-5ms column (20 m x 180 μm x 0.18 μm , Part 221-5522LTM, Agilent Technologies, Inc.). The oven was programmed as follows: initial temperature 35 °C for 3 min, then a ramp of 5 °C/min to 75 °C, then 2 °C/min to 220 °C and a final ramp of 30 °C/min to 300 °C, holding for 3 min. The transfer line was set at 280 °C, leading into an Agilent 5977A mass spectrometer. There was a solvent delay of 5 min. The MS scanned the range from 33 to 520 m/z. The MS source was set to 230 °C and the MS quad set to 150 °C. A standard mix of C₈-C₂₀ alkanes was analyzed with every batch of samples to calculate the Kovats Retention Indices and also to monitor the performance of the TDU-CIS-GC-MS system, in addition to the two internal standards used in every sample.

Data Analysis Workflow

The GC-MS data files were deconvoluted, integrated and aligned using MassHunter Profinder B.08.00 (Agilent Technologies, Inc.). Compounds with siloxane base peaks (73, 147, 207, 221 and 281 m/z) were removed as they are artefacts from the PDMS sorbent. Compounds found in less than 60% of replicates from one treatment were also excluded from analysis. Peak areas were normalized to the naphthalene-d8 internal standard. Compounds were tentatively identified by calculating their Kovats Retention Index in comparison to reported literature values and by comparison of extracted mass spectra to the NIST 2014 mass spectral library.

Data analyses were performed on MATLAB R2017a software (Mathworks, Natick, MA), PLS_Toolbox (Version 8.6, Eigenvector Research Inc., Manson, WA) and Agilent's GeneSpring (Version B.14.9), with p-value = 0.05 throughout. Specifically, PLS regressions were built using PLS_Toolbox. Presented are the results of cross validation only, in which the PLS model predicts the concentration of the oxidative stressor in the media (e.g. 10% CSE or 50 mM H₂O₂) based on the volatile profile of that sample. Predicted concentrations are plotted against the known, or actual, concentration.

Results and Discussion

H₂O₂ Effects on VOC Emissions

Viability Assay for H₂O₂ Exposure—The alamarBlue[®] Assay incorporates a fluorometric/colorimetric growth indicator based on detection of oxidation-reduction in cell metabolic activity. An ANOVA was applied to 6 treatments (5 H₂O₂ concentrations and one control; Figure 2). The assay found that the 0, 0.1 and 0.5 mM peroxidation exhibited similar levels of metabolic activity, indicating that there was no mortality in these three peroxidation treatments. Cells that received the 10 mM level of exposure exhibited a significant drop in metabolic activity relative to healthy controls, indicated that this concentration of H₂O₂ was stressful enough to cause mortality in some cells.

The viability assay results indicated significant cell death after 24 h in cells exposed to 50 mM H₂O₂ (absorbance value <0). Similar results in cell viability in response to oxidative stress were observed in various cell culture systems. For example, Ballinger *et al.* found that mitochondria DNA lesions occurred from 0.05 mM H₂O₂ in human retinal pigment epithelium cells [39]. Imlay and Linn reported that 10–30 mM H₂O₂ was significant to kill *Escherichia coli* due to the DNA damage incurred [40]. It is possible that a higher concentration of H₂O₂ caused major mitochondria DNA damage in our H₂O₂ peroxidation experiment as well. Also, Yamada *et al.* found exposure of human tracheal epithelial cells to 1 mM H₂O₂ resulted in an increase of permeability, likely related to cell stress and viability loss [7].

PLS Regression Model for H₂O₂ Exposure—We first wanted to determine the time point at which the HBE1 cells exhibited the strongest volatile response to H₂O₂ peroxidation. To do so, PLS regression models were built from the four measured time points (Figure 3) for the four concentrations of H₂O₂ (0.1, 0.5, 10, 50 mM). The t=12 h time point exhibited the best linear fit, indicating the strongest VOC profile exposure response to H₂O₂. The 36 and 48 h time points do not include the 50 mM exposure, as these cells were found to have suffered mortality after 24 h peroxidation (Figure 2).

The t=24 h mark resulted in the poorest linear correlation between predicted and measured H₂O₂ peroxidation. The 10 mM concentration was indistinguishable from the 50 mM concentration, suggesting these two peroxidation treatments had experienced similar volatile changes after 24 h of peroxidation. It was not possible to investigate this trend after 24 h since the 50,000 μM peroxidation treatment was removed from the rest of the analysis due to cell death. In our cell culture models, the H₂O₂ treatment would likely first affect the cellular membranes. Shestivska *et al.* stressed synthetic membranes with a H₂O₂ solution and observed volatile changes through the first 15 h of exposure [41].

In PLS regression, each variable (metabolite compound) is given a Variable Importance in Project (VIP) score, which estimates the importance of each variable to build the PLS model. Typically, compounds with a VIP score >1 are considered relevant to the PLS regression. In our analysis, 43 compounds were found to have a VIP score >1. These VOC metabolites were identified in the following compound chemical classes: alkanes or/and alkenes (20 %) and aldehydes (12 %), alcohols (8 %), aromatic hydrocarbons (8 %),

cycloalkanes (4 %), and acids (3 %). Hydrocarbons, aldehydes, alcohols and ketones are chemical classes known to be related to the lipid oxidation pathways [42, 43]. Therefore, the majority of the compounds which were important to build the PLS model may have strong relation to known oxidative stress response pathways.

