Wildfires and extracellular vesicles: Exosomal MicroRNAs as mediators of cross-tissue cardiopulmonary responses to biomass smoke

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Declaration of Competing Interest

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

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2022.107419.
Abstract

Introduction: Wildfires are a threat to public health world-wide that are growing in intensity and prevalence. The biological mechanisms that elicit wildfire-associated toxicity remain largely unknown. The potential involvement of cross-tissue communication via extracellular vesicles (EVs) is a new mechanism that has yet to be evaluated.

Methods: Female CD-1 mice were exposed to smoke condensate samples collected from the following biomass burn scenarios: flaming peat; smoldering peat; flaming red oak; and smoldering red oak, representing lab-based simulations of wildfire scenarios. Lung tissue, bronchoalveolar lavage fluid (BALF) samples, peripheral blood, and heart tissues were collected 4 and 24 h post-exposure. Exosome-enriched EVs were isolated from plasma, physically characterized, and profiled for microRNA (miRNA) expression. Pathway-level responses in the lung and heart were evaluated through RNA sequencing and pathway analyses.

Results: Markers of cardiopulmonary tissue injury and inflammation from BALF samples were significantly altered in response to exposures, with the greatest changes occurring from flaming biomass conditions. Plasma EV miRNAs relevant to cardiovascular disease showed exposure-induced expression alterations, including miR-150, miR-183, miR-223-3p, miR-30b, and miR-378a. Lung and heart mRNAs were identified with differential expression enriched for hypoxia and cell stress-related pathways. Flaming red oak exposure induced the greatest transcriptional response in the heart, a large portion of which were predicted as regulated by plasma EV miRNAs, including miRNAs known to regulate hypoxia-induced cardiovascular injury. Many of these miRNAs had published evidence supporting their transfer across tissues. A follow-up analysis of miR-30b showed that it was increased in expression in the heart of exposed mice in the absence of changes to its precursor molecular, pri-miR-30b, suggesting potential transfer from external sources (e.g., plasma).

Discussion: This study posits a potential mechanism through which wildfire exposures induce cardiopulmonary responses, highlighting the role of circulating plasma EVs in intercellular and systems-level communication between tissues.

Keywords
Exosomes; Extracellular vesicles; Environmental exposures; Mixtures; Systems biology; Wildfires

1. Introduction

Wildfires are increasing in both prevalence and intensity worldwide, a disastrous scenario that is steadily becoming more common alongside global climate change (Hurteau et al. 2014; UNISDR 2017; Westerling et al. 2006). This time-sensitive issue is significantly impacting public health, as smoke emitted from wildfires is known to cause respiratory disease, cardiovascular events, and mortality, among other adverse outcomes (Black et al. 2017b). The cardiopulmonary system is particularly vulnerable to the effects of wildfire smoke, as identified through several recent studies in humans. Wildfire-associated effects relevant to the heart include cardiac arrest, heart failure, and ischemic heart disease (Chen et al. 2021; Delfino et al. 2009; Haikerwal et al. 2015; Hanigan et al. 2008; Rappold et al. 2011; Wettstein et al. 2018). Effects with direct relevance to the lung include asthma,
bronchitis, dyspnea, chronic obstructive pulmonary disease, and increased risk of respiratory infections (Delfino et al. 2009; Dohrenwend et al. 2013; Hanigan et al. 2008; Johnston et al. 2006; Rappold et al. 2011). Despite these recognized relationships, the specific mechanisms that underlie such cardiopulmonary effects remain to be fully established, and there is an overall lack of knowledge surrounding the potential role circulating molecules in the blood may have in wildfire-associated effects.

An understudied mechanism that may be impacting cross-tissue toxicity associated with wildfire smoke exposures centers around extracellular vesicles (EVs). EVs are small, membrane-bound particles that play important roles in intercellular communication (Thery et al. 2018). These particles are derived from parent cells and, once released, can travel and communicate with nearby or distant cells, either imparting beneficial, neutral, or detrimental effects on recipient cells (Fu et al. 2020). Effects include the alteration of target cell gene/protein expression which can impact molecular pathways influencing cellular phenotypes, biological functions, and overall tissue pathology. The specific content of EVs depend on their cell of origin, but generally contain nucleic acids, proteins, amino acids, lipids, and metabolites, which remain functional and may be transferred to target cells (Carberry et al. 2022; Fu et al. 2020; Zhang et al. 2017).

EVs have been reported in many biological fluids, including blood. Peripheral blood EVs have the capacity to target distal tissues and alter biology. For example, miRNAs in peripheral plasma EVs have been shown to transfer into cardiomyocytes and alter cell signaling and function, in rodents and in vitro (Minghua et al. 2018). EV-based therapies are even being proposed for the prevention/treatment of cardiovascular disease, as evaluated based on blood delivery mechanisms; though there are notable concerns regarding side effects on other tissues (Davidson et al. 2019). In terms of environmental exposures, it is clear that the investigation of EVs will help to elucidate important intercellular communication mechanisms and associated disease etiologies relevant to public health (Carberry et al. 2022).

EVs contain different types of molecules, including microRNAs (miRNAs), which have a recognized role in intercellular communication (Makarova et al. 2016). miRNAs are small RNA molecules approximately 22 nucleotides in length, on average, that can significantly impact the regulation of gene expression via epigenetic processes (Clark and Rager 2020). miRNAs are also important posttranscriptional regulators of gene expression that alter final protein expression patterns by degrading target mRNA transcripts or inhibiting mRNA translation, largely through the (mi)RNA-induced silencing complex (RISC). These processes are highly reliant upon miRNAs recognizing target mRNA molecules through base pair sequence homologies and other complementary structural attributes (Clark and Rager 2020; Duchaine and Fabian 2019). Expression profiles of miRNAs have been shown to be disrupted by common air pollutants, including constituents present in wildfire smoke emissions, such as formaldehyde, benzene, and particulate matter, across model systems (humans, animal models, and in vitro models) (Cheng et al. 2020; Fenga et al. 2016; Rager et al. 2013; Rager et al. 2014; Rager et al. 2011b). A recent pilot study evaluating indoor biomass smoke exposures implemented gene-specific approaches to evaluate select miRNAs in circulating plasma, and found that the expression levels of two miRNAs (miR-126 and
miR-155) were increased in women cooking with wood vs women cooking with gas (Ruiz-Vera et al. 2019). The current research is among the first to identify relationships between miRNA expression signatures in circulating blood EVs and emissions from wildfire-relevant biomass burns.

Previous studies have shown that wildfire-relevant exposure conditions can cause inflammation in the lung and circulating blood, changes in overall immune function, and tissue injury within the lung and heart (Black et al. 2017a; Kim et al. 2019; Kim et al. 2014; Kim et al. 2018; Swiston et al. 2008). Molecular mechanisms that are known to lead to tissue injury and inflammation include changes in hypoxia and cell stress (Giussani et al. 2014; Lee et al. 2019; Li et al. 2019a). Hypoxia is characterized as a condition in which oxygen is limited, and when prolonged, hypoxia elicits oxidative cellular stress. A critical pathway known for its role in response to hypoxia is the hypoxia inducible factor 1 subunit alpha (HIF1A) pathway, where HIF1A activation leads to adaptive responses, including tissue protection and adaptation (Lee et al. 2019). HIF1A signaling notably overlaps with inflammation and other responses relevant to tissue injury and repair and has demonstrated functionality and disease implications in both the lung and heart (Lee et al. 2019). Because hypoxia and cell stress-related signaling play such critical roles in cell health and injury throughout the cardiopulmonary system, this study set out to evaluate the potential roles of these pathways in response to wildfire exposure conditions across tissues. We tested the specific hypothesis that miRNAs within peripheral blood EVs show differential expression in response to variable biomass smoke exposures, and that these patterns associate with altered hypoxia and cell stress signaling changes in the lung and heart.

