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RESEARCH ARTICLE



In vitro toxicity assessment of emitted materials collected during the manufacture of water pipe plastic linings

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ABSTRACT

Objectives: US water infrastructure is in need of widespread repair due to age-related deterioration. Currently, the cured-in-place (CIPP) procedure is the most common method for water pipe repair. This method involves the on-site manufacture of a new polymer composite plastic liner within the damaged pipe. The CIPP process can release materials resulting in occupational and public health concerns. To understand hazards associated with CIPP-related emission exposures, an *in vitro* toxicity assessment was performed.

Materials and Methods: Mouse alveolar epithelial and alveolar macrophage cell lines and condensates collected at 3 worksites utilizing styrene-based resins were utilized for evaluations. All condensate samples were normalized based on the major emission component, styrene. Further, a styrene-only exposure group was used as a control to determine mixture related toxicity.

Results: Cytotoxicity differences were observed between worksite samples, with the CIPP worksite 4 sample inducing the most cell death. A proteomic evaluation was performed, which demonstrated styrene-, worksite-, and cell-specific alterations. This examination of protein expression changes determined potential biomarkers of exposure including transglutaminase 2, advillin, collagen type 1, perilipin-2, and others. Pathway analysis of exposure-induced proteomic alterations identified MYC and p53 to be regulators of cellular responses. Protein changes were also related to pathways involved in cell damage, immune response, and cancer.

Conclusions: Together these findings demonstrate potential risks associated with the CIPP procedure as well as variations between worksites regarding emissions and toxicity. Our evaluation identified biological pathways that require a future evaluation and also demonstrates that exposure assessment of CIPP worksites should examine multiple chemical components beyond styrene, as many cellular responses were styrene-independent.

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Cured-in-place pipe; proteomics; macrophages; epithelial cells; styrene; cancer

Introduction

Millions of miles of US water pipes, which provide safe drinking water, drain excess water from roadways, and remove sewage require repair due to age related deterioration (Agency USEP 2002; Academies NRCotN 2006; Engineers ASOC 2013). To address this need, the cured-in-place pipe (CIPP) lining procedure is often applied because the old pipe does not have to be removed, therefore reducing time, disruptions, and expense. This method involves the on-site manufacture of a new polymer composite (plastic) lining within the damaged pipe. Use of the procedure is expected to expand globally, with the CIPP market exceeding \$2.5 billion by 2022, and accounting for 40% of the US pipe rehabilitation market (Stratview Research 2017). However, a growing body of evidence from federal and state agencies as well as Universities indicates that the CIPP

manufacturing process can release chemicals into the environment negatively impacting worker and public health (Tabor et al. 2014; Bourbour Ajdari 2016; Teimouri Sendesi et al. 2017; Health CDoP 2018; Ra et al. 2018).

The CIPP procedure involves the on-site use of raw chemicals as well as the generation and release of contaminants before, during, and after the plastic lining is manufactured resulting in worker and public health concerns. Briefly, the procedure comprises the insertion of a flexible raw chemical resin-impregnated tube into the damaged pipe, followed by the inflation of an uncured resin tube against the damaged pipe wall. Then resin is polymerized via curing to create a new plastic liner. Following establishment of the new liner, it can be air cooled and the ends are cut off. Resins are often referred to as either styrene or non-styrene based and popular curing methods include thermal (water or steam) or ultraviolet light processes. Currently, the

most popular procedure involves styrene-based resins and steam curing (Stratview Research 2017). Styrene is a prevalent chemical utilized in the CIPP process, and has been classified by the U.S. National Toxicology Program as reasonably anticipated to be a human carcinogen (National Toxicology Program 2011; Persoons et al., 2018). While prior CIPP occupational exposure studies with epoxy resin have involved dermal exposure (Anveden Berglind et al. 2012; Fillenham et al. 2012; Aalto-Korte et al. 2015), evidence shows that CIPP workers can be exposed to styrene through other routes including inhalation (Persoons et al. 2018). Specifically, an occupational study demonstrated styrene urinary metabolite levels were greater for CIPP workers than workers in the injection molding or other plastics manufacturing industries (Persoons et al. 2018). Prior studies from the composite sector where workers utilized styrene-based resins have demonstrated elevations in markers of lung toxicity and disease, decrements in lung function, and oxidative stress (Sati et al. 2011).

A number of indoor and ambient air public health incidents (>100) have been associated with CIPP worksites, with some prompting formal responses from government agencies (AirZone International 2001; Bauer and McCartney 2004, 20; Tabor et al. 2014; Teimouri Sendesi et al. 2017, 17; The Times 2017; Ra et al. 2018; Ra et al. 2019). Based on their own 2017 investigation, the California Department of Public Health issued two safety warnings related to the CIPP procedure due to public health concerns (Teimouri Sendesi et al. 2017, 17; Health CDoP 2018; Bertke et al. 2018, 24). In 2017, we compiled exposure events near CIPP installation sites in at least 32 states and many internationally (Teimouri Sendesi et al. 2017; Ra et al. 2019). Materials emitted from construction sites entered public spaces and nearby buildings, prompting the evacuation of affected or nearby schools, daycare centers, animal shelters, homes, and offices due to complaints of dizziness, headaches, eye and respiratory irritation, shortness of breath, and vomiting (Teimouri Sendesi et al. 2017; Ra et al. 2018). Persons, including children, were sometimes transported to a hospital or self-admitted after the exposures (AirZone International 2001, 20; The Times 2017; The Herald Bulletin 2017; Nyack News and Views 2017). Further, one fatality was recently associated with the CIPP process when a worker became trapped within a pipe (Rezin 2017). Postmortem toxicology detected elevated blood styrene levels, prompting the medical examiner to conclude chemical exposure was a significant contributing factor in the worker's accidental death within the pipe (Morrow 2017). In the US, emissions have been continued to be permitted to exit the worksite and air testing has often not been conducted (Teimouri Sendesi et al. 2017; Ra et al. 2018; Ra et al. 2019). A literature review revealed that few investigators have characterized what materials are emitted at CIPP worksites, and none probed emission-induced human health hazards (Ra et al. 2019; Li et al. 2019). Several observations were concerning, including: (1) styrene exhausted out of a manhole, at 250 to 1070 ppm_v (Bourbour Ajdari 2016), (2) 1 km downstream in the sewer the styrene level was unchanged (Environment,

RRvVeMNNIfPPhat 2005), and (3) emissions traveled above-ground 'kilometers from the worksite' (Bauer 2012).

Our own 2017 air sampling and analysis at worksites in Indiana and California revealed several discoveries of interest (Teimouri Sendesi et al. 2017; Ra et al. 2019). Emissions were condensed (condensate sample) and also were collected with Tedlar bags and sorbent tubes. Condensate samples were found to be a complex multi-phase mixture of chemicals consisting of partially cured resin, condensed phase droplets, water vapor, and a variety of vapors including styrene, acetone, phenol, phthalates, and other VOCs and SVOCs (Teimouri Sendesi et al. 2017). Differences were identified regarding condensate chemical composition and concentration, suggesting variations in operational procedures and conditions. A comparative *in vitro* cytotoxicity assessment of collected condensates was performed by normalizing samples based upon the primary condensate component, styrene, and utilizing mouse alveolar epithelial and alveolar macrophage cell lines. This assessment demonstrated differential reductions in cell viability that were CIPP worksite-specific and independent of styrene.

Emissions produced from the CIPP procedure represent a complex and variable exposure that could adversely affect workers and the public. As the CIPP procedure is increasingly being utilized to repair deteriorated water infrastructure systems, human exposures are expected to expand. Currently, evidence is lacking regarding potential disease development following inhalation, pathways and biomarkers of toxicity, and operational procedures that may mitigate CIPP emission related health effects. To begin to address these deficiencies, the authors exposed mouse alveolar epithelial and alveolar macrophage cell lines to CIPP emission condensates collected from three worksites or to styrene alone. Following exposure, cells were evaluated for altered cytotoxicity and transcription of genes related to inflammation and oxidative stress. Further, an untargeted proteomics approach was utilized to identify CIPP worksite-specific and styrene-dependent/independent cellular responses. Ultimately, our global evaluation of cellular responses following CIPP condensate exposure will assist in determining the potential hazards associated with an understudied and expanding manufacturing procedure.

Materials and methods

CIPP condensates

This study evaluated toxicity associated with CIPP worksite condensate samples collected at three CIPP installation sites in California. All installations used a styrene-based resin to fabricate the CIPP where styrene (32% wt) and talc (20%–30% wt) were the only disclosed resin ingredients (AOC 2008; Teimouri Sendesi et al. 2017). Another safety data sheet reported 0.5% Trigonox® KSM and 1% di-(4-tert-butylcyclohexyl) peroxy dicarbonate initiators were applied (AOC 2007; Teimouri Sendesi et al. 2017). Detailed procedures and methods related to the collection and characterization of CIPP worksite condensates, including identities and quantities of chemicals as well as chemical

characterization of the raw resin, are described in Teimouri Sendesi et al. (2017). Briefly, at each worksite, stainless steel air manifolds were placed at exhaust emission points for the capture and condensation of materials emitted from the process into air. Materials were removed from the air stream by ambient cooling and passage of the air through cold condensers prior to being collected in Pyrex® bottles. Condensates were then stored at 4°C until being characterized and evaluated in cellular response studies. Chemicals in condensates were identified and quantified via a GC/MS method, utilizing 1,4-dichlorobenzene-*d*₄ as the internal standard. In summary, styrene was determined to be the chemical in greatest abundance for all condensates using the approach applied. Additional chemicals identified within the condensates included butylated hydroxytoluene, benzaldehyde, benzoic acid, phenol, 1-tetradecanol, and others. These chemicals were at different concentrations across condensates. The uncured resin tube and new CIPPs were chemically extracted and residual chemicals that remained in those solid materials were identified.