Table 1 shows the top 10 VIPscore compounds tentatively identified from cell samples exposed to H₂O₂. The compounds we detected generally agree with the types of compounds found in similar cell culture models [26, 32, 34]. The alkane (2,9-dimethyl decane), certain alcohols [2-(2-Hydroxyphenoxy)-1-phenylethanol, 3-Methylbutyraldehyde oxime, 3,4-Dimethyl-2-hexanol], and a ketone (Oxolan-2-one) were identified as the top 10 VIP scored compounds. These compounds were potentially produced from lipid fragmentation as a result of the peroxidation treatment. Some of the compounds have been previously reported as oxidative stress biomarkers. Benzenecarbaldehyde and phenylacetonitrile have previously been reported in the headspace of cell cultures infected with viruses due to the lipid fragmentation by the oxidative stress [32, 42, 43]. Benzenecarbaldehyde is an aldehyde known to be produced during the inflammatory processes as a result of lipid peroxidation and oxidative stress [30].

To demonstrate the effects of H₂O₂ on HBE1 volatile emissions, the ten compounds with the highest VIP scores were plotted and grouped by concentration exposure (Figure 4). In these ten examples, we saw a majority exhibit a trend to increase in volatile abundance with peroxidation (compounds 270, 83, 4, 173, 176). There were also examples of VOCs that suddenly gained abundance or only appeared in the final 50 mM treatment (compounds 293, 84, 234). Compound 254 decreased with exposure concentration. We examined how these 10 compounds changed over 48 h within the 10 mM peroxide treatment (data not shown). The majority of these compounds peaked in abundance at either the 24 or 36 h time point.

We expect peroxidation of the cell culture media to produce artefact VOCs that are not endogenous to HBE1 cells. Both media controls and media with hydrogen peroxide controls were included in this work. Our cell culture analyses only included compounds that were measured with an abundance of at least three times greater in cell cultures compared to respective media controls. Examples of the types of VOCs found in media with hydrogen peroxide are presented in Supplemental Table 1 for reference by future researchers.

Smoke Extract Effects on VOC Emissions

Viability Assay for CSE Exposure—Per the viability assay, the four CSE peroxidation conditions (0, 10, 30, 60 %) demonstrated similar levels of metabolic activity (Figure 5). An ANOVA was performed on the assay data and found that cells did not show any differences in metabolic activity or cell death or metabolic differences after 48 h in the CSE concentrations ($p < 0.05$ significance level). Chang *et al.* also reported that they did not see any tissue damage in 5 % and 10 % CSE exposure among non-COPD lung cells using a lactate dehydrogenase detection assay [44]. However, when they measured mRNA expression, they observed upregulation of the pro-inflammatory cytokine IL-17A after 3 h of 5 % CSE peroxidation [44]. Thus, although our cell viability assay did not show any mortality, it is possible that cell metabolites are experiencing robust changes which could lead to alterations in VOC emissions.

Our viability assay did not demonstrate any differences in metabolic activity, as measured by AlamarBlue assay, for all four CSE peroxidation conditions (0, 10, 30, 60 %) after 48h (Figure 5). Similarly, Chang *et al.* reported that they did not see any tissue damage in 5 % and 10 % CSE exposure among non-COPD lung cells using a lactate dehydrogenase detection assay [44]. However, when they measured mRNA expression, they observed upregulation of the pro-inflammatory cytokine IL-17A after 3 h of 5 % CSE peroxidation [44]. Therefore, we anticipated robust metabolic changes, with significant alterations in VOC emissions, at the doses of CSE we used despite the uniform results from the viability assay.

PLS Regression Model for CSE Exposure—As in the H₂O₂ peroxidation experiment, we built PLS regression models to determine the time point at which the HBE1 cells exhibited the strongest change in their volatile emissions that relates to CSE. PLS regression models built at four time points post exposure to four concentrations of CSE (0, 10, 30, 60%) are shown (Figure 6). The t=12 h time point exhibited the best linear fit, indicating the strongest VOC profile response. The 36 h time point exhibited the worst linear correlation, and it is currently unclear why we observed this result. We suspect that some cellular repair mechanisms may be activated that are helping to remediate the oxidative stress effects on the metabolite profiles. However, additional future work is likely needed to understand these phenomena.

Cigarette smoke itself is a complex aerosol containing more than 4,400 chemicals [45]. Thus, the smoke extract itself confounds the volatile profile of the cell cultures. Using the CSE media controls, we were able to distinguish compounds that were emitted from the cell cultures and not exclusively originating from the smoke extract. We included only compounds that had an abundance of three times greater in cell culture samples than in respective media control samples. Supplemental Table 1 includes examples of the types of compounds found in CSE media controls. There were 35 compounds that had: a) a VIP score >1 per a PLS regression model at 12 h post exposure; and b) were found to be three times greater abundance in HBE1 cell culture samples than in respective CSE media controls. Most of these compounds are alkane or an alkene (20 %) and aldehydes (20 %). Also identified were: aromatic hydrocarbons (14 %), cycloalkanes (9 %), heterocyclic compounds (9 %), alcohol (11 %), acids (6 %), ketones (3 %), and terpenes (6 %).