2. Methods

2.1. Exposure conditions to simulate wildfire exposure scenarios

We previously developed laboratory methods to simulate variable wildfire exposure conditions through the burning of biomass materials at different combustion settings (Kim et al. 2019; Kim et al. 2014; Kim et al. 2018; Rager et al. 2021). For this study, peat and northern red oak were selected as prototypic fuels due to their prevalence and known associations with both pulmonary and cardiovascular outcomes (Burbank et al. 2019; Ghio et al. 2012; Kim et al. 2019; Kim et al. 2014; Kim et al. 2018; Rebuli et al. 2019). Peat represents an accumulation of partially decayed vegetation or organic matter, and gathers in environments where biological decay rates are higher than the capacity the environment has to destroy or recycle the biological decay (Stracher et al. 2015). Peat land ecosystems are common in areas throughout the world, including cold environments (e.g., the Arctic), temperate environments (e.g., Ireland), and subtropic environments (e.g., throughout the swamp and everglade regions across the southeastern U.S.). Due to their pervasive nature, smoldering peat fires are the largest type of fire on earth and are estimated to account for 15% of annual global greenhouse gas emissions (Stracher et al. 2015). Northern red oak is one of the most dominant species of biomass in North America, with high prevalence throughout the eastern and central parts of the U.S. (Abrams 1992). Red oak is also becoming more prevalent over time in areas prone to wildfire, as wildlands that experienced fire tend to favor the regeneration and recruitment of this type of biomass over
other more shadetolerant species (Dey and Guyette 2000). Biomass fuels used in this study were specifically pocosin peat acquired from the East coast of North Carolina and northern red oak acquired from the Air and Energy Management Division at the U.S. Environmental Protection Agency (US EPA), as previously detailed (Kim et al. 2018).

These biomasses were evaluated under either flaming or smoldering conditions to capture effects resulting from different combustion environments, yielding four separate biomass smoke exposures (i.e., flaming peat, smoldering peat, flaming red oak, and smoldering red oak). Exposure conditions were generated using a tube furnace, comprised of a quartz tube and electric heater ring, which was configured to generate consistent combustion conditions (e.g., a stable flame or smolder) (Kim et al. 2018; Rager et al. 2021). Biomass fuels were burned for 60 min, resulting in the emissions of particle matter (PM) and gaseous compounds. PM and condensable gas-phase semi-volatile compounds were captured using a multistage cryotrap system consisting of three impingers set at cooling temperatures of −10, −50, and −70 °C. This design allowed for a broader collection of particles and semi-volatiles which often pass through more traditional filter-based air mixture collection methods. Acetone was then used to extract smoke condensate samples from the impingers, and then samples were concentrated and dried under nitrogen gas. These smoke condensate samples were analyzed for chemical composition and used for in vivo exposures. Chemical components within these exposure samples have been previously characterized and reported (Kim et al. 2018; Rager et al. 2021; Ragerlab-Dataverse 2021), and are re-summarized here (Table A.1).

2.2. In vivo exposures

Mice were exposed to biomass smoke and tissues collected as previously detailed (Kim et al. 2018; Rager et al. 2021). All experiments received approval by the U.S. EPA Institutional Animal Care and Use Committee. In summary, female CD-1 mice (adult, pathogen-free) were evaluated, representing an outbred strain that is commonly used in the field of air pollution research as it does not promote one specific genotype. We have also used female CD-1 mice previously, which allows for direct comparisons between studies (Cho et al. 2009; Kim et al. 2014; Kim et al. 2015; Tong et al. 2010). Use of female mice further increased study feasibility by adding increased power to detect differences in expression between exposure groups using fewer mice, though we recognize that inclusion of both sexes in future studies will further enhance this research topic, as we have identified sex-dependent responses to biomass smoke exposures through in vitro models using human donor cells (Broke et al. 2022; Reboli et al. 2019). Mice were weighed and weight-randomized into groups of six for each exposure. Animal inclusion/exclusion criteria was based on weight (requiring approximately 20 g) and overall health status. A vehicle control group, exposed to saline, was included as well as a lipopolysaccharide (LPS)-treated group as a positive pulmonary inflammation response indicator.

Biomass smoke exposures were prepared through solvent exchange of PM samples in acetone to saline, yielding particle concentrations of 2 mg/mL in saline. Samples were sonicated using an ultrasonicator for 4 min. A total of 50 μL of sample (amounting to a total PM mass of 100 μg) was used as the final exposure administered into the lungs of mice.
through oropharyngeal aspiration. This specific dose was used to represent a potential peak 24 h exposure condition that can occur during wildfires. To detail, concentrations of PM proximal to wildfires have reached peak concentrations ranging 2–2.8 mg/m$^3$ (Naeher et al. 2007), which convert to PM lung deposition rates across 24 h that are estimated to overlap with the PM dose used here (Kim et al. 2018). The positive pulmonary inflammation control group was exposed to 2 μg of LPS in 50 μL saline, and the negative vehicle control group was exposed to 50 μL saline. All mice (including biomass smoke exposed, saline control, and LPS) were exposed on the same day.

2.3. Tissue Collection, hematology Measures, and cardiopulmonary phenotyping

Biological samples were collected 4 and 24 h post-exposure, as previously described (Kim et al. 2018; Rager et al. 2021). Briefly, for each mouse, blood was collected by cardiac puncture using a 1 mL syringe, and about 0.5 mL blood was collected in a microfuge tube containing 17 μL of 4% sodium citrate. Collected samples were centrifuged at 4 °C (10 min and 3200 rpm) and aliquots of citrated plasma were collected and stored at −80 °C. Hematology measures were collected on these samples, including counts of total white blood cells, lymphocytes, total red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, and platelet counts using a Coulter® AcT10 Hematology Analyzer (BeckmanCoulter). For each mouse, the left mainstem bronchus was isolated and clamped with alligator clips, and the trachea exposed, cannulated, and tied off with suture thread. The right lung lobes were lavaged and resulting bronchoalveolar lavage fluid (BALF) samples were placed in 2 mL tubes and kept on ice. The left lung and heart were collected and snap-frozen in liquid nitrogen for subsequent storage at −80 °C. Frozen lung, plasma, and heart tissues were transferred to UNC Chapel Hill and similarly stored at −80°C until further processing.

BALF samples were further processed to evaluate potential changes in cardiopulmonary toxicity markers resulting from exposures. Markers included immune cell counts, cytokine expression levels, and lung injury markers. Specific cell counts included macrophages and neutrophils. Cytokines evaluated in BALF samples included interleukin-6 (IL-6), macrophage inhibitory protein-2 (MIP-2), and tumor necrosis factor-α (TNF-α). Lung injury markers included albumin, y-glutamyl transferase (GGT), lactate dehydrogenase (LDH), and total protein concentrations, as well as N-acetyl-β-D-glycosaminidase (NAG) activity. Hematology and cardiopulmonary measures were statistically evaluated for significant differences between exposed vs control (saline) samples using one-way ANOVA followed by the Dunnett’s multiple comparison test, as previously detailed and reported (Kim et al. 2018; Ragerlab-Dataverse 2021), with data re-summarized here (Table A.2). Cardiopulmonary toxicity markers were also evaluated for potential association with hematology measures using the Spearman Rank Correlation test through the corr.test function in R Software (v4.0.3), and resulting p-values were corrected for multiple testing through false discovery rate (FDR) adjustment (Storey JD 2019).

2.4. EV measurements and analyses

2.4.1. EV terminology and reporting standards—For this study, EV terminology usage is based upon suggestions in the most recent Guidelines for Minimal Information
for Studies of Extracellular Vesicles published in 2018 (MISEV2018) by the International Society for Extracellular Vesicles (Thery et al. 2018). The term ‘EV’ is thus defined here as any particle that is naturally released by a cell that is surrounded by a lipid bilayer and cannot replicate (and therefore, does not contain a functional nucleus). EV subtypes are currently defined based upon how each particle was released from the parent cell. Exosomes originate through endocytosis pathways, which involves the folding inwards of a cell’s plasma membrane and forming vesicles through membrane budding. Ectosomes (also referred to as micro-vesicles or microparticles) are generated through exocytosis pathways, which involves the budding of vesicles directly from the plasma membrane that are then directly shed into the extracellular space. Apoptotic bodies are also considered an EV subtype, released in response to programmed cell death. There is notably a lack of specific markers that can effectively differentiate between EV subtypes (Thery et al. 2018). EV subtypes also overlap in size, with diameters ranging between 30 and 200 nm for exosomes, 100–2000 nm for microvesicles, and 1000–5000 nm for apoptotic bodies (Carberry et al. 2022). Therefore, the MISEV2018 advocates for the overall reporting of detected size range, as opposed to using size information to make author-dependent conclusions on EV subtypes. Despite nuances surrounding EV subtype definitions, the functions of most EVs are considered to be very similar (Cocucci and Meldolesi 2015). Thus, we referred to the analysis of membrane-bound particles isolated from plasma as EVs throughout this study, and also report corresponding physical characteristics and biochemical composition to aid in future delineation between particle types.