Since condensates were environmentally collected samples, endotoxin levels were evaluated for each sample utilizing a commercially available kit (Thermo Scientific Pierce Chromogenic Endotoxin Quant Kit, Thermo Fisher Scientific, Hampton, NH). Endotoxin levels for all CIPP condensates were undetectable (<0.1 EU/ml) (data not shown). Since styrene was the most abundant chemical within each of the collected condensates Site 1: 4329 ± 937 ppm; Site 3: 3590 ± 800 ppm; and Site 4: 1819 ± 504 ppm (mean ± standard deviation) all CIPP condensates were diluted to equivalent styrene concentration for toxicity evaluations. This allowed for comparisons between condensates as well as the assessment of styrene-dependent and independent responses through the use of cells exposed to only styrene (ACROS Organics, Thermo Fisher Scientific, Hampton, NH) at the same concentration.

Cell culture and exposures

Mouse alveolar macrophages (RAW 264.7) and mouse alveolar type II (C10) cell lines were cultured individually in DMEM medium supplemented with 10% FBS and 100 U/ml penicillin-streptomycin. Cells were maintained in culture dishes under standard conditions at 37°C and 5% CO₂. All experiments to evaluate toxicity were performed at 90% confluency and in serum-free media conditions. Prior to exposures condensates were removed from refrigerator storage and mixed vigorously by hand to make a homogeneous solution. Condensates were immediately diluted to form stock solutions with equivalent styrene concentrations in serum-free media. These stock solutions were mixed by pipetting and added to cells that had previously been transferred to serum-free media conditions. Cell culture plates were then sealed and returned to cell culture incubators until time points for assessments of endpoints were reached. No change in media volumes was visually observed at any sample collection time point. Cells were also viewed via bright field microscopy to visually confirm presence,

morphology, and changes in density prior to sample collections.

Assessment of CIPP condensate-induced concentration- and time-dependent cytotoxicity

To determine concentration- and time-dependent alterations in cytotoxicity, cells were exposed to condensate samples diluted to equivalent styrene concentrations of 10, 100, 250, 500, or 1000 ppm, styrene only at matching concentrations, or serum-free media only (controls) for 1, 4, 8, or 24 h. Following the exposure, changes in cell viability were assessed via the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis MO) via manufacturer instructions. Absorbance values from exposed cells were compared to control cells to determine alterations in cell viability. Cytotoxicity data are presented in graphs as mean ± standard error of the mean and an *n* = 4/group, with each sample consisting of three technical replicates. Significant differences between exposures and controls were determined by two-way ANOVA with a Dunnett's *post-hoc* test (*p* < 0.05). Limited cytotoxicity was observed at 24 h for all condensate samples at normalized styrene concentrations of 500 ppm or less, therefore concentrations of 250 and 500 ppm were utilized for subsequent experiments.

CIPP condensate modifications in the expression of genes related to inflammation and oxidative stress

Cells were grown to 90% confluency in 24-well plates and exposed to CIPP condensate samples or styrene alone at concentrations of 250 or 500 ppm for 1 h and 24 h, while untreated cells were used as the control group. Total RNA was isolated from cells using Direct-zol RNA MiniPrep (Zymo Research Corp. Irvine, CA) via manufacturer instructions. Following quantification via nanodrop, total RNA was reverse transcribed to cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (control), interleukin-6 (IL-6), monocyte chemoattractant protein 1 (MCP1), and hemeoxygenase-1 (HO-1) utilizing SsoAdvanced™ SYBR Green Supermix (Bio-Rad) and inventoried QuantiTect primer assays (Qiagen, Balencia, CA). Relative mRNA fold changes were calculated considering untreated cells as control and normalized to GAPDH as the internal reference. Gene expression data are presented in graphs as mean ± standard error of the mean and an *n* = 4–6/group. Significant differences between exposures were determined by one-way ANOVAs within each time point with a Tukey's *post-hoc* test (*p* < 0.05).

Proteomic evaluation of CIPP condensate-induced cellular effects

Cells were again grown in 24 well plates to 90% confluency and exposed to CIPP condensate samples or styrene alone

at 250 ppm for 24 h ($n=3-4$ /group). Following exposure, cells were collected, lysed for protein extraction, and digested to isolate peptides for proteomic analysis. Briefly, following a series of PBS washes, cells were collected with trypsin, trypsin was neutralized with cell culture media containing 10% fetal bovine serum, and cells were pelleted via centrifugation. The cell pellets were then washed multiple times with ice-cold PBS to remove any residual culture media before re-suspending in 8M urea. Cells were lysed using a barocycler at 4°C, 35,000 psi, for 90 cycles (20 s at high pressure and 10 s at low pressure). Concentration of proteins in each sample were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA). Fifty micrograms of total protein (equivalent sample volume) from each sample was digested using trypsin and LysC mix using a method similar to our previous publications (Shannahan et al. 2013; Raghavendra et al. 2017; Kobos et al. 2018). Following digestion, peptides were desalted using UltraMicroSpin columns (The Nest Group, Southborough, MA) as described by manufacturer's instructions. Peptide concentrations were then determined using a BCA assay. One microgram of peptides from each sample were analyzed by reverse-phase HPLC-ESI-MS using a Dionex UltiMate 3000 RSLC Nano System (Thermo Scientific) which was directly connected to a Q-Exactive HF Hybrid Quadrupole-Orbitrap MS (Thermo Scientific) and a Nanospray Flex Ion Source (Thermo Scientific). Peptides were loaded onto a trap column (300 μ m ID \times 5 mm, 5 μ m 100 Å PepMap C18 medium) at a 5 μ L/min flowrate using 98% purified water/2% ACN/0.01% FA solvent system. After 5 min, the trap column was switched in-line with the AcclaimTM PepMapTM RSLC C18 (75 μ m \times 15 cm, 3 μ m 100 Å PepMap C18 medium, Thermo Fisher Scientific) analytical column for peptide separation at a 0.3 μ L/min flowrate for 120 min. A 5–30% linear gradient of solvent B was run for 80 min, followed by 11 min of 45% solvent B and 2 min of 100% solvent B with an additional 7 min of isocratic flow. Solvent A was then applied at 95% for 20 min for column equilibration. A Top20 data-dependent MS/MS scan method was used to acquire the MS data. Injection time was set to 100 ms, resolution to 120,000 at 200 m/z, spray voltage of 2 eV and an AGC target of 1×10^6 for a full MS spectra scan with a range of 400–1650 m/z. Precursor ions were fragmented at a normalized collision energy of 27 eV using a high-energy C-trap dissociation. Acquisition of MS/MS scans were done at a resolution of 15,000 at m/z 200. To avoid repeated scanning of identical peptides, dynamic exclusion was set at 30 s.

Raw data files from LC-MS/MS analysis were processed using a MaxQuant computational proteomics platform version 1.6.2.3 (Cox and Mann 2008; Cox et al. 2011). The peak list generated was searched against the *Mus musculus* sequences from UNIPROT retrieved on 08-01-2018 (83,916 proteins). To compare differences in proteins due to exposures, a label-free quantification (LFQ) method was utilized. LFQ values were used to generate fold changes by comparing individual protein LFQ values between exposed and

control cells to generate fold changes. LFQ values for all proteins identified in the proteomic analysis of C10 (alveolar type II cells) and RAW 264.7 (alveolar macrophages) are included within [Supplemental Tables 1 and 2](#). Fold changes from groups were then averaged and statistically compared using *t*-tests ($p < 0.05$). This statistical approach has been utilized in previous evaluations employing untargeted proteomic approaches (Cox et al. 2014; Ganief et al. 2017; Pan et al. 2017; Yan et al. 2018). Specifically, Pascovici et al. demonstrated that *t*-tests are appropriate as opposed to multiple-comparison corrections due to a number of statistically limiting factors (few replicates, ratio compression, effect size, and other constraints) related to exploratory proteomic studies resulting in the exclusion of all true positives (Pascovici et al. 2016). Only proteins determined to be significantly altered ($p < 0.05$) by *t*-tests and present in at least 3 samples from each group were utilized for the generation of Venn diagrams comparing exposure responses. These lists of proteins along with *p* values were imported into Ingenuity Pathway Analysis (IPA, Qiagen, Germantown, MD) for gene ontology and molecular pathway analysis. This same procedure was applied to determine differences between styrene only exposed cells and those exposed to CIPP condensates as well as between individual CIPP condensate exposures. Proteins determined to be modified independent of styrene but shared between condensates were also assessed via Ingenuity Pathway Analysis to determine shared CIPP-specific proteomic responses.

To determine quantitative differences in proteins due to exposures, individual fold changes, computed by comparing exposures to controls, were compared using a one-way ANOVA with a Dunnett's *post-hoc* test ($p < 0.05$). Graphs and analyses were performed using GraphPad prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). Data from replicates were averaged and used to produce a standard error of the means ($n=3-4$ /group).