Similar to the H₂O₂ peroxidation experiments, known oxidative stress-related chemical classes were found in these CSE peroxidation experiments such as: hydrocarbons, aldehydes, alcohols and ketones. In contrast, terpenes were not found in the H₂O₂ peroxidation experiments, but were found in the CSE exposures. Terpene compounds have been reportedly related to antioxidant activity. Oxidative stress impairs the prooxidant-antioxidant balance in favor of the former by increasing the production of reactive oxygen species, in part through a NAD(P)H oxidase-dependent mechanism [9]. Additionally, CSE is a known activator of the Nrf-2/Keap signaling pathway and AP-1 transcription factor [46], both of which are highly responsive to oxidative changes in the environment of the cell. Time-sensitive biphasic responses to CSE have been seen with inflammatory cell profiles and Nrf-2/Keap-dependent heme oxygenase 1 regulation in the lung [47, 48], consistent with our biphasic dose-response observed in these experiments. The precise link between

Nrf2 or AP1-induced signaling pathways and specific metabolic compounds remains to be determined, but future experiments can be designed to look at specific activation of these pathways and the resultant metabolic profile that results.

A common terpene mammalian metabolomic pathway is isoprene metabolism, namely the reaction of isopentenyl pyrophosphate and dimethylallyl pyrophosphate to form the 10-carbon geranyl pyrophosphate [49]. It is possible that terpenes were produced from cellular metabolism due to the defense response of peroxidation treatment. However, the terpene antioxidant defense response of mammalian cells has not been reported and further experiments will be needed to confirm the identity of these potential cell defense compounds.

The top 10 VIP score compounds from cell samples exposed to CSE were tentatively identified (Table 2). Aldehydes (5-Methyl-2-phenylhex-2-enal), ketones (isoshyobunone), and alcohols (4-(4-methylphenyl)pentanal) were identified as the top 10 VIP-scored compounds. These compounds are potentially produced due to the lipid fragmentation of peroxidation treatment. Also, 4,6,8-trimethylnon-1-ene was reported as a potential breath biomarker of active pulmonary tuberculosis by Phillips *et al.* [50], and the alkanes and methylated alkane are putatively products of oxidative stress. Ramya *et al.* reported that plant extract 2-methyltetracosane had strong activity as a free radical scavenger [51]. Phenolic compounds also have been reported as efficient antioxidant compound due to the function of free radical terminators and metal chelators [52]. Yoon *et al.* reported that 2,4-Di-tert-butylphenol showed efficient antioxidant effects in thiobarbituric acid reactive substance assay [53].

A PLS regression showed ten compounds at t=12 h CSE peroxidation that were deemed important (Figure 7), and these are tentatively identified (Table 2). In these examples, there were not as many trends that correlated VOC abundances with CSE concentration. Compound 289 decreased in abundance with exposure, while 161 clearly increased with exposure. Compounds 98, 145, 228, 258, 148 and 159 generally see a trend of VOCs increasing with concentration, but the abundances of these compounds for the 30 % CSE suddenly drop and increase again for the 60 % peroxidation. The internal standards for these samples did not suggest any technical issues.

Comparison of H₂O₂ and smoke extract on VOC emissions

We saw oxidative stress-related VOCs from both the H₂O₂ and CSE peroxidation experiments. We found more antioxidant related compounds emitted from the cells in the CSE peroxidation experiment, compared to H₂O₂ peroxidation. This could partially be due to cell viability differences between the two peroxidation treatments. In H₂O₂ peroxidation, we observed slightly lower viability compared to the CSE experiments. Hence, cells in H₂O₂ were experiencing strong oxidative stress and might have contributed to an imbalance of the antioxidative defense response. However, in the CSE peroxidation treatment, cells were still metabolically active and they showed a strong antioxidant defense response, which resulted in the long chain carbon VOCs that we detected. We believe these differences in the cell response contributed to the changes of the VOC profile differences between the two peroxidation experiments. Furthermore, in H₂O₂ peroxidation, we saw shorter chains

and smaller VOCs which are more related to membrane lipids and fatty acid fragmentation, likely due to the high level of peroxidation. In contrast, heavier molecular weight VOCs were emitted in the CSE peroxidation experiment, suggesting these compounds could be related to cytokine or protein damage produced due to the cell antioxidant defense response.

The comparative, parallel findings with H₂O₂ and CSE was expected and somewhat reassuring. Recent evidence points to the generation of H₂O₂ by CSE as an important cause of airway epithelial cell injury[54, 55]. Bronchial epithelial cells exposed to five percent CS extract generated maximal amounts of H₂O₂ at four hours followed by cellular injury and impaired wound healing[55]. Both the antioxidant, N-acetylcysteine, and the transfection of cells with a vector encoding superoxide dismutase to block H₂O₂ improved wound healing after CS extract exposure. Further studies have revealed that the mechanism of CSE/ H₂O₂ mediated airway epithelial injury is via upregulation of DUOX1[54]. Given the causal relationship, we would expect similar VOC patterns from such cells.

Conclusions

To our knowledge, our study is the first to show a specific cellular VOC production response to different oxidants concentrations and time points in bronchial epithelial cells, under two different peroxidation paradigms (H₂O₂ and CSE). From our PLS regression models, we found 12 h was the most effective peroxidation time point for our cell culture system. Previously in the literature, peroxidation studies were typically confined to shorter time courses (< 6 h) and longer duration oxidative stress experiments are not common. However, oxidative stress is likely a chronic cellular response, and our understanding of our data is that longer time course studies, at lower oxidant levels, are needed to more closely mimic a clinical microenvironment for the cells.