2.4.2. Isolating EVs from plasma—EVs that were exosome-enriched were extracted and purified from mouse plasma samples using Invitrogen’s Total Exosome Isolation (from plasma) kit, according to manufacturer protocol (ThermoFisher Scientific, Waltham, MA). In brief, plasma samples were centrifuged to pellet cell debris, and the clarified supernatant was transferred to a new tube. Filtered PBS and isolation reagent were added to samples according to manufacturer’s guidelines, incubated for 10 min, and centrifuged at 10,000 × g for 5 min to pellet exosome-enriched EVs. Supernatant was removed completely, and samples were resuspended in 100 μL filtered PBS. EVs were stored at −20°C until further analysis. This isolation approach yielded intact EVs that were used for: (1) image analysis; (2) quantification and characterization through Nanoparticle Tracking Analysis (NTA); and (3) further processing to extract small RNA for miRNA signature analysis.

2.4.3. Imaging and NTA of plasma EVs—A subset of isolated EV samples was characterized through microscopy and NTA approaches, with the goal of supporting that the implemented methods successfully isolated particle samples enriched for EVs from mouse plasma. These methods are described in supplemental material (see Appendix Methods). These approaches yielded the following information: images of EVs, particle count and size distribution of EVs, and percentages of EVs stained with cell membrane dye and select protein antibodies to further characterize the types of EVs collected and analyzed.

2.4.4. MiRNA signature analysis of plasma EVs—Total RNA (including small RNA) was extracted from isolated EVs using the Invitrogen Total Exosome RNA and Protein Isolation kit according to manufacturer protocol. RNA was purified using the RNA
Clean and Concentrator kit (Zymo), quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific), and stored at −80°C until analysis. Small RNA yield and quality was measured using the LabChip® Small RNA Assay (PerkinElmer), with example results shown in Fig. A.1. Isolated small RNAs were evaluated using the HTG EdgeSeq miRNA Whole Transcriptome Assay (HTF Molecular Diagnoses), with resulting libraries sequenced through single end sequencing using the HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina) at the UNC High-Throughput Sequencing Facility. Sequencing counts were processed through an established miRNA Profiling Pipeline (Chu et al. 2016) with associated code publicly available (BCGSC 2019). Data were aligned to the UCSC Genome Browser Assembly GRCm38/mm10 and annotated to miRBase 22.1.

2.5. mRNA signature analysis of lung and heart tissues

Lung and heart tissues were processed by slicing approximately 30 mg of tissue from each sample and homogenizing in Buffer RLT Plus for 4 min at 20 Hz using the TissueLyser II (Qiagen). Total RNA was extracted using the AllPrep DNA/RNA/miRNA kit (Qiagen). RNA was quantified with a Nanodrop 1000 spectrophotometer (Thermo Scientific) and quality-verified on a representative subset of 30 samples using the 2200 TapeStation Automated Electrophoresis System (Agilent Technologies). Samples showed RNA integrity numbers (RINs) that greatly exceeded requirements for the employed sequencing technologies, with RINs ≥ 8.8. RNA samples were then analyzed using the TempO-Seq Mouse Whole Transcriptome assay (BioSpyder) with resulting libraries sequenced using the HiSeq 2500 Ultra-High-Throughput Sequencing System (Illumina). Sequencing data were processed using the Tempo-SeqR pipeline (BioSpyder 2021), with QA/QC metrics including the average number of mapped reads in positive controls (i.e., requiring > 6×10^6 mapped reads), low signal-to-noise ratios (i.e., requiring total number of mapped reads in the positive control divided by the total number of mapped reads in negative controls > 20:1), and the percentage of mapped reads in positive control (i.e., requiring > 80%). Count data were aligned to the Ensemble database v98 and summarized as number of counts per gene.

2.6. MiRNA and mRNA signature data processing and statistical analysis

The miRNA and mRNA sequencing data were processed separately through similar pipelines in R Software (v4.0.3). A background filter was applied to the count data by requiring that transcripts be expressed at signals greater than the overall median in at least 20% of the samples, paralleling our previously published methods (Eaves et al. 2020a; Payton et al. 2020; Ring et al. 2021). This filter resulted in a final panel of 155 miRNAs across the plasma EV samples and 17,356 mRNAs across the tissue samples. Potential sample outliers were visualized through principal component analysis using the prcomp function, as well as hierarchical clustering based on Euclidean distance metrics using the hclust function. One lung mRNA sample and one plasma EV miRNA sample demonstrated outlying data distributions through both approaches and were excluded from the analysis to minimize potential outlier interference. Count data were then normalized by median-of-ratio estimates using sample-specific size factors, as implemented through the DESeq2 package (v1.30.0) (Love et al. 2014). Resulting variance stabilized expression data were carried forward in the statistical analysis. For the lung mRNA data, these originated from tissues of mixed cell populations that could have shifted in response to exposure (as indicated in part
by the cardiopulmonary toxicity changes); thus, potential sources of sample heterogeneity were addressed through the remove unwanted variation (RUVASeq) package (v1.24.0), in which a surrogate variable was empirically estimated and added to the statistical model. RUVA was not incorporated in the EV miRNA analysis, as hematological changes were extremely minimal and shifts in EV signatures occurring from potential shifts in originating cell types represented trends that we aimed to identify. Potential batch effect was addressed in the miRNA analysis by incorporating processing date as a covariate in the statistical model.

Statistical models were implemented to identify miRNA and mRNA signatures associated with each of the exposure conditions separately. This between-group statistical comparison design was implemented to identify miRNAs and mRNAs that were altered by each individual exposure condition, since each biomass smoke sample contained varying exposure chemistries (Table A.1), and thus, different molecular responses could be initiated by these exposures. Models were based on negative binomial generalized linear models comparing samples from exposed vs unexposed mice, using the DESeq2 package with associated shrunken logarithmic fold changes in expression (Love et al. 2014). Z-statistics were calculated by dividing the resulting fold changes by standard error values and then used to compare against normal distribution curves to derive Wald test p-values (Love et al. 2014). To control for the large number of transcripts evaluated, multiple test corrected p-values were calculated using the Benjamini Hochberg (BH) procedure (Benjamini and Hochberg 1995). The miRNAs and mRNAs with BH p < 0.10 were identified as significantly differentially expression in association with an exposure condition, resulting in lists of differentially expressed miRNAs and differentially expressed genes. These statistical filters notably parallel previously implemented criteria when analyzing miRNA and mRNA expression signature alterations (Clark et al. 2021; Eaves et al. 2020b; Payton et al. 2020).

2.7. Relationships between plasma EV miRNAs and cardiovascular disease

Differentially expressed miRNAs within plasma EVs were evaluated for known relationships to cardiovascular disease and function, to further evaluate the plausibility of EV miRNAs playing a role in wildfire-associated disease outcomes. Here, lists of miRNAs were queried against the Ingenuity Pathway Analysis knowledgebase (Ingenuity Systems®, Redwood City, CA) for relationships to disease signatures. The significance of enriched disease signatures was calculated using a modified Fischer’s Exact test, with p-values adjusted for multiple testing (BH procedure), resulting in the identification of miRNA disease signatures that were overrepresented amongst the miRNAs with altered expression associated with exposure (BH p-value < 0.10) (IPA 2019; Payton et al. 2020). MiRNAs with published roles in cardiovascular disease were identified from these recognized signatures, and all miRNA-disease signature relationships were reported for completeness.

