Exploratory analysis of the impact of sex on sputum proteomic response to inhaled wood smoke in humans

Morgan Nalesnik[1,2], Elise Hickman[1,2] (https://orcid.org/0000-0001-6152-050X), Martha Almond[2], Laura Herring[3,4], Angie L. Mordant[4], Allie C. Mills[4], Alexis Payton[2,6], Julia E. Rager[1,2,6] (https://orcid.org/0000-0002-2882-5042), David B. Peden[1,2,5] (https://orcid.org/0000-0003-4526-4627), Ilona Jaspers[1,2,5] (https://orcid.org/0000-0001-8728-0305), Neil E. Alexis[1,2,5] (https://orcid.org/0000-0002-9417-8269), Meghan E. Rebuli[1,2,5] (https://orcid.org/0000-0003-1918-2257)

[1]Curriculum in Toxicology & Environmental Medicine, University of North Carolina, Chapel Hill, NC
[2]Center for Environmental Medicine, Asthma, and Lung Biology, University of North Carolina School of Medicine, Chapel Hill, NC
[3]Department of Pharmacology, University of North Carolina School of Medicine, Chapel Hill, NC
[4]Michael Hooker Metabolomics and Proteomics Core, University of North Carolina School of Medicine, Chapel Hill, NC
[5]Department of Pediatrics, University of North Carolina School of Medicine, Chapel Hill, NC
[6] Department of Environmental Sciences and Engineering, University of North Carolina School of Medicine, Chapel Hill, NC

Corresponding author
Meghan E. Rebuli, PhD
4004A Mary Ellen Jones Building; CB 7325
116 Manning Drive
University of North Carolina at Chapel Hill
Chapel Hill, NC 27599
meradfor@email.unc.edu

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ABSTRACT

Wildfires have surged in frequency and severity and in 2022 contributed to nearly 30% of the fine inhalable particulate matter ($PM_{2.5}$) in the United States. Health effects from wildfire-induced wood smoke (WS) exposure include worsened pre-existing lung diseases and lung function, increased emergency room visits, and increased risk of premature death. Evidence suggests that males and females have unique responses to air pollutants, but sex-specific responses to WS remain understudied. To evaluate whether males and females differentially respond to WS, we analyzed induced sputum in humans following a controlled chamber exposure to WS. A total of 79 participants were exposed to 500μg/m^3 of WS for 2 hours with intermittent exercise, and a subset of participants' samples were analyzed for cellularity and cytokine concentrations and protein expression in the sputum supernatants. Cell differentials were compared between pre-, 6 hours, and 24 hours post-exposure, and proteomic and cytokine signatures were compared between pre- and 24 hours post-exposure. 368 proteins were significantly different in females and 27 were significantly different in males post exposure. Pathway analysis revealed inhibition of leukocyte extravasation signaling, phagosome formation, and macrophage nitric oxide and reactive oxygen species pathways in females versus males. Females had a lower percentage of iNOS+ and higher percentage of CD301+ sputum macrophages versus males. Overall, this exploratory analysis suggests that in response to acute WS exposure, different pathways are affected in females compared with males. Future studies are needed to determine whether this confers an immune advantage and to understand the mechanisms of sex-specific WS-induced respiratory effects.