From both the H₂O₂ and CSE peroxidation experiments, known oxidative stress biomarkers were detected in our system. Moreover, in CSE peroxidation, we observed VOCs likely related to the cell antioxidant defense response. The research field of cell VOC oxidative stress markers is still developing, and specific VOCs related to the overall cell antioxidant defense response remains largely unknown. In future studies, we plan to better define the link between specific oxidative stress cellular pathway alterations to specific VOC generation. We believe our initial findings will help steer future VOC oxidative stress research towards understanding the cellular origin of this category of breath biomarkers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Partial support was provided by: Revelar, Inc. funded industry contract #A18-1712 [CED, NJK, RHH, SEE, MS]; NIH award U01 EB0220003-01 [CED, NJK]; NIH National Center for Advancing Translational Sciences (NCATS) through grant#UL1 TR000002 [CED, NJK]; NIH award UG3-OD023365 [CED, NJK]; NIH award 1P30ES023513-01A1 [CED, NJK]; NIH-National Heart, Lung and Blood Institute 1K23HL127185 [MS]; and NIH training grant award T32 HL007013 [MSY]. The contents of this manuscript are solely the responsibility of the authors and do not necessarily represent the official views of the funding agencies.

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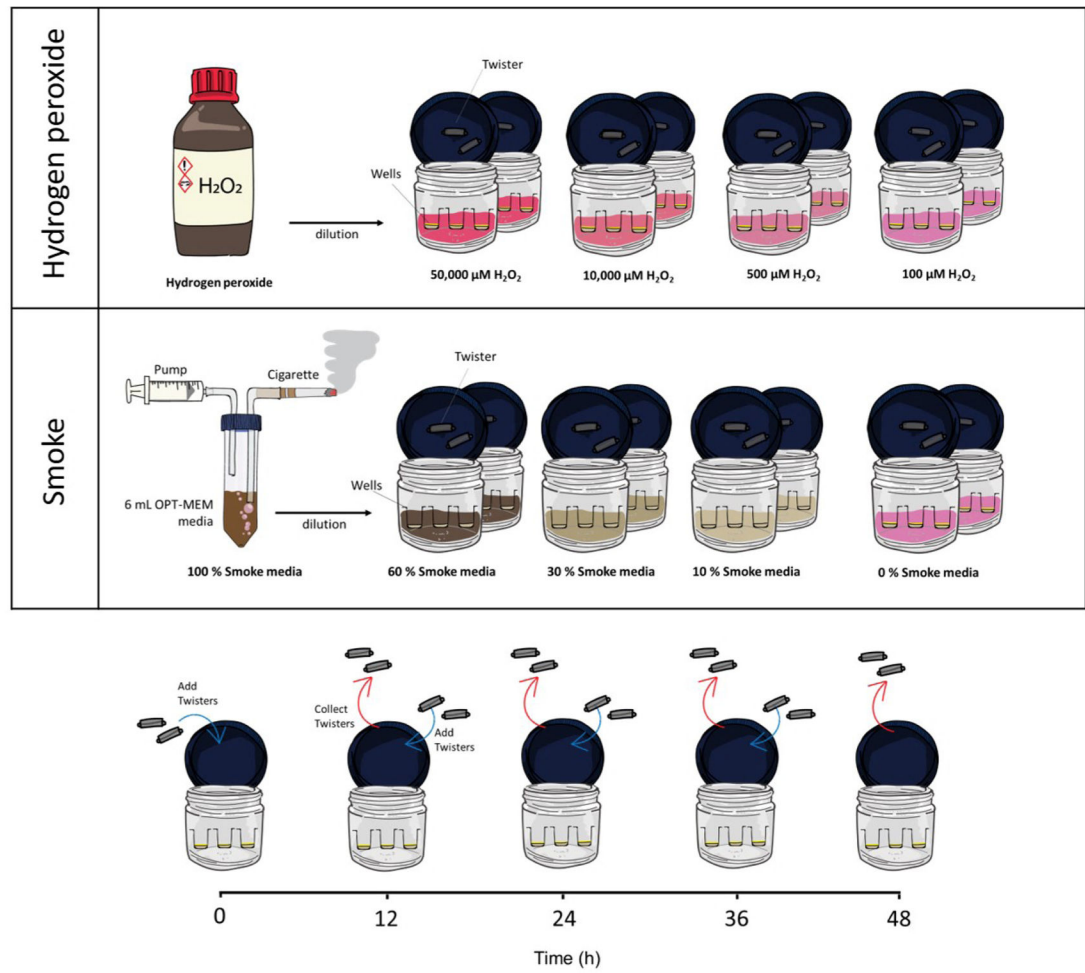