2.8. Systems level analyses of transcriptional changes and predicted miRNA-mRNA interactions

The molecular mechanisms underlying wildfire-associated cardiopulmonary responses were further evaluated through systems level analyses of transcriptional response signatures. Here, pathway enrichment analyses were first carried out on the lists of differentially expressed
genes identified in the lung and heart. Enrichment analyses were organized separately per tissue and exposure condition, and all enrichment results were compared to identify common pathways that were consistently disrupted across tissues and biomass burn exposure conditions. Gene lists were specifically analyzed for relationships to canonical pathways, as aggregated within the Ingenuity Pathway Analysis Knowledgebase (Ingenuity Systems). Statistical enrichment of each pathway was determined using a modified Fischer’s Exact test, where over-represented categories were defined as those containing more molecules than expected by random chance after correction for multiple testing through the BH procedure (BH p-value < 0.10) (Benjamini and Hochberg 1995). These methods parallel those implemented in our recent studies (Klaren et al. 2019; Payton et al. 2020; Ring et al. 2021).

An additional systems-level analysis was carried out through the prediction of potential miRNA-mRNA interactions. Here, interactions between plasma EV miRNAs and heart mRNAs were the focus of analysis, as we hypothesized that wildfire exposures modulate molecular profiles in circulating EVs that eventually impact distal targets, including the heart. Interactions were predicted between molecules identified as differentially expressed by wildfire exposure conditions. miRNA-mRNA interactions were predicted in silico based on experimentally validated interactions curated from literature, gathered from TarBase, providing > 670,000 unique miRNA-mRNA interactions supported through peer-reviewed literature (Karagkouni et al. 2018). Interactions were also predicted in silico through base pairing homologies between the miRNA seed sequencing and 3’ untranslated mRNA regions, organized through TargetScan (v7.2) (Agarwal et al. 2015). These in silico predictions were filtered to focus on predictions with ‘high confidence’, as defined as those with cumulative weighted context scores ≤−0.4. This filter is based on a cumulative score across factors that influence the likelihood of miRNA-mRNA interactions, including binding site location, binding site type, local adenine and uracil content, target site abundance, stability of seed-pairing, and supplementary pairing factors (WIBR 2019).

Relationships between predicted miRNA-mRNA pairs were further evaluated statistically by correlating the expression profiles of each plasma EV miRNA against the expression profile of each potential target mRNA in the heart. Correlations were carried out using the Spearman Rank Correlation test in R (corr.test function) with a significance filter of p < 0.05. miRNA-mRNA pairs were lastly filtered for those showing expression change directions that support the current miRNA consensus, that miRNAs largely regulate protein expression signatures by silencing genes (Clark and Rager 2020; Duchaine and Fabian 2019). Therefore, significant pairings that included miRNAs with increased expression with target mRNAs at decreased expression, and pairings that included miRNAs with decreased expression with target mRNAs at increased expression were the focus of the results and corresponding biological interpretation.

### 2.9. Gene-Specific evaluation of miRNA vs. pri-miRNA expression changes in the heart

The expression levels of select miRNAs in the heart were evaluated as follow-up using gene-specific qRT-PCR. Here, RNA (including small RNA) was extracted from separate slices of tissue from six vehicle control and six red oak flame-exposed mice (selected
based upon their robust transcriptional changes) using ZYMO Direct-zol RNA MiniPrep and quantified using the Nanodrop 2000. cDNA synthesis was performed on 10 ng of total RNA for miR-30b, miR-125b, and miR-U6 and on 1 µg of total RNA for pri-miR-30b and pri-miR-125b using a TaqMan miRNA cDNA Synthesis kit (ThermoFisher Scientific). miRNA qPCR reactions were performed in triplicate on a QuantStudio 12 K Flex Real-Time PCR system using TaqMan qPCR Master Mix according to the manufacturer’s instructions. TaqMan miRNA assays were evaluated for mature miR-30b (ID 000602), precursor pri-miR-30b (ID Mm03306200_pri), mature miR-125b (ID 000449), precursor pri-miR-125b (Mm03307273_pri), and U6 snRNA (ID 001973). Resulting qRT-PCR cycle times (Ct) were normalized against the U6 housekeeping miRNA, and fold changes in expression were calculated based off delta delta Ct values and evaluated for significance using a two-sided t-test comparing exposed versus unexposed sample distributions (Livak and Schmittgen 2001). Results were illustrated using GraphPad Prism and BioRender Software.

2.10. Data availability

All data generated and analyzed in this study are publicly available. Specifically, the exposure chemistry and mouse cardiopulmonary toxicity markers were published through the Ragerlab-Dataverse (Ragerlab-Dataverse 2021). MiRNA and mRNA sequencing data were deposited into the National Center for Biotechnology Information Gene Expression Omnibus repository and made publicly available under accession numbers GSE173561 and GSE164542 (Edgar et al. 2002). Follow-up gene-specific PCR data were published through the Ragerlab-Dataverse (Carberry et al. 2021).

3. Results

3.1. Study overview

This study was designed to evaluate the cross-tissue impacts of wildfire-associated exposure conditions, through the evaluation of four biomass/combustion conditions: flaming peat; smoldering peat; flaming red oak; and smoldering red oak. Female CD-1 mice were exposed via oropharyngeal aspiration to 100 µg of a smoke condensate sample and evaluated for potential changes in hematology and markers of cardiopulmonary toxicity. These exposures and phenotypic profiling data have been previously published (Kim et al. 2018; Rager et al. 2021). The current study expanded upon this foundational data and banked tissues to generate and analyze new molecular-level data across the lung, peripheral blood, and heart. A summary of how each sample was analyzed is provided in Table 1. Plasma EV miRNA and heart mRNA transcriptomic analyses focused on the 24 h post-exposure responses to better capture later changes in the blood/heart rather than more immediate responses occurring mostly in the lung; while the lung mRNA analyses included the 4 h timepoint to capture earlier events that may precede later distal effects.

3.2. Hematology and cardiopulmonary responses to biomass smoke exposures

Hematology and cardiopulmonary responses were evaluated in relation to each of the biomass burn exposure scenarios. Overall, hematology changes were minimal (Fig. 1A). There were only two instances where total white blood cell and lymphocyte counts were measured at significantly decreased levels in mice (associated with flaming peat at 4 h
post-exposure), though these values may have been influenced by relatively higher values in the negative control mice from the 4 h group. There were no other instances of hematological changes observed across total white blood cells, lymphocytes, total red blood cells, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, and platelet counts. Markers of cardiopulmonary toxicity showed exposure-induced changes, particularly those relevant to tissue injury and inflammation (Fig. 1B). To detail, BALF albumin levels were significantly increased in response to flaming peat (24 h post-exposure) and flaming red oak (4 h post-exposure). Neutrophil counts were also significantly increased in response to flaming peat (4 and 24 h), smoldering peat (4 h), and flaming red oak (4 h). Other changes in cardiopulmonary toxicity markers included IL-6, MIP-2, TNF-α, LDH, NAG, and total protein concentrations in association with select exposure conditions (e.g., flaming peat and flaming red oak). These changes in cardiopulmonary toxicity markers notably lacked significant correlation with hematology measures (Table A.3), further supporting that the observed changes in toxicity resulted from biomass exposures. Overall, flaming biomass conditions were associated with much greater changes in cardiopulmonary toxicity phenotypes in comparison to smoldering conditions.

Our group has notably carried out parallel experimentation focusing on cardiopulmonary toxicity resulting from peat smoke condensate samples collected from wildfires in Eastern North Carolina using the same mouse strain, sex, and exposure design (i.e., 100 μg/mouse via oropharyngeal aspiration) (Kim et al. 2014). This study evaluated cardiac function through several hemodynamic parameters and found that exposures induced low recovery of post-ischemic left ventricular developed pressure, among other cardiac effects evaluated through a heart perfusion system. Additionally, visual inspection of heart sections stained with 2,3,5-triphenyltetrazolium chloride found exposure-induced tissue infarction (Kim et al. 2014). These findings further support that cardiopulmonary effects occur in mice exposed to similar wildfire-relevant exposure conditions, including changes in heart function and tissue injury.

3.3. Confirmation of EV isolations from mouse plasma

EVs were isolated from a subset of mouse plasma samples and further characterized using imaging and NTA approaches. Imaging analysis confirmed the presence of EVs, resulting in visual representation of particles largely < 200 nm in diameter (Fig. 2). Notably, samples analyzed from frozen (Fig. 2A–C) vs freshly isolated (Fig. 2D) aliquots produced similar images, supporting the utility of different isolation and storage approaches for future research applications.