Results

Time- and concentration-dependent assessment of CIPP condensate cytotoxicity

To evaluate site-specific cytotoxicity and to establish parameters for subsequent evaluations, alveolar type II epithelial (C10) cells and alveolar macrophages (RAW) were exposed to collected styrene only or CIPP condensates across a variety of concentrations (0, 10, 100, 250, 500, or 1000 ppm) and time points (1, 4, 8, or 24 h) ([Figure 1](#)). For comparisons, all samples were normalized based upon styrene concentration. No overt cytotoxicity (<75% cell viability) was observed in C10 or RAW cells following exposure to styrene only or condensate samples collected from CIPP worksites 1 and 3 at concentrations up to 1000 ppm styrene through 24 h ([Figure 1](#)). Exposure to the condensate sample from CIPP worksite 4 resulted in increasing cytotoxicity across time and concentration for both cell types ([Figure 1](#)). Based upon this data, concentrations of 250 and 500 ppm were utilized for subsequent evaluations of cellular response to collected CIPP worksite condensates.

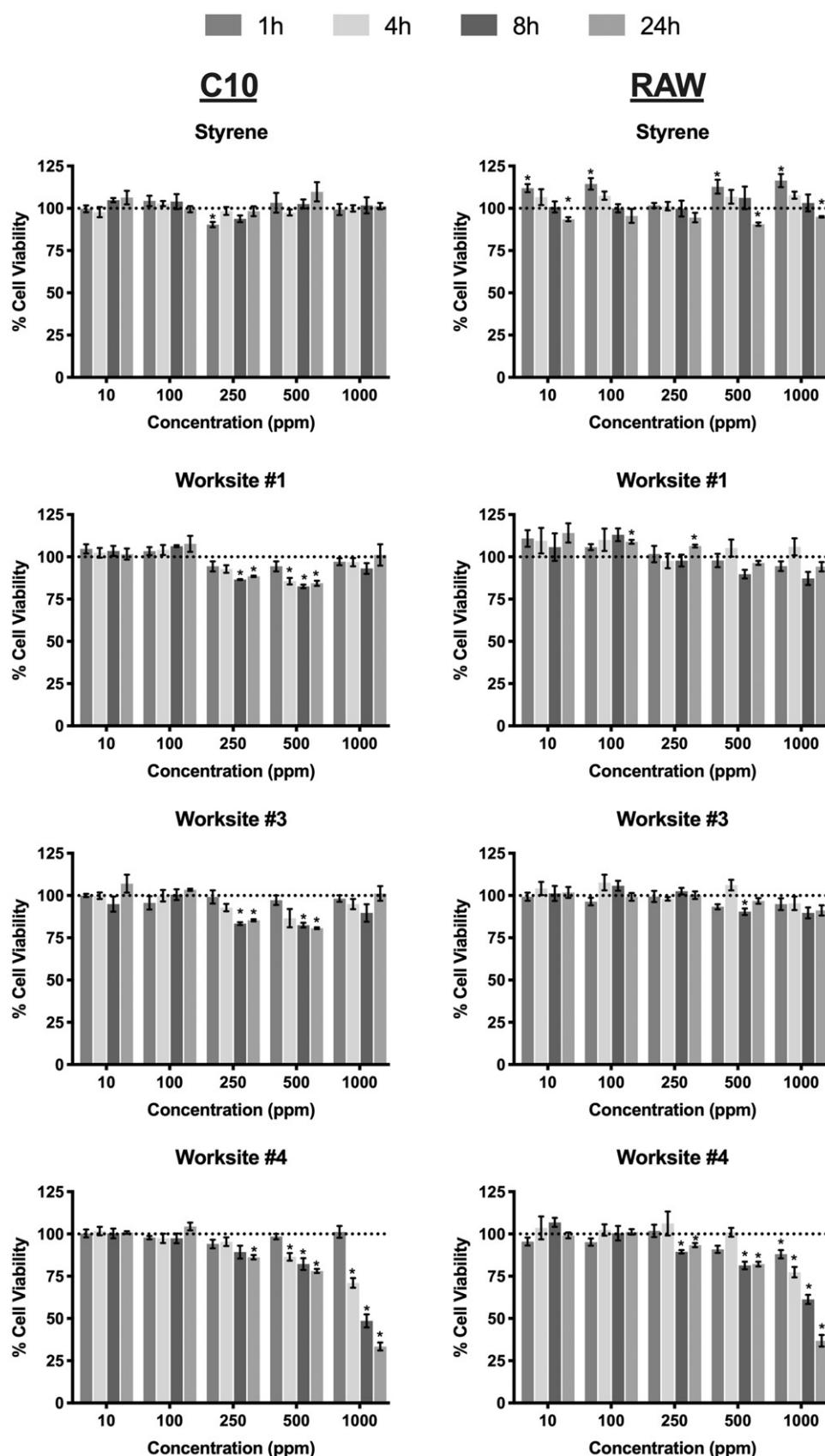


Figure 1. Alveolar epithelial (C10) and macrophage (RAW) cell viability following exposure to styrene-only or collected CIPP condensates from 3 worksites. CIPP condensates were normalized based upon styrene content. Cells were exposed to either CIPP condensates or styrene at concentrations of 0, 10, 100, 250, 500, or 1000 ppm of styrene for 1, 4, 8, or 24 h. Viability was evaluated via the MTT assay and normalized to control (untreated) cells represented by the dotted line. Data are presented as mean \pm standard error of means, $n = 4/\text{group}$. *Statistical significance compared to control (untreated) cells (one-way ANOVA with a Dunnett's post-hoc analysis, $p < 0.05$).

CIPP condensate-induced alterations in gene expression

C10 and RAW cell activation were assessed through measuring modulations in expression of genes related to inflammation, interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), as well as oxidative stress, hemoxygenase-1 (HO-1) following exposure to styrene-only or CIPP condensates at normalized styrene concentrations of 250 or 500 ppm for 1 or 24 h (Supplemental Figures 1 and 2). IL-6 gene expression was induced in C10 cells exposed to styrene (250 and 500 ppm), and CIPP condensates from worksites 1 and 4 (500 ppm) following a 1 h exposure (Supplemental Figure 1). At 24 h post-exposure, IL-6 mRNA levels were only elevated following exposure to CIPP condensate from worksite 4 (250 and 500 ppm). MCP-1 expression was induced only in C10 cells exposed to CIPP condensates from worksite 4 at 1 h and reduced at 24 h due to exposure to worksite 3 condensate. C10 cells demonstrated reduced expression of HO-1 following 1 h exposure to styrene (500 ppm), and CIPP condensate from worksite 3 (250 and 500 ppm). At 24 h, HO-1 levels remained reduced in C10 cells exposed to CIPP condensates from worksite 3 (500 ppm) but were elevated due to exposure to CIPP condensate from worksite 4 (250 and 500 ppm). RAW cells exposed to styrene (500 ppm) demonstrated elevated IL-6 mRNA expression at 1 h, while at 24 h elevations were observed only in cells exposed to condensate from CIPP worksite 1 (250 and 500 ppm) (Supplemental Figure 2). MCP-1 gene expression was induced in RAW cells 1 h following exposure to styrene (500 ppm), and condensates from CIPP worksites 3 (500 ppm), and 4 (250 and 500 ppm). By 24 h these responses had returned to baseline while MCP-1 expression was elevated in RAW cells exposed to condensates from CIPP worksite 1 (500 ppm). RAW cells exposed to styrene (500 ppm) demonstrated decreased expression of HO-1 mRNA; however, by 24 h, cells exposed to condensate from CIPP worksite 1 (250 and 500 ppm) exhibited increased HO-1 expression.

Proteomic evaluation of CIPP condensate-induced responses

Following a 24 h exposure of alveolar epithelial (C10) cells or alveolar macrophages (RAW) to collected CIPP worksite condensates at a normalized styrene concentration of

250 ppm, a global proteomic assessment was performed. The complete list of all the proteins identified in this study as well as their corresponding intensity values can be found in Supplemental Tables 1 (C10 cells) and 2 (RAW). To be included in the data analysis, proteins had to be present in at least 3 of the 4 replicates. For C10 cells there were 3835, 3848, 3869, 3686, and 3833 distinct proteins identified for controls, styrene-only exposed, CIPP worksite 1 condensate, CIPP worksite 3 condensate, and CIPP worksite 4 condensate, respectively (Supplemental Table 1). For RAW cells there were 3512, 3591, 3522, 3570, and 3533 distinct proteins identified for controls, styrene-only exposed, CIPP worksite 1 condensate, CIPP worksite 3 condensate, and CIPP worksite 4 condensate, respectively (Supplemental Table 2). These proteins were then evaluated to compare (1) CIPP condensate exposures to controls, (2) CIPP condensate exposures to styrene-only exposed cells, and (3) differences between worksites. Only proteins found to be significantly altered ($p < 0.05$) were utilized for these comparisons.