Figure 1.
Scheme of the work

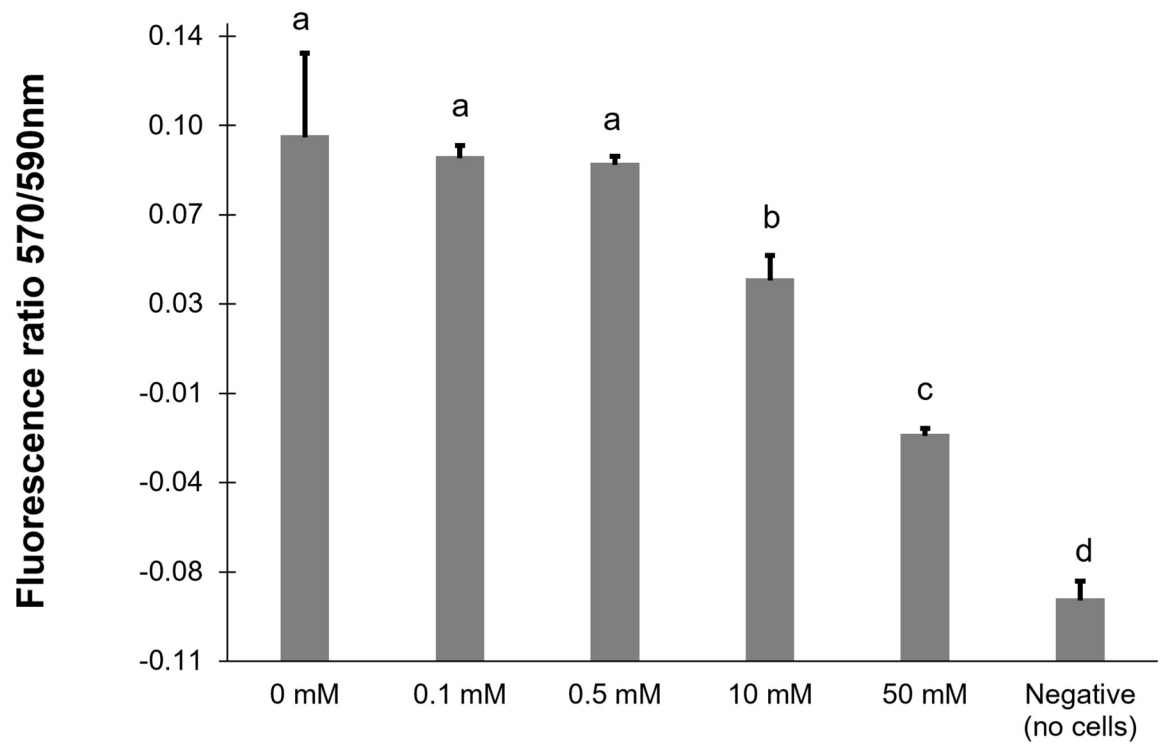


Figure 2. Cell viability from an alamarBlue assay taken after 24 h of hydrogen peroxide exposure analyzed using ANOVA for differences (letters indicate significance grouping).

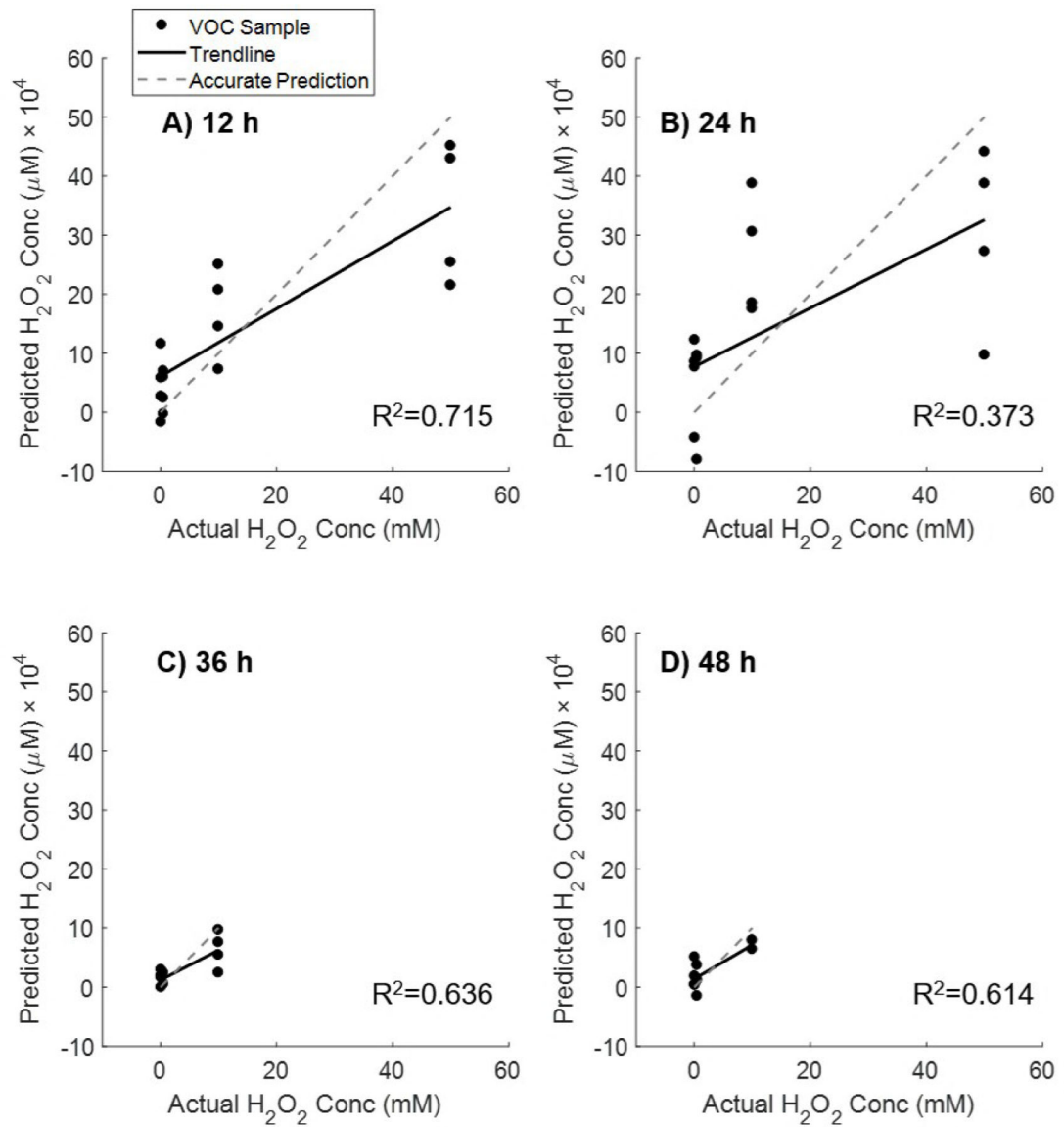


Figure 3.