INTRODUCTION

Exposure to air pollution causes adverse health effects and is estimated to be responsible for over 7 million premature deaths per year (Lelieveld et al. 2020; Manisalidis et al. 2020). Humans are exposed to a wide range of air pollutants, but one pollutant that stands out as a major public health concern is wood smoke (WS), generated from burned tree biomass. Woodsmoke exposure is primarily derived from wildfire events and indoor wood burning. Due to climate change, the length, scale, and duration of wildfire events has increased (Grant and Runkle 2022; Schoennagel et al. ; Xu et al. 2020), creating substantial amounts of respirable ($PM_{2.5}$ - PM_{10}) wildfire smoke particles. In 2022 alone, nearly 30% of $PM_{2.5}$ in the United States originated from wildfires (US EPA 2025). During wildfire events, local ambient $PM_{2.5}$ levels can abruptly reach > 500 μg/m^3, well above the current single day National Ambient Air Quality Standard of 35 μg/m^3. Indoor wood burning is also a considerable source of WS exposure, with over 2 billion people around the world relying on burning wood for indoor cooking and heating (Smith et al. 2000). Although indoor wood burning is commonly associated with developing countries, it is also popular in the United States, where over 2 million households use wood as the main fuel source for heat (National Institute of Environmental Health 2022). Burning biomass indoors can also serve as a significant source of PM_{10} WS exposure, with mean 24-hour PM_{10} levels in households burning biomass often ranging from 300 to 3,000 μg/m^3, well above the National Ambient Air Quality Standard of 150 μg/m^3 (Salvi and Barnes 2010).

WS exposure is associated with increases in hospitalizations of people with asthma, chronic obstructive pulmonary disease, pneumonia, acute bronchitis, and cardiovascular disease (Delfino et al. 2009; Franchini and Mannucci 2012; Rappold et al. 2011; Reid et al. 2016; Reid and Maestas 2019). As wildfires continue to increase in frequency and incidence, increases in morbidity, mortality, and premature deaths among susceptible populations are also anticipated to increase (Orru et al. 2013; Silva et al. 2017; West et al. 2007), highlighting the importance of studying the mechanisms of WS health effects. Previous studies of specific molecular pathways have revealed increased oxidative stress and inflammation following WS exposure, along with decreases in lung function (Barregard et al. 2008; Bloomer et al. 2009; Kinney 2008; Reid and Maestas 2019). Our group has previously reported that WS exposure *ex vivo* increases gene expression of IL-1β and IL-6 in primary nasal epithelial cells and that controlled exposure to WS in healthy human participants increases sputum percentages of polymorphonuclear cells (PMNs (neutrophils)) and concentrations of IL-6, IL-8, and IL-1β, and decreases TNFα (Alexis et al. 2022; Brocke et al. 2022). Another study found decreases in bronchial wash metalloproteinases and ICAM-1 post WS exposure (Muala et al. 2015). Although the results from these studies have demonstrated the effects of WS on pulmonary inflammatory biomarkers, these studies were limited in scope, as they only assessed a small number of endpoints.

Moreover, it is increasingly recognized that there are additional factors, such as sex, that may place healthy individuals at increased risk from WS exposure (Rebuli 2021). For example, males exposed to WS followed by live attenuated influenza virus primarily produced an inflammatory response while females exhibited decreased host-defense markers in response to dual exposure to pollutant and virus (Rebuli et al. 2019). Similarly, in a study of human nasal epithelial cells exposed *ex vivo* to WS, cells from females exhibited more robust downregulation in gene expression than cells from males (Brocke et al. 2022). Animal studies have also shown sex-specific differences in inflammatory and cell signaling responses following exposure to

wildfire or ozone (Black et al. 2017; Buford et al. 2024; Cabello et al. 2015). However, key knowledge gaps remain in understanding how sex differences influence respiratory susceptibility to adverse responses to WS exposure due to the limited number of endpoints measured in previous studies and the complexity of the response to WS, which is hypothesized to involve a myriad of mediators and cells.

Here, we examined the airway proteomic response to WS exposure using induced sputum samples collected pre- and 24 hours post-WS exposure in healthy human adult participants. We found significantly different protein and biological pathway responses to WS exposure between male and female participants. We further demonstrated that these findings were supported by sputum inflammatory cell and macrophage phenotype analyses. Overall, this study demonstrates the importance of sex-stratified analyses and provides insights into differing mechanisms of response to WS in males and females using an unbiased proteomics approach.