Proteomic assessment of alveolar epithelial (C10) cell responses to CIPP condensates

C10 cells exposed to CIPP condensates and styrene-alone exhibited differential protein expression compared to untreated controls (Table 1; Supplemental Table 3 contains the list of proteins used to generate Table 1). The CIPP condensate sample collected at worksite 4 was demonstrated to induce the greatest number of altered proteins compared to controls, while worksite 3 induced the least (Table 1). Exposure of C10 to styrene-only resulted in alterations of 129 proteins, with the majority being down-regulated (Table 1). Of the proteins altered following exposure to all three CIPP condensates, the majority were up-regulated. Comparing responses between C10 cells exposed to CIPP condensates and cells exposed only to styrene demonstrated alterations in responses (Table 1). The CIPP condensate sample from worksite 4 induced the greatest number of proteins changed compared to styrene-only (Table 1, data available in Supplemental Table 3). The majority of proteins altered were found to be up-regulated in C10s exposed to CIPP condensates compared to styrene only. Comparisons of worksites demonstrated that condensates from worksites 1 and 4 had the greatest differences in the number of

Table 1. Comparisons of proteins altered between exposure groups in C10 alveolar epithelial cells.

C10 Cells	Styrene	Site 1	Site 3	Site 4
Control vs	129 ↑ 53 ↓ 76	335 ↑ 189 ↓ 146	307 ↑ 173 ↓ 134	636 ↑ 382 ↓ 254
Styrene vs		333 ↑ 192 ↓ 141	322 ↑ 200 ↓ 122	648 ↑ 395 ↓ 253
Site 1 vs			142 ↑ 75 ↓ 67	388 ↑ 230 ↓ 158
Site 3 vs				224 ↑ 130 ↓ 94

All proteins included are statistically significant for the comparison ($p < 0.05$).

proteins altered (Table 1, data available in Supplemental Table 3). Worksites 1 and 3 were determined to be the most similar, with only 142 statistically different proteins identified.

To contrast global responses to exposures in terms of altered C10 protein expression, a Venn diagram was produced (Figure 2; Supplemental Table 4). All exposures (styrene-only and all CIPP worksite collected condensates) were determined to alter 6 proteins (Figure 2). These proteins

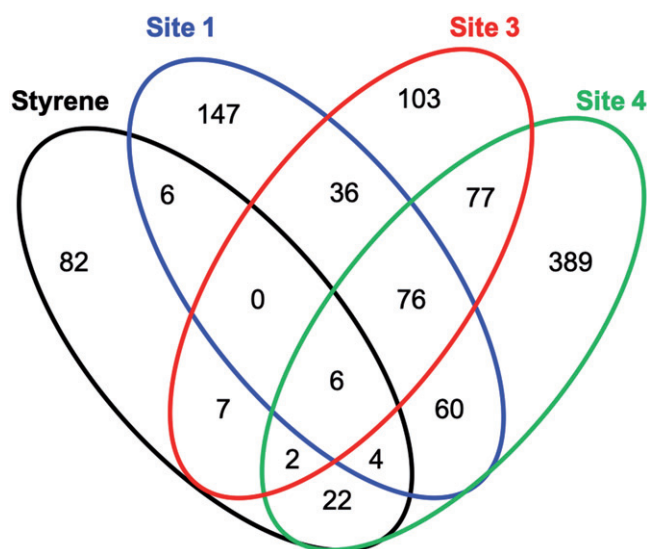


Figure 2. Comparison of proteins found to be significantly altered due to each exposure condition compared to controls (untreated) alveolar epithelial (C10) cells. To be included within the comparison proteins had to have been identified to be statistically altered ($p < 0.05$; $n = 4/\text{group}$). The Venn diagram was created to illustrate similarities and differences in the cellular response to each exposure. The list of specific proteins used to generate the Venn diagram is in Supplemental Table 4.

included aldose reductase, transglutaminase 2, collagen alpha-2(V) chain, fibronectin 1, vascular adhesion molecule 1, and N-acetylglucosamine-6-sulfase (Supplemental Tables 3 and 4). In all exposures, aldose reductase was significantly decreased, while collagen alpha-2(V) chain and vascular adhesion molecule 1 were increased compared to untreated controls (Figure 3). Specifically, CIPP condensates from all worksites significantly reduced aldose reductase levels compared to styrene-alone, while collagen alpha-2(V) levels were significantly increased compared to styrene-alone. Further, worksite 4 induced greater collagen alpha-2(V) levels compared to worksites 1 and 3. Transglutaminase 2 and N-acetylglucosamine-6-sulfase were significantly decreased following exposure to styrene-only; however, exposure to all CIPP condensates resulted in significant increases compared to controls. A significant difference in transglutaminase 2 induction was also observed between CIPP condensates collected from worksites 1 and 4, while N-acetylglucosamine-6-sulfase was significantly different when comparing worksites 1 and 3 with worksite 4 (Figure 3). Fibronectin 1 levels were significantly increased following exposure to styrene-only, and decreased following exposure to all CIPP condensates (Figure 3). Worksite 4 condensate resulted in the most inhibition of fibronectin 1 levels compared to worksites 1 and 3.

Each exposure was also determined to induce distinct alterations in protein levels, with condensate from CIPP worksite 4 being the most unique, with 389 proteins distinctly modified (Figure 2). Together, CIPP condensates altered 76 proteins in common that were not changed following styrene-only exposure (Figure 2). All of these 76 proteins were found to be modulated in the same direction regardless of CIPP worksite (45 up-regulated and 31 down-

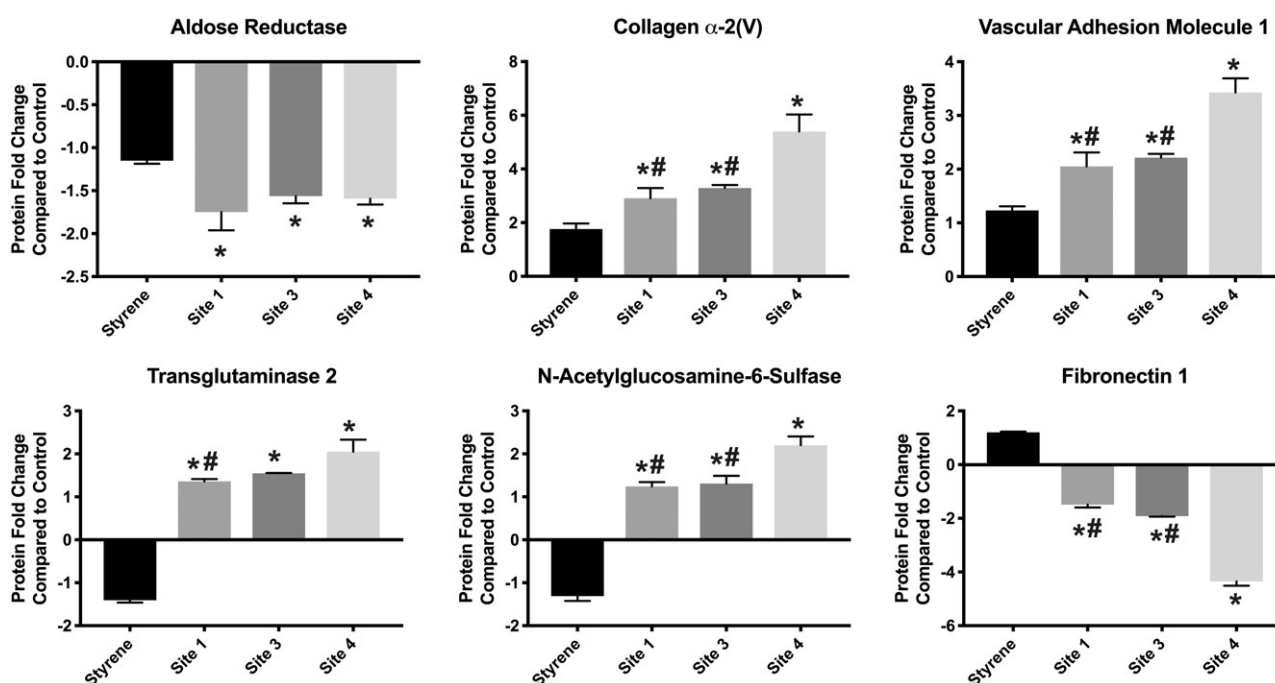


Figure 3. Quantitative differences in proteins determined to be shared following all exposures in alveolar epithelial (C10) cells. Data are presented as protein fold change compared to control (untreated) cells (mean \pm standard error of means; $n = 4/\text{group}$). *Statistical significance compared to styrene-only exposed group (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$). #Statistical significance compared to CIPP worksite 4 response (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$).

Table 2. Gene ontology assessment of CIPP condensate-induced protein alterations in C10 alveolar epithelial cells.