PLS regression models built from VOC profiles at four time points from cells exposed to four concentrations of H_2O_2 (100, 500, 10,000, 50,000 μM). Cells exposed to 50 mM experienced mortality after 24 h. The $t=12$ h time point exhibited the best linear fit, indicating the strongest VOC profile response to exposure to hydrogen peroxide.

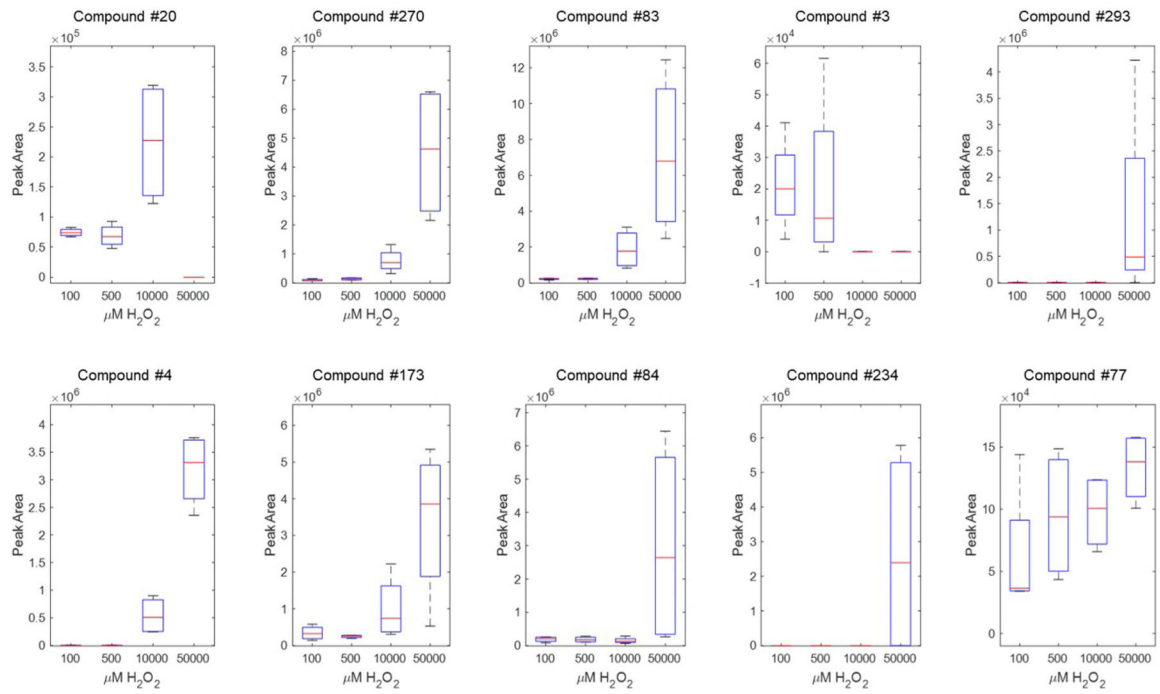


Figure 4. Ten compounds associated with H₂O₂ exposure per a PLS regression at 12 h H₂O₂ exposure. Compounds are identified in Table 01