MATERIALS AND METHODS

Study Protocol

This study represents an extended analysis of banked samples from a previously conducted study by Alexis et al. (Alexis et al. 2022). The study design is summarized in Figure 1. 79 healthy adults (29 males and 50 females) aged 18-40 years were recruited for this study. Participants were either non-smokers with an FEV1 of >80% of predicted or individuals with physician-diagnosed mild asthma with an FEV1 of >75% predicted. Mild asthmatics were defined as participants only using a bronchodilator as needed and who were not using inhaled or oral corticosteroids. Participants underwent the consent process (in accordance with the Declaration of Helsinki) and then began study procedures. Full inclusion and exclusion criteria and the study protocol have been described in prior publications (Alexis et al. 2022). Briefly, participants received physical exams by the study physician and baseline samples were collected, including induced sputum with spirometry. Participants then entered into a wood smoke exposure chamber and were exposed to 500µg/m^3 of WS derived from kiln dried red oak for 2 hours with intermittent exercise as described previously (Alexis et al. 2022; Burbank et al. 2019; Ghio et al. 2012). Notably, our group has previously demonstrated no difference between participants' sputum cell counts, sputum cell surface marker expression, and cytokine expression at baseline and air exposure with exercise; therefore, baseline samples were used as control samples for each participant (Lay et al. 2007). Lung function (reported previously in (Alexis et al. 2022)) was measured immediately post exposure, while induced sputum was collected at 6 and 24 hours post exposure (Alexis et al. 2022; Hernandez et al. 2012; Kobernick et al. 2016). As shown in Figure 1 and detailed below, of the 79 recruited participants, 49 provided adequate sputum samples which allowed for evaluation of sputum cellularity. From these 49 participants, samples from 30 volunteers were randomly selected for proteomic analyses at pre and 24 hours post exposure based on sample volume remaining and balancing the cohort by sex. 24 hours post exposure was chosen for proteomic analysis because the largest changes in cell differentials were previously observed at this time point (Alexis et al. 2022). The study protocol was approved by the University of North Carolina at Chapel Hill Institutional Review Board (study number #15-1775) and is listed in Clinicaltrials.gov (NCT02767973). All study procedures and analyses were performed in accordance with relevant guidelines and regulations.

Sputum Induction, Sample Processing, and Cell Counts

Sputum samples were collected and processed using established methods (Alexis et al. 2022; Lay et al. 2011). Briefly, participants underwent sputum induction with three 7-minute periods of hypertonic saline inhalation. Sputum samples were kept on wet ice until the cell-enriched mucus plugs were picked manually from each sputum sample by trained personnel and then processed to reduce dilution by saliva and squamous epithelial cell contamination. The plugs were weighed using a standard lab balance, treated with Dulbecco's phosphate-buffered saline (Sigma-Aldrich Cat. No. D8537-500ML), homogenized on a rotating tumbler, centrifuged and cell free supernatants captured and stored at -80°C. The sputum sample cell pellets were homogenized (15 min.) with 0.1% dithiothreitol (Thermo Scientific Cat. No. R0861) and filtered, then total cell count and cell viability were assessed using hemocytometry. Cytospin slides were prepared and stained with Hema 3 stain for differential cell count analysis excluding squamous epithelial cells.

Immunohistochemical staining for iNOS (antibody from R&D Systems, Minneapolis, MN 55413) was used as a marker for M1-like/pro-inflammatory macrophages (Golden et al. 2021; Xue et al. 2018), and CD301 (antibody from Miltenyi Biotec, San Diego, CA) was used as a marker for M2-like/alternatively activated macrophages (Abdelaziz et al. 2020; Xiang et al. 2022). Slides were visualized using light microscopy, and cell percentages were calculated based on a minimum of 100 macrophages counted per slide. Isotype controls were used, and samples were counted in a blinded fashion. For representative images of CD301+, iNOS+, and CD301+/iNOS+ macrophages, see Figure S1. Cell counts were reported as percentages of total cells and cells per mg mucus plug to reflect the nature of the response (ratio of cell types present) and magnitude of response (total cells), respectively.

Multiplexed Cytokine Analysis

Cell-free sputum supernatants pre and 24 hours post exposure were analyzed for IL-1β, IL-6, IL-8, and TNFα protein concentrations using a Meso Scale Discovery (Rockville, MD) V-PLEX Human Proinflammatory Panel II as previously published according to the manufacturer instructions (Alexis et al. 2022).