Styrene	Worksite 1	Worksite 3	Worksite 4
(A) Top upstream regulators			
PDGF BB (4.07E-06)	MYC (1.27E-16)	MYC (7.79E-20)	TP53 (2.93E-30)
TP53 (7.62E-06)	KRAS (5.37E-14)	TP53 (6.36E-15)	MYC (8.83E-18)
Zinc (1.33E-04)	D-glucose (3.47E-10)	OSM (2.66E-11)	HNF4A (3.46E-16)
(B) Top enriched diseases and disorders			
Organismal injury and abnormalities (5.67E-03– 5.70E-09; 123)	Organismal injury and abnormalities (3.05E-03– 1.10E-15; 322)	Cancer (2.64E-03– 7.96E-16; 287)	Organismal injury and abnormalities (4.35E-04– 1.57E-23; 595)
Cancer (5.67E-03– 5.70E-09; 122)	Cancer (3.05E-03– 1.10E-15; 317)	Endocrine System Disorders (2.03E-03– 7.96E-16; 251)	Cancer (4.11E-04– 1.57E-23; 586)
Gastrointestinal Disease (5.67E-03– 6.70E-7; 111)	Gastrointestinal Disease (3.03E-03– 1.08E-10; 281)	Organismal Injury and Abnormalities (2.64E-03– 7.96E-16; 291)	Gastrointestinal Disease (4.14E-04– 264E-13; 513)
(C) Top molecular and cellular functions			
Cellular function and maintenance (4.39E-03–2.81E-05; 39)	Cell death and survival (2.69E-03–2.13E-09; 139)	Cell death and survival (2.12E-03–6.06E-13; 129)	Cell death and survival (4.14E-04– 6.59E-21; 265)
Cellular Assembly and Organization (5.67E-03–1.49E-05; 36)	Cellular Function and Maintenance (3.04E-03–4.23E-08; 107)	Cellular Compromise (2.04E-03–5.75E-11; 53)	Cellular Development (4.02E-04–1.18E-11; 158)
Cell Morphology (5.67E-03–2.41E-05; 24)	Cellular Assembly and Organization (3.06E-03–2.74E-08; 98)	Cellular Development (2.30E-03–2.74E-10; 96)	Cellular Growth and Proliferation (4.02E-04–1.18E-11; 153)

regulated) (Supplemental Table 3). Specifically, NADPH cytochrome P450 reductase, gelsolin, cyclin-dependent kinase 2, fatty acid synthase, tensin-2, and prostacyclin synthase were reduced, while calumenin, catalase, signal transducer and transcription activator 6 (STAT6), ribonuclease T2, collagen alpha-1 (I) chain precursor, collagen type III alpha-1 chain, and cold inducible RNA-binding protein were increased (Supplemental Tables 3 and 4). Exposure to CIPP condensates collected at worksites 3 and 4 uniquely shared alterations in 77 proteins that were not altered following exposure to worksite 1 or styrene-only (Figure 2). Worksite 4 shared 22 proteins with styrene that were not found to change with exposure to CIPP condensates from worksites 1 and 3.

Pathway analysis was performed on all proteins found to be significantly altered in C10 cells following individual exposures compared to controls. The proteins identified to be significantly altered were related to a variety of upstream regulators (Table 2(A)). Tumor protein p53 (TP53) was the most significantly identified upstream regulator in our assessment, and was related to exposure to CIPP condensates collected from worksite 4 (Table 2(A)). The myelocytomatosis oncogene (MYC) was the second most significantly identified upstream regulator of protein changes observed in our study (Table 2(A)). Other upstream regulators related to alterations in proteins following exposure included kirsten rat sarcoma viral oncogene (KRAS), hepatic nuclear factor 4 (HNF4A), platelet-derived growth factor beta polypeptide b (PDGFBB), oncostatin-M (OSM) and glucose. The top enriched diseases and disorders associated with the proteins altered following exposures included organismal injury and abnormalities, cancer, gastrointestinal disease (styrene-only, worksite 1 and worksite 4), and cancer (worksite 3 only) (Table 2(B)). Further, the top molecular and cellular functions related to the altered proteins included cellular function and maintenance, cellular assembly and organization, cell morphology, cellular development and cell death and survival (Table 2(C)). The top network for each exposure were as follows: RNA post-transcriptional modification,

cancer, connective tissue disorders for styrene-only; cell morphology, cellular assembly and organization, auditory disease for worksite 1 condensate; RNA post-transcriptional modification, cell morphology, cellular assembly, and organization for worksite 3 condensate; and cancer, gastrointestinal disease, hepatic system disease for worksite 4 condensate (Supplemental Figure 4).

To further evaluate responses specific to CIPP condensate exposures, the 76 proteins determined to be altered in common between all condensates and not styrene only were examined (Figure 2). Although all were identified to be altered similarly (Supplemental Table 3), quantitative differences were identified between CIPP condensates (Figure 4). For example, changes in collagen, type 1 alpha 1, stat6, and NADPH P450 reductase were exacerbated by exposure to worksite 4 condensate compared to worksites 1 and 3, while all altered NFκB2 similarly (Figure 4). When these styrene-independent processes were globally examined via Ingenuity Pathway analysis, immunological disease, organismal injury and abnormalities, cell death and survival was determined to be the primary disease pathway induced.

Proteomic assessment of alveolar macrophage (RAW) responses to CIPP condensate exposure

Alveolar macrophages (RAW) also demonstrated differential protein expression following CIPP condensate or styrene-only exposures compared to controls (Table 3; Supplemental Table 5 contains a list of proteins used to generate Table 1). It was determined that the CIPP condensate collected from worksite 4 induced the greatest number of changes in protein expression while worksite 1 induced the least. The majority of changes in protein levels were reduced compared to control for worksite 1, while most were increased for worksites 3 and 4 (Table 3). Exposure to styrene-only demonstrated an equal number of increased and decreased proteins compared to control. A number of differentially expressed proteins were identified between individual CIPP worksite condensates and RAW cells exposed to only

styrene. Specifically, worksite 4 demonstrated the most differences when compared with styrene, whereas the response from worksite 1 was the most similar to styrene-only. Worksite-specific responses were also identified in terms of protein responses. CIPP condensates collected from worksites 3 and 4 were determined to be the most different, whereas worksites 1 and 3 were the most similar (Table 3).

To globally compare alterations in proteins due to exposures, a Venn diagram was produced (Figure 5, Supplemental Table 6). Exposure to styrene-only as well as all CIPP worksite condensates was determined to alter 6

proteins in common, advillin, methanethiol oxidase, glyoxylate reductase/hydroxypyruvate reductase, mitochondria ribosomal recycling factor, ran GTPase activating protein 1, and cathepsin S. Advillin and mitochondria ribosomal recycling factor were determined to be increased following all exposures, while others were decreased following exposures (Figure 6, Supplemental Tables 5 and 6). Specifically, exposure to all CIPP condensates resulted in increased advillin levels compared to styrene alone. Methethiol oxidase protein levels were statistically decreased following exposure to condensates collected at worksite 1 and 4 compared to styrene only. Further, worksite 4 demonstrated significantly elevated levels of methethiol oxidase compared to worksite 3. Mitochondrial ribosomal recycling factor was elevated following exposure to worksite 4 condensate compared to styrene-only exposed RAW cells. Ran GTPase activating protein 1 levels were reduced in cells exposed to condensate from worksite 4 as compared to styrene only. Further, worksite specific alterations in the magnitude of Ran GRPase activating protein 1 levels were observed as worksite 4 reduced

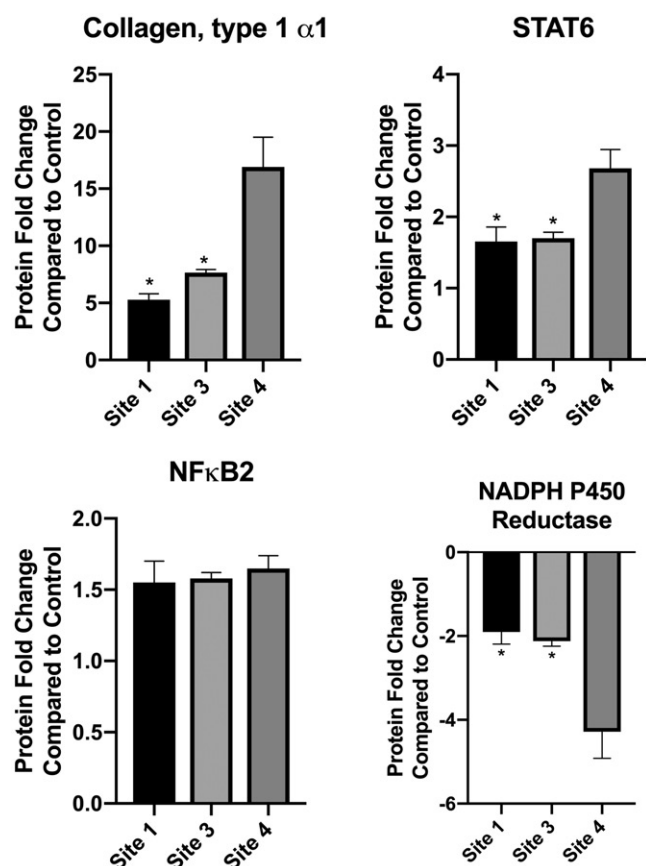


Figure 4. Assessment of proteins altered due to CIPP condensate exposure independent of styrene in alveolar epithelial (C10) cells. Quantitative differences in proteins shared only between CIPP worksite condensate exposures. Data are presented as protein fold change compared to control (untreated) cells (mean \pm standard error of means; $n = 4/\text{group}$). *Statistical significance compared to worksite 4 (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$).