NTA demonstrated particle diameters between an average of 120 and 150 nm (Table A.4, Fig. A.2A), within range of the EV subtype of exosomes (Carberry et al. 2022). The overall particle charge was between −18.10 and −12.01 mV (Table A.4, Fig. A.2B), well within range of expected particle charge for EVs successfully isolated from plasma samples (Rupert et al. 2017). Detected particle concentrations yielded concentrations between 2.8 and 6.6x10^{11} particles / mL PBS, after correcting for dilution (Table A.4). Results showed that, on average, 72.2% of particles were found to contain cell membrane dye, indicating the presence of particles with lipid bilayers (Table A.4), an attribute that is specific to
cells/EVs (as opposed to other particles) (Hartjes et al. 2019; Huang et al. 2020; Thery et al. 2018). The remaining particles without cell membranes could indicate the presence of other types of particles and/or protein aggregates, which are also expected given the complex starting sample material. The average particle diameter stained with cell membrane dye was slightly higher than the full particle samples (260–270 nm, on average), also indicating presence of smaller coeluting particles that were not membrane-bound (Table A.4, Fig. A.2C). An average of 45.6% of particles were found to contain the transmembrane protein, CD63 (Table A.4, Fig. A.2D). This percentage is consistent with data from another research group that isolated exosome-enriched EVs from peripheral plasma using parallel protocols (Matsumoto et al. 2016). Similar to the cell membrane dye NTA findings, particles labeled with CD63 showed slightly higher diameters (215–260 nm, on average) compared to all particles contained within the samples (Table A.4, Fig. A.2D). Importantly, CD63 is not always present on EVs and there is current debate surrounding the specificity of CD63, as well as other protein markers, in informing EV presence/subtypes (Hartjes et al. 2019; Huang et al. 2020; Thery et al. 2018). Collectively, these EV characterization results support the enrichment of EVs, likely containing a high proportion of the EV subtype, exosomes, within processed plasma samples based on imaging, particle size distribution, particle charge, cell membrane dye measurements, and CD63 protein measurements.

3.4. MiRNAs altered by biomass smoke exposures in plasma EVs

MiRNA expression signatures were evaluated in EVs isolated from mouse plasma for all 24 h post-exposure samples through sequencing technologies. A total of 35, 6, 27, and 3 miRNAs were identified with significant differential expression associated with flaming peat, smoldering peat, flaming red oak, and smoldering red oak smoke exposures, respectively (Table A.5). Thus, a higher number of miRNAs were altered in response to flaming vs. smoldering combustion conditions. Flaming conditions notably produced higher concentrations of inorganics and ionic constituents (Kim et al. 2018; Rager et al. 2021) (Table A.1); and therefore, exposure to these components may associate with greater disruptions in plasma EV miRNA expression signatures that warrants further investigation.

Combining results across exposure conditions, 53 miRNAs were differentially expressed in at least one exposure condition. Many miRNAs (n = 14 out of 53 [26%]) showed alterations associated with two or more exposure conditions. These miRNAs included miR-183 (consistently increased in expression), miR-30b (consistently increased in expression), and miR-378a (consistently decreased in expression). Note that miRNA signatures were altered in the absence of overt changes in hematology, indicating that these signals were responsive to the exposure conditions/cardiopulmonary molecular signals as opposed to changes in circulating blood cell counts or other hematological changes. It is also notably that the number of EV miRNA alterations are in range of previous publications, including a recent study evaluating the effects of ozone inhalation exposure in mice (Smith et al. 2021).

3.5. Roles of plasma EV miRNAs in cardiovascular disease

To further evaluate the plausibility of EV miRNAs influencing diseases associated with wildfire exposure, miRNAs were analyzed for known relationships to cardiovascular disease. Cardiovascular disease signatures were significantly enriched amongst all lists of exposure-
responsive miRNAs (Table A.6). Of the 53 total exposure-responsive miRNAs, 28 (53%) have recognized roles in cardiovascular disease and/or cardiovascular system development and function (Fig. 3). Notable miRNAs include miR-30b, which was increased in expression association with flaming peat and flaming red oak smoke exposures and has recognized properties that are pro-angiogenic and protective against hypoxia-induced cell injury in blood and heart tissues (Gong et al. 2017; Li et al. 2015; Shen et al. 2015). Other differentially expressed miRNAs associated with cardiovascular disease include miR-150, miR-223-3p, miR342-3p, and miR-378a, all with decreased expression and previous evidence showing decreased expression in blood associated with cardiovascular disease, in either diseased animal models or humans (Kaneko et al. 2017; Ray et al. 2020; Saadatian et al. 2019; Taibi et al. 2014; Zhao et al. 2020). Notable additional diseases and functions were also enriched amongst the miRNAs, including gene expression, inflammatory disease, and cardiovascular system development and function (Table A.6).

3.6. Lung and heart transcriptomic responses to biomass smoke exposures

Transcriptomic analyses of lung and heart tissues were carried out to identify mRNAs involved in common pathways that were altered upon exposure. To detail exposure-specific mRNA responses in the lung, a total of 2613, 1664, 228, and 96 mRNAs were identified with significant differential expression associated with flaming peat, flaming red oak, smoldering peat, and smoldering red oak, respectively, at 4 h post-exposure. At 24 h post-exposure, a total of 376, 8, 8, and 30 mRNAs were identified with significant differential expression associated with flaming peat, flaming red oak, smoldering peat, and smoldering red oak, respectively (Table A.7). These gene counts are notably in range of previous inhalation toxicology research evaluating complex air pollutant exposures (Chang et al. 2021; Rager et al. 2011a).

These lung transcriptomic results indicated that a much more robust transcriptional response was identified 4 h post-exposure vs. 24 h post-exposure, suggesting that the lung experienced more immediate-level responses to biomass smoke exposures that largely did not persist over time. To detail, an average of 1150 genes demonstrated significant differential expression across biomass smoke exposures 4 h post-exposure, while only 105 genes, on average, demonstrated differential expression 24 h post-exposure. As a comparison, transcriptional responses to the known pulmonary inflammatory agent, LPS, were very robust at both timepoints, with 5312 and 6098 differentially expressed genes at 4 and 24 h post-exposure, respectively.

Comparing lung transcriptomic responses across exposure conditions, flaming combustion conditions generally induced greater responses in comparison to smoldering. To detail, applying statistical thresholds of BH p-value < 0.10, flaming peat exposure was associated with the altered expression of 2613 genes at 4 h, while smoldering peat exposure was associated with 228 genes at 4 h. Similarly, flaming red oak exposure was associated with the altered expression of 1664 genes at 4 h, while smoldering red oak exposure was associated with 96 genes at 4 h. Similar trends were apparent at 24 h post-exposure for peat, while red oak responses were overall minimal by this timepoint in the lung (Fig. 4).
mRNA analyses of the heart focused on the 24 h post-exposure responses to biomass smoke, based on a priori data supporting tissue-level responses occurring during that time in the heart (Kim et al. 2014). The condition that caused the least transcriptional response in the heart was smoldering red oak exposure, associated with the differential expression of 52 genes. Flaming peat was associated with 180 differentially expressed genes, and smoldering peat with 1249 differentially expressed genes, indicating that this particular flaming condition was not the most potent at the timepoint evaluated. The condition causing the most transcriptional response in the heart however was flaming red oak, associated with the differential expression of 2516 genes. All differentially expressed genes in lung and heart tissues and their associated statistics are provided (Tables A.7 and A.8).

3.7. Systems level responses across tissues

Transcriptional responses in the lung and heart were evaluated at the systems level first through pathway enrichment analysis. Some of the most frequently altered pathways across tissues were relevant to hypoxia and cell stress (addressing the study’s hypothesis), including the following: NRF2-mediated Oxidative Stress Response, Hypoxia Signaling in the Cardiovascular System, and HIF1A Signaling (Fig. 3 and Fig. 4, Table A.9). Other pathways were notably altered across tissues, including those relevant to inflammatory response (e.g., Acute Phase Response Signaling, Interleukin-3 [IL-3] Signaling, Interleukin-8 [IL-8] Signaling, Interleukin-15 [IL-15] Signaling, and Janus Kinase [JAK] / Signal Transducer and Activator of Transcription Proteins [Stat] Signaling).