Proteomics

Proteomic sample preparation, mass spectrometry, and raw data processing were performed by the UNC Michael Hooker Metabolomics and Proteomics Core. The protein concentration of each cell-free sputum supernatant sample was quantified using the Pierce BCA colorimetric assay (Thermo), and an estimated 150 µg of sample was aliquoted for further processing. The 150 µg aliquots were dried down via lyophilization, resuspended, and prepared using S-trap mini (Protifi) according to manufacturer protocol. Tryptic peptides were cleaned using Pierce desalting spin columns (Thermo), dried and quantified using Pierce fluorometric peptide quantification assay (Thermo). All samples were normalized to 0.1 µg/µl. Samples were analyzed by LC-MS/MS using an Ultimate3000 coupled to anExploris480 (Thermo Scientific). Individual samples from each participant were injected onto an IonOpticks Aurora series 2 C18 column (75 µm id × 15 cm, 1.6 µm particle size; IonOpticks) and separated over a 90 min gradient. The gradient for separation consisted of 3-41% mobile phase B at a 250 nl/min flow rate, where mobile phase A was 0.1% formic acid in water and mobile phase B consisted of 0.1% formic acid in 80% ACN. The Exploris480 was operated in product ion scan mode for Data Independent Acquisition (DIA). A full MS scan (m/z 300-1300) was collected; resolution was set to 120,000 with a maximum injection time of 20 ms and AGC target of 300%. Following the full MS scan, a product ion scan was collected (30,000 resolution) and consisted of stepped higher collision dissociation (HCD) set to 25.5, 27, 30; AGC target set to 3000%; maximum injection time set to 55 ms; variable precursor isolation windows 300-1300 m/z. As a quality control metric, a separate pooled control sample was analyzed intermittently during LC-MS/MS to assess instrument variability. The pooled control sample was created by combining 1 µg from all 27 samples and then diluting to 0.1 µg/µl.

Raw data files were processed in Spectronaut (v. 16.1.220730.53000, Biognosys) using a library-based method. The spectral library was created using the pooled replicates searched against a Uniprot Human reviewed database (UP000005640, containing 20,360 protein sequences, downloaded Janaury 2022). The library contained 2816 proteins. Next, the samples

were analyzed against the library in Spectronaut to identify and quantify proteins. The following settings were used: enzyme specificity set to trypsin, up to two missed cleavages allowed, cysteine carbamidomethylation set as a fixed modification, methionine oxidation and N-terminal acetylation set as variable modifications. Precision iRT calibration was enabled. A false discovery rate (FDR) of 1% was used to filter all data. Single hits were removed. This resulted in retention of 2244 features. Supernatants from three of these volunteers were contaminated with blood, as indicated by high detection of blood proteins and a low number of proteins detected, and were removed from the dataset for downstream analysis, allowing for proteomic analyses of samples from 27 participants.

Data Analysis

Analysis Software and Reproducibility. All analyses were conducted using R version 4.5.0 (R Core Team 2025) using methods similar to those we have previously published (Vitucci et al. 2023). Packages used throughout the analysis included *tidyverse* (Wickham et al. 2019), *janitor* (Firke 2024), *ggpubr* (Kassambara 2025), *rstatix* (Kassambara 2023), and *table1* (Rich 2023). See Data Links section for links to script and data.

Sputum Cell Differentials and Cytokines. Raw and log2-transformed data were tested for normality and homogeneity of variance using the Shapiro-Wilk test, histograms, Q-Q plots, and Levene's test. All endpoints with the exception of percentage cell metrics were log2-transformed prior to analysis. A mixed repeated measures ANOVA was used to determine the effect of sex and timepoint (pre, 6 hours post exposure, 24 hours post exposure) on sputum cell differentials and cytokine concentrations while accounting for variation between participants. Paired pairwise t-tests with Benjamini-Hochberg correction were used to follow up on endpoints with overall $p < 0.05$ for either sex or timepoint. Data were also stratified by sex and compared between timepoints using pairwise paired t-test with Benjamini-Hochberg correction. Data were missing for some participants at one or more timepoints; thus, for each endpoint, only participants with complete data across all three timepoints were retained when testing for significant differences for that endpoint (see Supplemental Tables S1-S3 for detailed N per endpoint and factor).