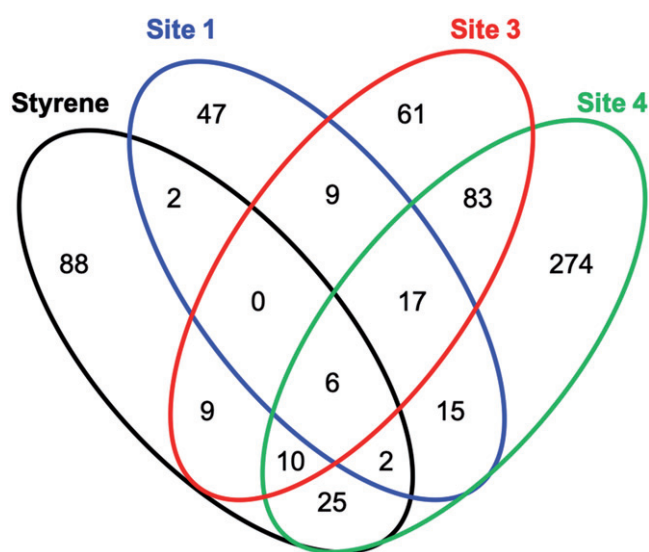


Figure 5. Comparison of proteins found to be significantly altered due to each exposure condition compared to controls (untreated) alveolar macrophages (RAW). To be included within the comparison proteins had to have been identified to be statistically altered ($p < 0.05$; $n = 4/\text{group}$). The Venn diagram was created to illustrate similarities and differences in the cellular response to each exposure. The list of specific proteins used to generate the Venn diagram is in Supplemental Table 6.

Table 3. Comparisons of proteins altered between exposure groups in RAW macrophages.

RAW Cells	Styrene	Site 1	Site 3	Site 4
Control vs	142 ↑ 71 ↓ 71	98 ↑ 47 ↓ 51	195 ↑ 99 ↓ 96	432 ↑ 220 ↓ 212
Styrene vs		127 ↑ 52 ↓ 75	317 ↑ 167 ↓ 150	436 ↑ 211 ↓ 225
Site 1 vs			69 ↑ 41 ↓ 28	140 ↑ 87 ↓ 53
Site 3 vs				192 ↑ 88 ↓ 104

All proteins included are statistically significant for the comparison ($p < 0.05$).

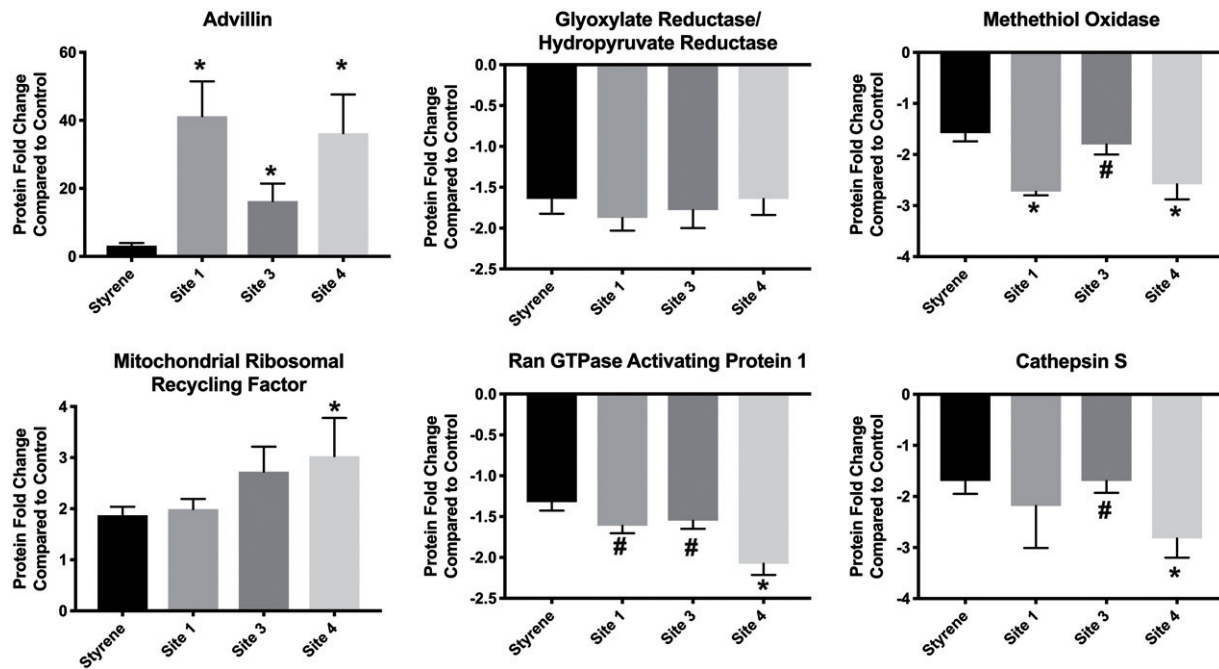


Figure 6. Quantitative differences in proteins determined to be shared following all exposures in alveolar macrophages (RAW). Data are presented as protein fold change compared to control (untreated) cells (mean \pm standard error of means; $n = 4$ /group). *Statistical significance compared to styrene-only exposed group (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$). #Statistical significance compared to CIPP worksite 4 response (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$).

Table 4. Gene ontology assessment of CIPP condensate-induced protein alterations in RAW macrophages.

Styrene	Worksite 1	Worksite 3	Worksite 4
(A) Top upstream regulators			
MYCN (1.40E-05)	PRL (3.60E-07)	MYC (1.7E-10)	MYC (4.66E-12)
HNF4A (2.04E-05)	FOS (6.81E-07)	APOE (6.58E-10)	TP53 (9.58E-12)
ESR1 (6.65E-04)	CTSB (5.40E-06)	Interferon alpha (2.42E-09)	Ciprofloxacin (3.98E-11)
(B) Top enriched diseases and disorders			
Organismal injury and abnormalities (1.56E-02–1.26E-08; 133)	Cancer (8.52E-03–3.56E-10; 94)	Organismal injury and abnormalities (1.15E-03–3.86E-08; 181)	Organismal injury and abnormalities (8.89E-04–1.08E-11; 399)
Cancer (1.53E-02–1.26E-08; 132)	Organismal injury and abnormalities (8.52E-03–3.56E-10; 94)	Cancer (1.07E-03–3.86E-08; 179)	Cancer (8.67E-04–1.08E-11; 394)
Endocrine system disorders (1.22E-02–1.67E-05; 104)	Endocrine system disorders (8.52E-03–4.38E-06; 82)	Inflammatory response (1.14E-03–1.70E-11; 72)	Endocrine system disorders (5.95E-04–5.48E-11; 328)
(C) Top molecular and cellular functions			
Cell Death and Survival (1.36E-02–1.76E-06; 60)	Cell Death and Survival (8.52E-03–1.47E-05; 44)	Cell Death and Survival (1.16E-03–3.06E-08; 79)	Cell Death and Survival (8.89E-04–1.26E-12; 178)
Cellular Compromise (1.22E-02–1.58E-08; 26)	Cell-To-Cell Signaling and Interaction (8.52E-03–2.60E-06; 24)	Cell-To-Cell Signaling and Interaction (1.11E-03–6.86E-11; 52)	Protein Synthesis (5.28E-04–5.38E-12; 98)
Protein Synthesis (1.22E-02–2.33E-08; 26)	Cellular Compromise (8.52E-03–1.39E-09; 21)	Molecular Transport (8.49E-04–4.36E-08; 51)	Cellular Compromise (8.11E-04–7.78E-19; 79)

levels beyond worksite 1 and 3. Cathepsin S was significantly reduced compared to styrene only following exposure to worksite 4 condensates. This reduction was exacerbated in comparison to the response observed following exposure to condensate from worksite 3. Each exposure demonstrated alterations in distinct proteins, with the CIPP condensate from worksite 4 being the most unique (Figure 5). A total of 17 proteins (5 up-regulated and 12 down-regulated) were determined to be altered in all RAW cells exposed to CIPP worksite condensates but not changed due to styrene exposure only. These proteins included cyclin-dependent kinase 4, CD14 antigen, E-selectin ligand 1, perilipin, tensin-3, phosphoserine phosphatase, and others. CIPP condensate from worksite 4 shared 25 proteins with styrene-

only that were not changed with the other worksite condensate exposures.

Pathway analysis was performed on all proteins found to be significantly altered following individual exposures compared to controls for RAW cells. The proteins identified to be significantly altered were related to a variety of upstream regulators, with MYC being the most significantly identified for worksite 4 (Table 4(A)). Other upstream regulators included TP53, apolipoprotein E (APOE), HNF4A, and others. The top enriched diseases and disorders associated with the proteins altered following exposures include organismal injury and abnormalities, cancer, endocrine system disorders (styrene only, worksite 1 and worksite 4), and inflammatory response (worksite 3) (Table 4(B)). The top