To further evaluate potential cross-tissue mechanisms of these responses, interactions between plasma EV miRNAs and potential target mRNAs in the heart were bioinformatically predicted and evaluated for transcripts associated with flaming red oak exposure. This biomass smoke condition was selected for this in-depth molecular profiling because it produced (1) the most robust changes in mRNA expression profiles in the heart (n = 2516 genes with differential expression) that were also enriched for hypoxia/cell stress; (2) changes in miRNA expression profiles in circulating EVs (n = 27 miRNAs with differential expression); (3) changes in markers of cardiopulmonary inflammation and tissue injury (Fig. 1 and Fig. 4). In silico miRNA-mRNA target predictions were based on experimentally validated interactions coupled with sequencing homology-based predictions between plasma EV miR-NAIs and heart tissue mRNAs that were significantly differentially expressed upon exposure. This analysis yielded 1146 miRNA-mRNA potential interactions that spanned 16 unique miRNAs in plasma EVs and 782 unique mRNAs in heart tissue (Table A.10). Filtering these miRNA-mRNA interactions for directionality and statistical significance, 9 represented pairings with miRNAs at exposure-induced increased expression and associated target mRNAs at decreased expression that were correlated at p < 0.10 across exposed mice that did not demonstrate significant correlation across control mice. Conversely, 18 predicted miRNA-mRNA interactions represented pairings with miRNAs at decreased expression and associated target mRNAs at increased expression that were correlated at p < 0.10 across exposed mice that did not demonstrate significant correlation across control mice (Table A.10). Of the predicted miRNA-mRNA interactions, 58 were identified to include mRNAs involved in Hypoxia Signaling in the Cardiovascular System, HIF1A Signaling, and/or
NRF1-mediated Oxidative Stress Response (Table A.10), some of which were highlighted through network-level interactions involved in hypoxia and cell stress (Fig. 5).

3.8. **Gene-Specific evaluation of miRNA vs. pri-miRNA expression changes in the heart**

MiR-30b was identified as significantly increased expression in association with flaming red oak exposure (as well as flaming peat), has known roles in cardiovascular disease, and protects against cell injury in the lung, blood, and heart (Gong et al. 2017; Li et al. 2015; Shen et al. 2015; Yi et al. 2019). Furthermore, there is evidence demonstrating intercellular transfer of miR-30b via secreted EVs, specifically from mesenchymal stem cells to endothelial cells, resulting in the promotion of angiogenic effects (Gong et al. 2017). As a result, we set-out to evaluate whether there was evidence from this study supporting the transfer of miR-30b from plasma EVs into target heart tissue. To further evaluate this potential effect, we implemented gene-specific qRT-PCR to measure for: (i) expression changes of miR-30b occurring in exposed heart tissues; and (ii) whether these changes occurred in the absence of changes in its molecular precursor, pri-miR-30b. Another miRNA was included as a negative control, namely, miR-125b which was detected though showed no changes in plasma EV miRNA expression associated with exposure. Including both mature and precursor miRNAs was important, as miRNA biogenesis occurs through a series of steps first involving the transcription of pri-miRNA. Pri-miRNA then undergoes levels of processing, including cleavage into pre-miRNA, translocation outside of the nucleus, further cleavage resulting in a double-stranded miRNA duplex, and final incorporation into the RISC as mature miRNA (Clark and Rager 2020). Therefore, if the expression of a mature miRNA increases, it should coincide with increased expression of its precursor molecules (e.g., pri-miRNA) when arising from the same cell.

Evaluation of flaming red oak-exposed vs control mouse heart tissue resulted in an exposure-associated increased expression of miR-30b that was highly significant (p < 0.0005), which occurred in the absence of changes in its molecular precursor, pri-miR-30b. When evaluating the negative control, miR-125b, no changes in the mature miRNA or its precursor was identified. To evaluate the potential for miRNA processing in the heart to be generally influenced by exposure, the expression levels of dicer 1, ribonuclease III (Dicer1) were investigated within the previously detailed mRNA analysis. Dicer1 was important to evaluate as it represents a major regulator of miRNA precursor processing into mature miRNA molecules (Michlewski and Caceres 2019). Notably, Dicer1 expression was not significantly altered upon exposure in the heart (Table A.8), thus supporting external mechanisms of miRNA expression changes. Though findings would be further supported if additional timepoints were included, data support the potential for miR-30b expression changes occurring in the heart due to miRNA transfer from external tissue (e.g., plasma) (Fig. 6).

4. **Discussion**

This research aimed to identify relationships between miRNAs traveling in circulating plasma EVs and pathways that are altered across the lung and heart, in response to wildfire-relevant exposures. Smoke condensate conditions included flaming peat, smoldering peat,
flaming red oak, and smoldering red oak, selected to include fuel types that are prevalent in wildfire prone areas across the world (Abrams 1992; Dey and Guyette 2000; Stracher et al. 2015) and have known association with pulmonary and cardiovascular outcomes (Burbank et al. 2019; Ghio et al. 2012; Kim et al. 2019; Kim et al. 2014; Kim et al. 2018; Rebuli et al. 2019). We previously found that these biomass smoke condensate exposures caused changes in markers of cardiopulmonary tissue injury and inflammation in mice, with minimal changes in hematology (Kim et al. 2018; Rager et al. 2021). Plasma and tissues from these same mice were further evaluated at the molecular-level to produce the following new findings: First, miRNAs with known involvement in cardiovascular disease and hypoxia/cell stress were identified with differential expression in circulating plasma EVs (enriched for exosomes) in association with biomass burn conditions. Second, mRNA expression profiles within the lungs and heart showed common modulation of hypoxia and cell stress-related pathways in association with exposures, with generally more robust changes occurring in response to flaming vs. smoldering combustion conditions. Third, the flaming red oak smoke exposure condition induced the most robust changes in the heart and was thus further evaluated for potential miRNA-mRNA interactions across plasma EV and heart tissues. Here, heart hypoxia and cell stress-related transcriptional changes were predicted to be regulated by plasma EV-encapsulated miRNAs. An example hypoxia/cell stress-regulatory miRNA (miR-30b) was found at increased expression in the heart in the absence of changes in its molecular precursor. These data support a new mechanism underlying cross-tissue communication resulting from wildfire-relevant exposures, with miRNAs in circulating plasma EVs linking cross-tissue responses related to hypoxia and cell stress.

Important miRNAs within exosome-enriched EVs isolated from plasma showed exposure-associated differential expression, including those with direct relevance to cardiovascular disease. MiRNA profiling resulted in the identification of 53 miRNAs that were altered by at least one exposure condition, a large portion of which have known roles in cardiovascular disease. Previous studies have identified relationships between air pollutant exposure and altered expression of miRNAs within circulating blood (Cheng et al. 2020; Fenga et al. 2016; Rager et al. 2014; Ruiz-Vera et al. 2019). However, no studies have evaluated miRNA expression alterations associated with wildfire-relevant exposure conditions, particularly within EVs. EVs are important to evaluate since they can more stably transfer molecules across tissues due to their outer protective lipid bilayer and biocompatibility with target cells (Fu et al. 2020; Thery et al. 2018). In terms of biomarker utility, a recent review identified 32 studies that compared exosomal miRNA signatures vs non-exosomal miRNA signatures from circulating blood (mostly plasma), and concluded that the majority of articles (75%) recommended an exosomal source of miRNAs over non-exosomal miRNAs for providing improved biomarker information (Nik Mohamed Kamal and Shahidan 2019). These findings collectively suggest that plasma EV miRNAs may provide improved sensitivity/specificity towards informing exposure/disease status, and these miRNAs likely have increased probability of impacting the biology of distal cells due to their protective and biocompatible encasing. Therefore, the finding that wildfire-relevant exposure conditions alter the expression levels of plasma EV miRNAs is novel and likely informs downstream health consequences.
Transcriptomic profiling identified that pathways relevant to hypoxia and cell stress were frequently altered by exposures in the mouse lung and heart, and that responses were largely more robust in association with flaming conditions in comparison to smoldering conditions (at the same PM dose). One specific pathway of interest was HIF1A signaling, which a few studies have related to cardiopulmonary toxicity resulting from air pollutant exposures (Proper et al. 2014; Saini et al. 2008). Furthermore, there is strong evidence supporting the role of HIF1A signaling in cardiovascular disease and responses to hypoxia, which notably overlap with molecular signals involved in inflammation and injury (Lee et al. 2019; Semenza 2014; Sousa Fialho et al. 2019). The finding that these molecular pathway alterations are more pronounced in response to flaming combustion conditions, in comparison to smoldering, also parallels our previous research that focused on responses at the phenotypic-level (Kim et al. 2019; Kim et al. 2018). These enhanced biological responses likely occurred as a result of the formation of potentially more toxic compounds during flaming combustion conditions, including polycyclic aromatic hydrocarbons, ionic constituents, and inorganic compounds (Rager et al. 2021). This study uniquely highlights the role of HIF1A signaling, and general hypoxia and cell stress, in cross-tissue responses to wildfire-relevant exposure conditions throughout the lung and heart that become enhanced in association with flaming combustion conditions.