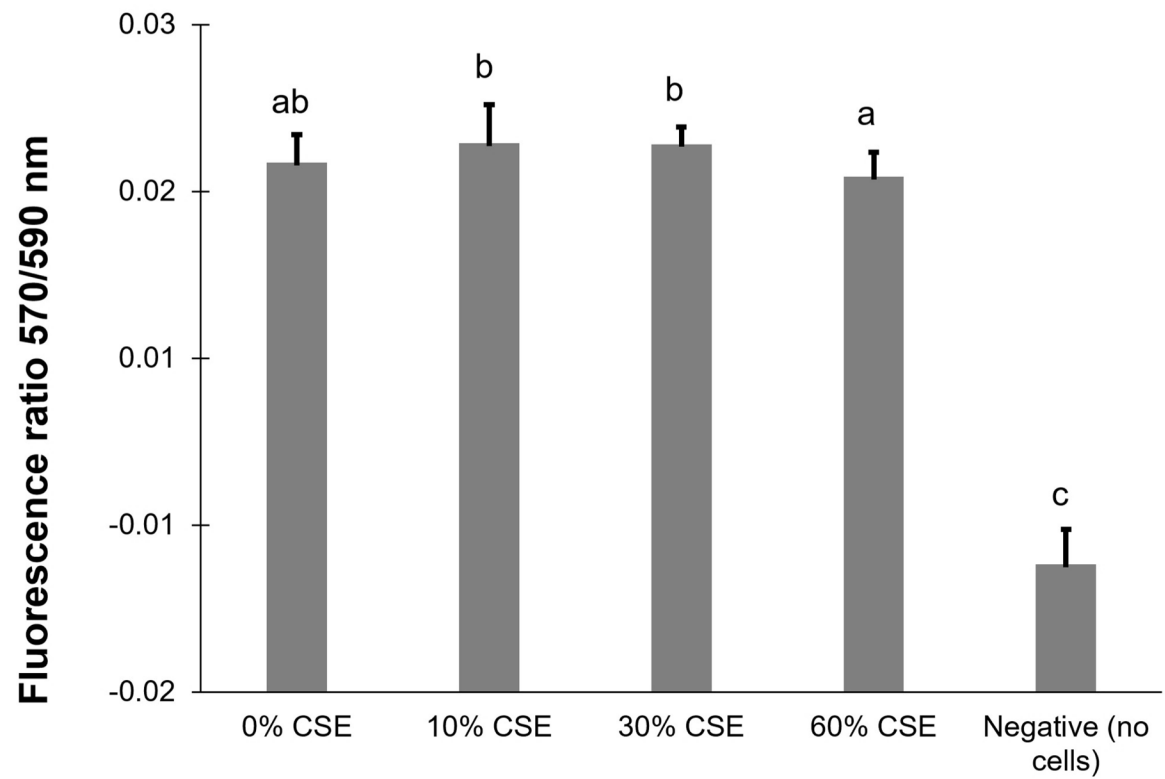


Figure 5. Cell viability from an alamarBlue assay taken after 48 h of cigarette smoke exposure (CSE). Letters indicate significance grouping per an ANOVA (letters indicate significance grouping)

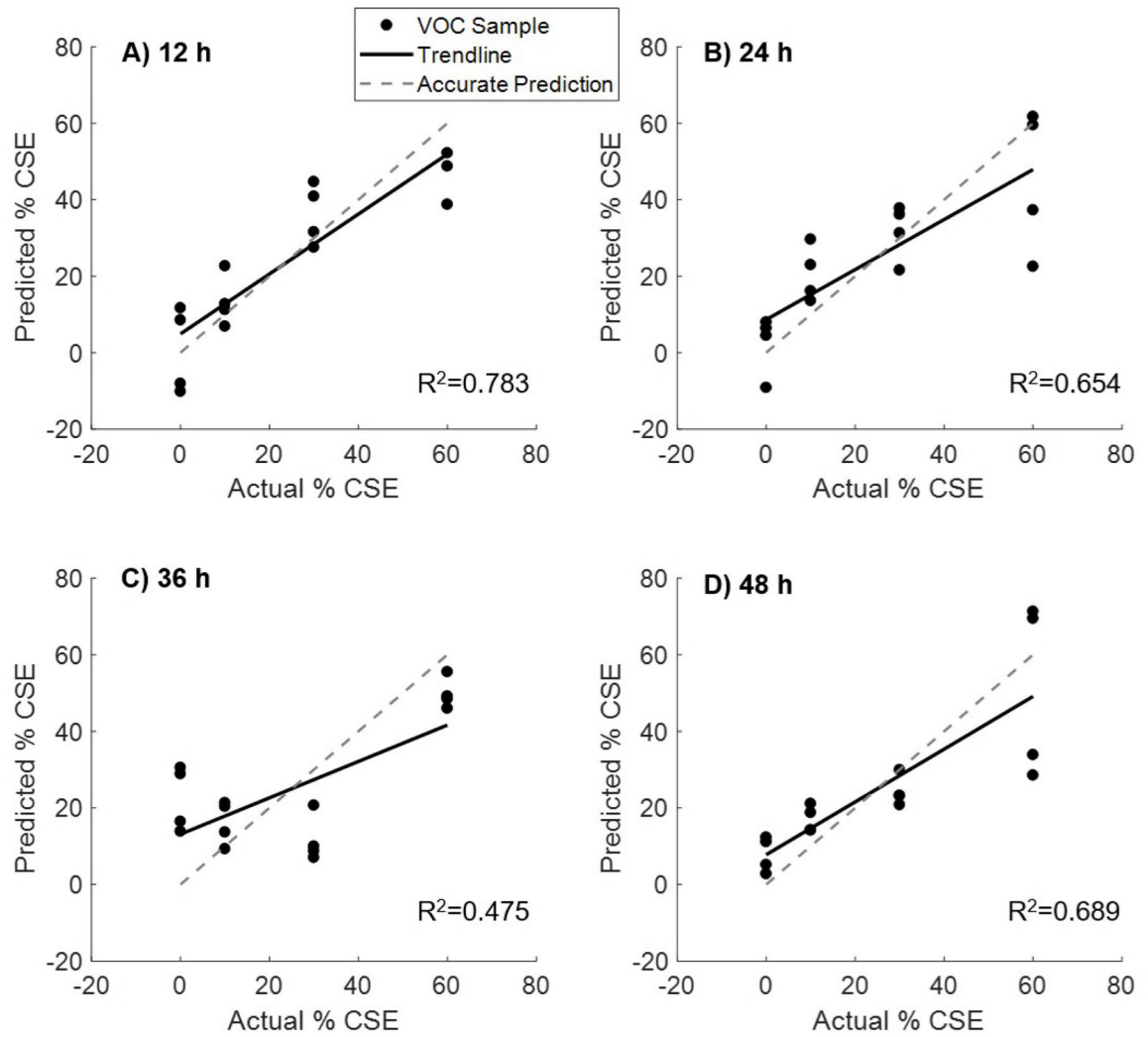


Figure 6. PLS regression models built from VOC profiles at four time points from cells exposed to four concentrations of cigarette smoke extract (0, 10, 30, 60%). The t=12 h time point exhibited the best linear fit, indicating the strongest VOC profile response to exposure to cigarette smoke.