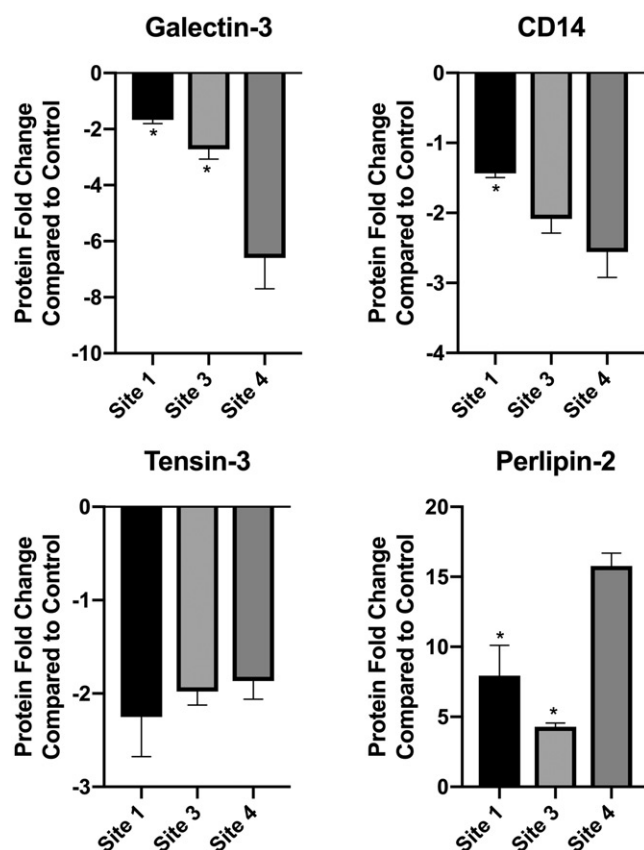


Figure 7. Assessment of protein altered due to CIPP condensate exposure independent of styrene in alveolar macrophages (RAW). Quantitative differences in proteins shared only between CIPP worksite condensate exposures. Data are presented as protein fold change compared to control (untreated) cells (mean \pm standard error of means; $n = 4$ /group). *Statistical significance compared to worksite 4 (one-way ANOVA with a Dunnett's *post-hoc* analysis, $p < 0.05$).

molecular and cellular functions related to the proteins altered in RAW cells included cell death and survival, cellular compromise, protein synthesis, cell-to-cell signaling and interaction, and molecular transport (Table 4(C)). The top network for each exposure were as follows: RNA post-transcriptional modification, cell cycle, DNA replication, recombination, and repair for styrene only exposed RAW cells; cellular movement, cell death and survival, cell morphology for worksite 1 condensate; infectious diseases, antimicrobial response, inflammation response for worksite 3 condensate; and infectious diseases, cell morphology, organismal development for worksite 4 condensate (Supplemental Figure 4).

To further evaluate RAW cell responses that were specific only to CIPP condensate exposures, the 17 proteins determined to be altered in common between all condensates and not styrene only were examined (Figure 5). Although all were similarly altered in terms of directionality differences in abundance were identified between CIPP worksite condensates (Figure 7 and Supplemental Table 5). For example, changes in galectin-3, perlpin-2, and CD14 were exacerbated by exposure to worksite 4 condensate compared to worksites 1 and 3, while all altered tensin-2 similarly (Figure 7). When these styrene-independent processes were globally examined via Ingenuity Pathway analysis, cardiovascular disease, inflammatory disease, organismal injury, and abnormalities were determined to be the primary disease pathway induced.

Discussion

In this study, the authors investigated potential toxicity related to the CIPP procedure by utilizing previously collected and characterized worksite air emission condensates. This examination was performed in mouse alveolar type II (C10) cells and alveolar macrophages (RAW) to understand lung cell responses following inhalation. To investigate the toxicity of a complex mixture and for appropriate comparisons to be made, samples were normalized based on styrene concentration. Normalization allowed for comparisons between CIPP worksites as well as for an understanding of styrene-specific responses through the use of cells exposed styrene alone. Overall, results demonstrated differential cytotoxicity, alterations in gene expression, and variations in protein responses between worksites that were not solely styrene-dependent.

Although a number of public health incidents have occurred near CIPP worksites, there exists limited information regarding CIPP emission exposure hazards. All of the three condensate samples utilized within our toxicity evaluation were from worksites utilizing the same styrene-based resin (L713) and installed by the same contractors during the same 3-day period. However, differences were identified in emission contents likely due to variations in the onsite CIPP manufacturing process. As we have recently reported, each CIPP was exposed to steam resulting in a different maximum temperature and duration of cure (Ra et al. 2019). No prior studies were found that documented the thermal manufacturing process with chemical air emissions analysis. Differential chemical contents of the emissions likely impact toxicological responses such as those observed in the present study. The condensed material from CIPP worksite 4 was determined to induce the greatest cellular responses (cytotoxicity and proteomic alterations) compared to other CIPP worksite condensate samples. The primary component of all collected CIPP worksite condensates was styrene. Therefore, because styrene was held constant, variations in observed responses were likely dependent on non-styrene components of the condensates.

Previous characterization of the worksite condensates revealed worksite 4's specimen contained greater amounts of benzaldehyde, benzoic acid (oxidized benzaldehyde), phenol, and 1-tetradecanol compared to the other sites (Teimouri Sendesi et al. 2017). Butylated hydroxytoluene (BHT) was present within condensates collected from worksites 1 and 3, while tripropylene glycol diacrylate (TGDA) was only present in condensate from site 4. In comparison to worksites 3 and 4, the condensate from worksite 1 contained the least amount of benzaldehyde, benzoic acid, and phenol. Condensate from worksite 1 was the only sample to contain dibutyl phthalate (DBP). Ultimately, these emissions produced by CIPP were unique between worksites and have not been previously evaluated in terms of hazard. Specifically, the exacerbated responses demonstrated by cells following exposure to worksite 4 condensates may be explained by these differences in components. Past research has demonstrated that many of the condensate components elevated in worksite 4 condensate (benzaldehyde, phenol, 1-

tetradecanol, TGDA) have the ability to act as respiratory, ocular, and/or skin irritants (Andersen 2006; National Toxicology Program 2011; Sati et al. 2011; Information NCfB 2019a; Information NCfB 2019b; Information NCfB 2019c; Information NCfB 2019d). Based upon our previous characterization of emissions from worksite 4, benzaldehyde levels were determined to be at 0.7% of styrene (Teimouri Sendesi et al. 2017). Therefore at the styrene concentration of 1000 ppm used for examination of cytotoxicity in our study, benzaldehyde levels were ~7 ppm. Previously, lymphocytes exposed *in vitro* to concentrations of benzaldehyde ranging from 1–50 ppm exhibited concentration-dependent increases in apoptosis (Ulker et al. 2013). Therefore increased benzaldehyde levels in worksite 4 condensate could contribute to the cytotoxicity we observed. Additionally, phenol levels were determined to be elevated in worksite 4 condensates. Phenol has been shown to be noncytotoxic to erythrocytes at concentrations up to 100 ppm (Michalowicz et al. 2018). Based on our calculations the phenol content of worksite 4 emissions was 0.25% of styrene (Teimouri Sendesi et al. 2017). This means at 1000 ppm styrene, the phenol concentration was ~2.5 ppm, which likely did not contribute significantly to the cytotoxicity we observed. Styrene, although held constant in our study, is known to cause elevations in markers of lung toxicity and disease, decrements in lung function, and oxidative stress in exposed workers (Sati et al. 2011). Further, it has been classified by the National Toxicology Program as reasonably anticipated to be a human carcinogen (National Toxicology Program 2011). Exposures at worksites are more complex than the condensates collected and utilized in our current study. During onsite monitoring of CIPP worksites, additional chemicals were determined in the CIPP emissions that were not detected in the collected condensates such as methylene chloride and others (Ra et al. 2019). The presence of these additional chemicals may further influence toxicity following inhalation. CIPP emissions represent a complex mixture of chemicals and exposure responses likely cannot be separated based upon individual chemical component contributions.

This study utilized two cell lines representative of cells that would interact with CIPP emissions following inhalation: alveolar epithelial cells and alveolar macrophages. Overall, the proteomic evaluation demonstrates variations in cellular responses to these exposures. Specifically, only 6 proteins were found to be modified in common between styrene-only exposed cells and all CIPP worksite condensates in both C10 and RAW cells. None of these proteins were found to be shared between cell types, demonstrating specific cellular responses to exposures. CIPP condensate exposure was determined to exacerbate these changes in proteins compared to styrene-only exposure based upon calculated fold changes. Advillin was determined to have the highest fold change and exhibit the greatest differential between styrene-only and CIPP condensate exposures. Advillin is an actin regulatory protein that belongs to the gelsolin/villin family and is involved in macrophage phagocytosis and cytoskeletal remodeling (Shibata et al. 2004;

Zhang et al. 2008). Advillin was not found to be significantly modified in alveolar epithelial cells due to any exposure condition. The elevation of advillin only in macrophages and not alveolar epithelial cells within our study suggests that it is a biomarker specific for innate immune cell activation within the lung. Of the chemicals identified within the CIPP emissions, to date, none have been linked in studies to altered advillin expression. However, advillin is known to be elevated during active macrophage phagocytosis, suggesting that the particulates present within the condensates are being internalized by the macrophages (Tan et al. 2015). Conversely, transglutaminase 2 was determined to only be modified in alveolar epithelial cells but not macrophages. Interestingly, styrene-only exposure was determined to reduce transglutaminase 2 protein expression, whereas all CIPP worksite condensates enhanced expression. This demonstrates that elevation of transglutaminase 2 may be a biomarker specific for CIPP emission exposure and responses which is not sensitive to styrene alone. Transglutaminase 2 is a cross-linking enzyme that has a role in inflammation and the development of fibrotic disease via extracellular matrix remodeling (Beninati et al. 2017; Penumatsa et al. 2017; Szondy et al. 2017). Research has demonstrated that lung transglutaminase 2 levels correlate with obstructive pulmonary disease severity, as well as decrements in lung function (Ohlmeier et al. 2016). The elevation of transglutaminase 2 in alveolar epithelial cells following exposure to CIPP condensates and not styrene alone suggests that CIPP emission exposures have the potential to be more fibrogenic than individual exposure to styrene. Additionally, vascular adhesion molecule 1, a marker of inflammation, was significantly elevated due to CIPP condensate exposure compared to styrene-only in epithelial cells. Vascular adhesion molecule 1 is involved in the recruitment of inflammatory cells to sites of exposure and suggests an exacerbated pro-inflammatory response due to CIPP exposure compared to styrene-alone. Together these findings identify the potential of CIPP exposures to modulate cellular immune responses. Although proteins were shared between cells exposed to styrene alone and CIPP emissions, alterations were observed in magnitude and directionality of responses. This further demonstrates that non-styrene components of the CIPP emission are of importance in terms of biological responses following the exposure.