Further analysis of the red oak burning condition, which induced the greatest transcriptomic response in the heart, revealed that EV-encapsulated miRNAs within circulating plasma were predicted to regulate the expression of exposure-responsive genes involved in hypoxia/cell stress. One notable miRNA, miR-30b, is involved in cardiovascular disease; protects against cell injury in the lung, blood, and heart; and has evidence supporting its ability to transfer from external cells into target cells via EVs and regulate downstream cardiovascular properties (Gong et al. 2017; Li et al. 2015; Shen et al. 2015; Yi et al. 2019). While many miRNAs were altered in expression by biomass burn exposures, follow-up expression analysis of miR-30b and its molecular precursor, pri-miR-30b, was carried out in heart tissues due to its statistical significance and its previously reported mechanistic role in hypoxia signaling and cardiomyocyte processes relevant to cardiovascular disease (Gong et al. 2017; Li et al. 2015; Shen et al. 2015). Here, the expression levels of mature miR-30b were found to increase in exposed tissues in the absence of changes to its precursor molecule, pri-miR-30b. This trend notably did not occur for another miRNA, miR-125b, that was included as a negative control, which was measured in plasma EV miRNAs though did not show exposure-induced disruptions in expression. An alternative mechanistic explanation is that elevated miR-30b could result from general increased miRNA processing in the heart. Precursor miRNAs have previously been shown to be loaded directly into EVs and thus may be transferred to other tissues for further processing (Gracia et al. 2017; Julou-Schaeffer et al. 1990; Melo et al. 2014). However, no changes in pri-miRNA molecules in heart tissues were identified using gene-specific approaches. Furthermore, a major regulator of precursor miRNA processing into mature miRNA, Dicer1, showed no significant transcriptional increase in the heart. This finding supports that increased Dicer1-mediated transcription of precursor miRNAs into mature miRNAs within the local tissue was not likely happening at this timepoint. These findings provided additional support for the notion of exposure-induced formation of EVs carrying miRNAs as mediators of systemic
changes. Additionally, these findings warrant further evaluation of the other significantly altered plasma miRNAs, especially those with previously reported cardiovascular relevance such as miR-378a.

When interpreted in the context of published literature, data from the current study support a potential mechanism of cross-tissue communication occurring in response to wildfire-relevant exposure conditions (Fig. 7). This mechanism begins with wildfire-relevant exposure conditions eliciting changes in lung hypoxia and cell stress signaling. This first key event is supported by data in the current study demonstrating that biomass smoke exposure conditions induced alterations in hypoxia/cell stress response pathways in the lung. These pulmonary responses are then proposed to cause the release of EVs into circulating blood, containing key regulatory miRNAs. There is published evidence supporting that lung hypoxia and cell stress causes the release of EVs into circulating plasma (Chen et al. 2017; Su et al. 2020; Zhang et al. 2021). Furthermore, data from the current study support that miRNA profiles in plasma EVs are altered in association with lung hypoxia/cell stress. Next, it is proposed that miRNAs circulating in plasma EVs are capable of transferring to distal target tissues, including the heart, and impacting target cell biology. There is published evidence supporting the transfer of functional miRNA molecules from plasma to the heart and/or cultured cardiomyocytes (Gong et al. 2017; Minghua et al. 2018). In addition, data from the current study demonstrated that plasma EV miRNAs were bioinformatically predicted to regulate a portion of the observed mRNA responses involved in hypoxia and cell stress in the heart. Specific miRNAs with known roles in these kinds of intercellular communication include miR-150, miR-223-3p, miR-30b, and miR-378a (Gong et al. 2017; Li et al. 2019b; Shen et al. 2015; Shi et al. 2016; Yi et al. 2019; Zhao et al. 2020; Zheng et al. 2020). These miRNAs were predicted to play a role in hypoxia and cell stress responses in the heart in the current study, and have been implicated in mediating hypoxia-related cell injury in vitro, in cardiomyocytes (Liu et al. 2018; Zhao et al. 2020), and/or hypoxia-related cardiovascular injury in vivo (Li et al. 2019b; Shen et al. 2015; Shi et al. 2016; Zheng et al. 2020). This mechanism is notably not restricted to unidirectional responses; instead, results suggest common patterns of modulation across tissues involved in hypoxia and cell stress, where changes in the heart could also be impacting molecular signatures within circulating blood which could influence other tissues. Indeed, there is evidence to support heart tissues release EVs containing miRNAs that then enter circulation in blood plasma (Das and Halushka 2015; Davidson et al. 2019; Emanueli et al. 2016). Collectively, this mechanism represents a possible set of key events linking wildfire exposures to cross-tissue communication that may ultimately impact resulting disease outcomes.

We recognize that further evaluation of this mechanism is needed, where the field of EV research within environmental health / toxicology is just now emerging (Carberry et al. 2022). In this study, for example, it is difficult to elucidate what specific event(s) impacted plasma EV miRNA composition. It is notable that other tissues besides the lung and heart were likely impacting the observed changes in EV molecular composition, including signals inherent within the blood. For example, platelets are known to be a major source of circulating EVs (Davidson et al. 2019). Still, changes in plasma EV miRNA expression profiles are currently being evaluated as promising biomarkers of exposure and mediators of biological responses in relation to air pollutant exposures (e.g., occupational metal
PM exposure (Pavanello et al. 2016)). In addition, miRNAs are naturally present at high concentrations across tissues. It remains unclear how transferred miRNAs can be effectively incorporated into the endogenous RISC complex, resulting in significant mediation of mRNA expression, when in competition with host miRNA profiles (Davidson et al. 2019). Still, there is evidence supporting miRNA transfer from plasma to cardiomyocytes, impacting host cardiomyocyte function (Gong et al. 2017; Minghua et al. 2018).

Overall, data from this study support changes in plasma EV miRNA expression with coinciding changes in hypoxia and oxidative stress signaling between the lung and heart. Future studies will also evaluate the impacts of biomass burn scenarios occurring via inhalation exposure routes. Inhalation-based exposure designs better capture gas-phase constituent components of biomass burn conditions (e.g., nitrogen oxides and smaller volatile organic compounds). For example, we recently evaluated peat and red oak smoke-associated responses through mouse inhalation exposure designs and found exposure-induced increased neutrophil count and changes in ventilatory timing (Kim et al. 2019). These designs could be leveraged in the future to test whether similar changes in plasma EV composition are observed. Additional tissue-specific histology measures could further enhance understanding of phenotypic endpoints resulting from exposures within lung and heart tissues, similar to our previous work which characterized heart tissue injury resulting from overlapping exposures in the same animal model (Kim et al. 2014). Future efforts could also leverage in vitro-based designs to more efficiently evaluate the wide variety of exposure scenarios relevant to wildfires (Zavala et al. 2020). Sex-specific responses are also important to evaluate, as we have identified sex-dependent differential molecular response patterns to biomass smoke exposures (Broke et al. 2022; Rebuli et al. 2019) that could be further evaluated through controlled animal experimentation. Further molecular characterization of EVs isolated from plasma samples of exposed mice will also enhance this growing area of research, including additional markers that inform EV origins in terms of biogenesis and host tissue (e.g., additional tetraspanins and tissue-specific markers) (Carberry et al. 2022). The continued expansion of research aimed at testing wildfire exposure-induced disease etiology will inevitably lead to the implementation of targeted methods to alleviate the global burden of disease associated with this growing exposure risk.