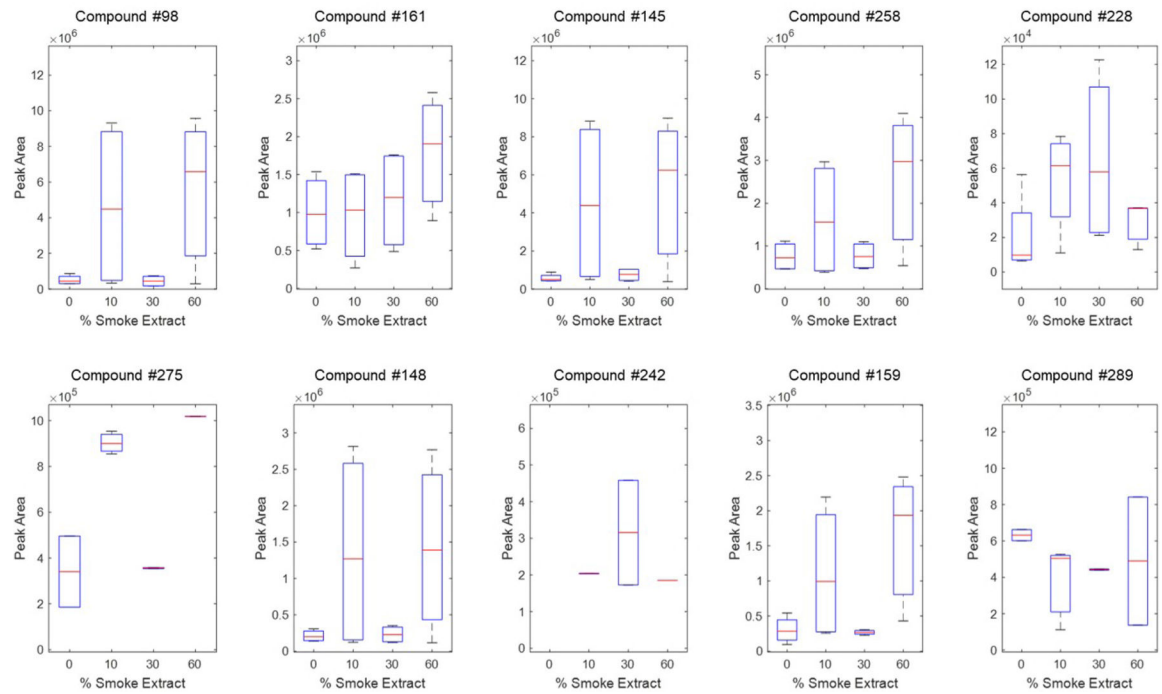


Figure 7.

Ten compounds associated with H₂O₂ exposure per a PLS regression at 12 h CSE exposure. Compounds are identified in Table 02

Table 1.Top 10 VIP score compounds tentatively identified from cell samples exposed to H₂O₂

Compound number	Base m/z	Retention Index (RI)	Name	Formula	MS Score	VIP H ₂ O ₂
20	117	1136	Phenylacetone nitrile	C ₈ H ₇ N	64.41	3.176
270	77	963	Benzenecarbaldehyde	C ₇ H ₆ O	91.55	2.858
83	40	900	1-Nitropentane	C ₅ H ₁₁ NO ₂	78.64	2.709
3	104.1	1297	1-Nitro-2-phenylethane	C ₈ H ₉ NO ₂	97.28	2.683
293	91.1	1050	2-Phenylacetaldehyde	C ₈ H ₈ O	91.97	2.646
4	104.1	1297	2-(2-Hydroxyphenoxy)-1-phenylethanol	C ₁₄ H ₁₄ O ₃	58.09	2.471
173	43.1	887	3-Methylbutyraldehyde oxime	C ₅ H ₁₁ NO	78.83	2.397
84	40	923	Oxolan-2-one	C ₄ H ₆ O ₂	61.48	2.250
234	45	887	3,4-Dimethyl-2-hexanol	C ₈ H ₁₈ O	73.8	2.068
77	43	1272	2,9-dimethyl decane	C ₁₂ H ₂₆	94.52	1.701

Table 2.

Top 10 VIP score compounds tentatively identified from cell samples exposed to cigarette smoke extract (CSE).

Compound number	Base m/z	Retention Index (RI)	Name	Formula	MS Score	VIP H ₂ O ₂
98	41.1	1322	3,7,11,15-Tetramethylhexadec-1-en-3-ol	C ₂₀ H ₄₀ O	77.72	2.113
161	43.1	1903	2-Methyltetracosane	C ₂₅ H ₅₂	83.08	2.057
145	43.1	1322	4,6,8-trimethylnon-1-ene	C ₁₂ H ₂₄	79	1.879
258	104	1418	5-Methyl-2-phenylhex-2-enal	C ₁₃ H ₁₆ O	85.87	1.771
228	57.1	1475	2,6-Di-tert-butylphenol	C ₁₄ H ₂₂ O	88.48	1.436
275	81.1	1776	Dodecan-3-ylbenzene	C ₁₈ H ₃₀	65.4	1.271
148	43.1	1389	(3S,6S)-6-Isopropyl-2-isopropylidene-3-methyl-3-vinylcyclohexanone	C ₁₅ H ₂₄ O	80.62	1.27
242	68.1	1024	A terpene compound			1.255
159	43.1	1707	A alkene compound			1.198
289	91.1	1821	4-(4-methylphenyl)pentanal	C ₁₂ H ₁₆ O	76.66	1.173