All protein alterations were globally evaluated to discover biological pathways that may be disrupted due to CIPP emission exposure. This comprehensive analysis was performed due to the lack of established toxicity data regarding exposures to CIPP emissions and is necessary for a broad understanding of biological implications. Upstream regulator analysis was used to elucidate mediators controlling the observed proteomic alterations. Specific upstream regulators found to be modified included the proto-oncogene, MYC, and the tumor suppressor, p53. Identification of these regulators suggests that CIPP emission exposure may modify normal cell cycle progression and induce cellular stress. Previous evaluation of styrene toxicity has demonstrated the induction c-myc expression in HepG2 cells while exposed

mice did not demonstrate gene expression changes consistent with activation of p53 (Diodovich et al. 2006; Andersen et al. 2017). However, exposure to the complex CIPP emissions was found to more significantly engage MYC and p53 compared to styrene alone. The presence of cancer as a top disease as well as cell death and survival as a top molecular and cellular function altered is consistent with elevated MYC and p53 signaling. Condensate from worksite 4 was determined to induce the most significant alterations in proteins associated with these pathways, likely due to its chemical composition. As discussed previously, worksite 4 emissions contained larger amounts of benzaldehyde, benzoic acid, phenol, and 1-tetradecanol compared to the other sites. To date information is lacking in C10 and RAW cells evaluating the induction of MYC and p53 mediated cancer pathways following exposure to these specific chemical and combination of chemicals. Therefore, connections cannot be made between elevations in these individual chemicals within worksite 4 emissions and cellular induction of MYC and p53 pathways. Ultimately, the initiation of pathways suggests that cancer-related endpoints specifically related to signaling via MYC and p53 should be evaluated in future studies assessing CIPP emission toxicity.

Distinct alterations in proteins were identified between cells exposed to CIPP emissions from different worksites, with worksite 4 condensates inducing the greatest number of unique proteins. It is likely that dissimilarities in CIPP emissions result from modifications in operational parameters, environmental factors, and batch-to-batch variations in the onsite formulation of resin materials. Although many proteins were found to be uniquely altered due to variations between worksites, a number of proteins (76 in alveolar epithelial cells and 17 in macrophages) were identified to be altered following all CIPP exposures and not following exposure to styrene alone. These alterations represent cellular responses that are styrene-independent while also being common to all CIPP emissions and could be considered as potential targets and/or general biomarkers of CIPP emission exposures. For example, gelsolin was reduced in C10 cells exposed to CIPP condensates, but unchanged due to exposure to styrene alone. Gelsolin is a regulator of actin and has been shown to inhibit inflammatory factors, therefore the decrease observed in our study may consequentially exacerbate the inflammatory response (Osborn et al. 2007). Calumenin, an endoplasmic reticulum calcium-binding protein, was increased in all C10 cells exposed to CIPP condensate. Increased calumenin levels have been shown to correlate with the increased metastatic potential of lung cancer cells and have been proposed as a target of cancer diagnosis and/or treatment (Nagano et al. 2015). These CIPP specific alterations in protein expression (76 proteins in C10 cells and 17 proteins in RAW), when viewed globally through gene ontology analysis, demonstrated commonalities in potential diseases and disorders related to all CIPP exposures. Specifically, C10 cells responded to all CIPP emission exposures by induction of the immunological disease, organismal injury and abnormalities, cell death and survival disease pathway. Individual protein components of

this pathway include collagen type 1, stat6, NFkB2, NADPH P450 reductase, and others. In general, the majority of the individual components of this pathway were induced to a greater degree by CIPP worksite 4 emissions. All proteins modified by CIPP emissions in common were found to induce the cardiovascular disease, inflammatory disease, organismal injury, and abnormalities disease pathway in RAW cells. Individual protein components of this pathway include galectin-3, CD14, tensin-3, perilipin, and others. Again, CIPP worksite 4 emissions were determined to exacerbate RAW responses in the majority of these shared proteins demonstrating greater engagement of the pathway. Alterations in proteins involved in the immune system are a commonality between these pathways induced by all evaluated CIPP emissions in both C10 and RAW cells. This suggests that the non-styrene components of the CIPP emissions may exacerbate immune responses following inhalation exposures. Further, these alterations in immune pathways may represent a biological response that is common between CIPP emission exposures and could be used as a signature of exposure.

Together, these initial findings demonstrate the potential for health effects following exposure to emissions from CIPP worksites and the need for future investigations. Specifically, CIPP emission exposures appear to modify proteins related to respiratory disease pathways. The use of cell culture systems which do not recapitulate the complex respiratory system, and the potential for species-specific differences that occur between humans and mice are limitations of the present study. However, the pathways determined to be altered in our cell culture-based assessment are conserved between mice and humans supporting the ability to translate our findings to human exposures. The complexity of the multi-phase emissions, their variable chemical composition, and the transient nature of CIPP worksites present unique challenges in toxicity assessment. Specifically, the current toxicity assessment did not utilize freshly generated samples, as they were stored following onsite collection. There may be differences in terms of the chemical components in the air at CIPP worksites and those collected in the condensates utilized in our study. As previously mentioned, the first known discovery and characterization of the multi-phase mixture were conducted in 2017 (Teimouri Sendesi et al. 2017) and very little information about emission characteristics exists (Ra et al. 2019). GC/MS and NMR methods applied were limited in the chemicals they could detect, and some undetected chemicals may contribute to worksite exposures and toxicity. Subsequent environmental sample characterizations using different methods have also revealed acetaldehyde, acetophenone, benzene, methylene chloride, (1-methylethenyl)-benzene, nonanal, and 1-pentanol in Tedlar Bag and/or sorbent tube air samples, which were not found in the collected condensate samples (Ra et al. 2019). Several of these chemicals were identified inside the new plastic liner further confirming their presence during liner creation. Based on these additional environmental sampling data the CIPP worksite exposures may be more complex than the condensates we

evaluated within our current study. Due to the transient nature of worksites and variations in the CIPP process, there is also a potential for the introduction of unique emission components between worksites due to cross-chemical contamination of equipment (Ra et al. 2019).

Controlled systematic studies are needed to understand how operational procedures (i.e. resin material, curing process, time, temperature, emission capture systems, emission monitoring, etc.) influence the potential for adverse health effects. Future studies should examine pulmonary and systemic responses following acute and chronic exposures in animal models that represent occupational and environmental exposures. Based upon the transient nature of CIPP worksites and variability in curing procedures these future studies should utilize laboratory-scale curing and exposure chambers to allow for greater control and reproducibility. These toxicity studies should be performed in conjunction with ongoing exposure assessment studies at actual worksites. Our current study evaluated emissions captured from three CIPP worksites and may not be representative of all worksites which vary on multiple parameters including resin types, environmental conditions, and installation practices such as curing methods (steam or UV), curing time, and curing temperature. It is unclear at this time how alterations in these CIPP parameters may influence emissions and biological responses. Based on our data demonstrating styrene-independent responses, alterations in chemical components of the initial resin material may have the greatest impact on emissions. Resins utilized for CIPP installations are similar to those used for boat manufacturing processes (Crandall and Hartle 1985; Carlo et al. 2007). Prior studies indicated that resins used in boat manufacturing caused liver toxicity and increased the risk of lung disease in exposed workers (Brodtkin et al. 2001; Strafella et al. 2013; Nett et al. 2017; Bertke et al. 2018, 66). These findings resulted in the implementation of controls to limit inhalation exposures in workers.

In conclusion, the CIPP repair process represents a unique exposure scenario due to the transient nature of the worksite, proximity to sensitive populations, and variability in the procedure itself. Due to the lack of foundational information regarding toxicological responses, our current study was necessary to identify cellular processes and pathways that might be perturbed due to CIPP emission exposures. Based on our findings, future studies should examine CIPP emission-induced modifications in pathways associated with cytotoxicity and cell injury, immune responses, and cancer. Further, while styrene is often the primary proposed emission component to evaluate at CIPP worksites, our study has demonstrated that toxicity is not dependent on styrene alone. Therefore exposure assessments performed at CIPP worksites may benefit from a more comprehensive evaluation that includes additional emission components such as benzaldehyde, phenol, 1-tetracecanol, particulates, and others. Currently, studies are underway to develop technologies/practices to monitor and mitigate CIPP worksite emissions via their capture as well as laboratory-based

toxicity assessment to examine biological implications of CIPP-emission exposures.

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Disclosure statement

AJW, BEB, JAH, and SMTS are named in a patent application (PCT/US18/28173) filed April 18, 2018 by the Purdue Research Foundation. The patent application pertains to the technologies for capturing, identifying, analyzing, and addressing emissions that are potentially hazardous to the environment and humans.

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