5. Conclusions

In conclusion, this study evaluated multi-tissue responses to wildfire exposure conditions to highlight a new potential mechanism connecting pulmonary and cardiovascular responses via circulating plasma EVs. Important miRNAs within plasma EVs were identified with differential expression associated with these exposures, with known roles in cardiovascular disease and hypoxia-induced injury across multiple tissues. These miRNAs, encapsulated in protective EVs, can serve as therapeutic targets for disease intervention, as well as biomarkers of exposure for firefighters and members of communities currently being impacted by wildfires. Transcriptomic responses in the lung and heart were enriched for hypoxia/cell stress signaling, with enhanced responses occurring in association with flaming vs. smoldering conditions. A notable miRNA, miR-30b, was identified at increased expression in the heart in the absence of changes in its molecular precursor, pri-miR-30b. These data were interpreted in the context of published literature and collectively provide a
weight-of-evidence for a new potential mechanism of cross-tissue communication occurring in response to wildfire-relevant conditions. The continued increased understanding of biological responses to wildfires is critical, as the prevalence and intensities of wildfires continue to grow worldwide.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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**Abbreviations:**

- **BALF**: bronchoalveolar lavage fluid
- **CMO**: cell membrane orange
- **EV**: extracellular vesicle
- **HIF1A**: hypoxia inducible factor 1 subunit alpha
- **IL-15**: interleukin-15
- **IL-3**: interleukin-3
- **IL-8**: interleukin-8
- **JAK**: janus kinase
- **miRNA**: microRNA
- **NTA**: nanoparticle tracking analysis
- **RISC**: (mi)RNA-induced silencing complex
- **Stat**: signal transducer and activator of transcription proteins
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Overall, hematology changes were minimal, while cardiopulmonary changes were present, including those related to tissue injury and inflammation. Abbreviations: MCH, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RBC, total red blood cells, WBC, total white blood cells. These data were previously generated/reported and evaluated for statistical significance via one-way ANOVA followed by the Dunnett’s multiple comparison test (Kim et al. 2018; Rager et al. 2021) and re-summarized here to address this study’s aim.
Fig. 2. Electron microscopy images confirming the isolation of particle samples enriched for EVs from plasma samples of mice exposed biomass burn conditions. Example images include those that are representative of (A-B) two samples from mice exposed to flaming peat condensate (isolated EVs frozen prior to analysis); (C) one sample from a mouse exposed to flaming red oak condensate (isolated EVs frozen prior to analysis); and (D) one fresh sample from a mouse exposed to red oak condensate (isolated EVs analyzed immediately after isolation without freezing). Negative stain transmission electron microscopy images show that the isolated EV samples are enriched for EVs, represented by the white circles. Smaller dots in the background likely represent general amorphous protein content. Example EVs are indicated by white arrows.
Fig. 3. Cross-tissue molecular responses to the wildfire-relevant exposures, including expression changes in plasma EV miRNAs involved in cardiovascular disease.
Molecular responses in mice exposed to flaming red oak smoke condensate samples are illustrated here as an example, though all biomass smoke conditions caused changes in hypoxia and cell stress pathways across tissues, and many miRNAs were also commonly modulated across conditions (as shown with red triangles).
Fig. 4. Summary of the number of genes with expression alterations, and pathways relevant to hypoxia and cell stress signaling associated with biomass smoke exposures in the mouse lung and heart.

Gene counts are summarized along the left y-axis, with values in green. Pathway enrichment values are summarized along the right y-axis, with values in blue, representing $-\log BH$ p-values; therefore, the higher the value, the greater significance. Exposure conditions are sorted to show the smoldering conditions first, followed by flaming, given that flaming conditions generally induced the more robust responses. The full canonical pathway name for hypoxia signaling was ‘hypoxia signaling in the cardiovascular system’, and for cell stress signaling, ‘NRF2-mediated oxidative stress response’.

\[ \text{Fig. 4.} \]
Fig. 5. Predicted molecular interactions between plasma EV miRNAs and heart mRNAs that were altered upon exposure to the biomass smoke condition produced from flaming red oak. miRNAs with exposure-induced increased expression (purple) and decreased expression (yellow) are shown. Proteins encoded by mRNAs with exposure-induced increased expression (green) and decreased expression (red) are shown, alongside proteins involved in related signaling (white). Predicted interactions between miRNAs and mRNAs are shown, as well as known interactions between proteins in the heart. These do not represent all potential interactions occurring in response to flaming red oak smoke exposure; rather, this network serves as an example highlighting miRNA-mRNA predictions involved in hypoxia/cell stress.
Fig. 6. qRT-PCR results showing significantly increased levels of mature miR-30b in the absence of changes in its molecular precursor, pri-miR-30b, supporting the possible transfer of miR-30b from external tissues (e.g., plasma) into heart tissues in response to biomass smoke exposures.

(A) Scenarios are presented depicting expected results if miR-30b expression changes were (left) and were not (right) influenced by external cellular communication mechanisms, such as EV miRNA transfer.

(B) qRT-PCR results are shown, produced from RNA analysis of heart tissues from mice exposed to flaming red oak smoke condensate (Exp) and corresponding saline controls (CT) collected 24 h post-exposure. Ct refers to raw qRT-PCR cycle threshold. Boxplot depicts median, quartile, and range of Ct values. (*) represents significance at p < 0.0005.
Fig. 7. Potential mechanism for cross-tissue communication through plasma EV changes associated with wildfire-relevant exposure conditions, supported by findings from this study and/or previous publications.

More details surrounding each connection and specific data / supporting publications are provided in the text, with select references detailed within the figure corresponding to each applicable mechanistic event.
Sample summary.

Each included endpoint is listed as a column, with each cell indicating whether it was evaluated (yes = evaluated; dash = not evaluated). The number of individual mouse samples included in each endpoint is also specified in parentheses. Note that an additional subset of EV samples were evaluated through microscopy and nanoparticle tracking analysis to confirm successful isolation of plasma EVs (see Appendix Methods and Table A.4). Abbreviations: EV, extracellular vesicle; qRT-pCR, real-time quantitative reverse transcription polymerase chain reaction.

| Exposure        | Sample Collection Post-Exposure (h) | Cardiopulmonary Toxicity Markers | Hematology | Plasma EV miRNA signature analysis | Lung mRNA signature analysis | Heart mRNA signature analysis | Heart miRNA-specific qRT-PCR |
|-----------------|-------------------------------------|----------------------------------|------------|-----------------------------------|-----------------------------|-----------------------------|-------------------------------|
| Saline          | 4                                   | yes (6)                          |            | yes (5)                           | -                           | yes (6)                     | -                             |
| Flaming Peat    | 4                                   | yes (6)                          | yes (5)    | -                                 | yes (6)                     | -                           | -                             |
| Smoldering Peat | 4                                   | yes (6)                          | yes (3)    | -                                 | yes (6)                     | -                           | -                             |
| Flaming Red Oak | 4                                   | yes (6)                          | yes (5)    | -                                 | yes (6)                     | -                           | -                             |
| Smoldering Red Oak | 4                                 | yes (6)                          | yes (5)    | -                                 | yes (6)                     | -                           | -                             |
| LPS             | 4                                   | yes (6)                          |            | -                                 | yes (6)                     | -                           | -                             |
| Saline          | 24                                  | yes (6)                          | yes (5)    | yes (6)                           | yes (6)                     | yes (6)                     | yes (6)                       |
| Flaming Peat    | 24                                  | yes (6)                          | yes (6)    | yes (6)                           | yes (6)                     | -                           | yes (6)                       |
| Smoldering Peat | 24                                  | yes (6)                          | yes (6)    | yes (6)                           | yes (6)                     | -                           | yes (6)                       |
| Flaming Red Oak | 24                                  | yes (6)                          | yes (6)    | yes (6)                           | yes (6)                     | -                           | yes (6)                       |
| Smoldering Red Oak | 24                               | yes (6)                          | yes (6)    | yes (6)                           | yes (6)                     | -                           | yes (6)                       |
| LPS             | 24                                  | yes (6)                          |            | -                                 | yes (6)                     | -                           | -                             |

1 These data were previously generated/reported (Kim et al. 2018; Rager et al. 2021) and re-summarized here to address this study’s aim.