Proteomic Data Processing. Intensity values were median normalization by summing the abundance values for each sample, calculating the median values across all of the summed abundance values, and then calculating the final feature abundance as the ratio of the summed abundance value over the overall median summed abundance value. Next, detection and protein identity filters were applied, where features were removed if there were fewer than three peptides detected or if the feature was mapped to multiple accession numbers, resulting in removal of 390 features (proteins or protein classes). Lastly, principal component analysis (PCA) using the *factoextra* package (Kassambara and Mundt 2020) was performed to identify potential sample outliers using qualitative visual inspection and quantitative flagging of outlier samples greater than 6 standard deviations away from the mean (Tan et al. 2016). No outliers were detected from either of these methods. After processing, 1854 proteins were retained and subsequently analyzed. Normalized and $\log_2$-transformed normalized data were then tested for normality and homogeneity of variance using the Shapiro-Wilk test, histograms, Q-Q plots, and Levene's test. The $\log_2$-transformed data were more normally distributed and had more equal homogeneity of variance than the non-transformed data, so $\log_2$ data were used for the proteomic differential expression analysis.

Proteomic Differential Expression. Paired t-tests were used to understand differences in protein expression pre- vs. 24 hours post-exposure, with the data analyzed in aggregate (all participants together) and stratified by sex. T-tests were also used to compare protein expression between males and females at baseline. A significance threshold of $p < 0.05$ was used for downstream interpretation.

Proteomic Pathway Analysis. To understand pathways altered by woodsmoke exposure, canonical pathway analyses were performed on the significantly differentially expressed proteins from each analysis (all participants together and stratified by sex) using Ingenuity Pathway Analysis (IPA)(Qiagen). Pathways were considered significantly enriched with $p < 0.05$. Pathways with z-scores > 2 were considered significantly activated, while pathways with z-scores < -2 were considered significantly inhibited.

RESULTS

Participant Demographics

Demographics for recruited participants, participants with sufficient sputum to assess sputum cellularity, and participants whose samples were analyzed via proteomics are presented in Table 1. Participants (n = 49) who were exposed to WS and produced adequate sputum samples were 55% female, 86% not Hispanic/Latino, 73% white. Demographics were similar for participants whose samples were analyzed via proteomics: 52% female, 96% not Hispanic/Latino, and 74% white. A small proportion of the participants were very mild asthmatics. There were no significant differences in sex, asthma status, race, ethnicity, age, or BMI between recruited participants, participants with sufficient sputum, and participants with samples analyzed via proteomics. Additionally, there were no notable differences between sexes in age, BMI, or asthma status within each of the participant subsets.

Sputum Cellularity and Cytokine Concentration

Using cell differential and cytokine data from the 49 participants who produced sufficient sputum for analysis, we found that there were significant differences in sputum cellularity and cytokine concentrations between sexes and across visits (Figure 2). In the aggregate analysis, the proportion of PMNs was increased at 24 hours post-exposure, while the proportion of macrophages and macrophages per mg was decreased. At baseline, males had significantly higher PMNs per mg than females, and at 24 hours post-exposure, males had significantly higher concentrations of IL-1β, IL-6, and IL-8 than females. When the analysis was stratified by sex, only IL-8 concentration was increased in males at 24 hours post exposure in comparison with males pre-exposure. In contrast, within females, the proportion of PMNs increased and proportion of macrophages decreased at both 6 and 24 hours post-exposure, and macrophages per mg decreased at 24 hours post exposure, suggesting that females were driving the aggregate results, as changes in cellularity were not observed in disaggregated male data. Summary statistics by group and full statistical results are available in Supplementals Tables S1-S3. Plots showing individual participant data connected by lines are show in Figure S2.

Differentially Expressed Proteins

In the aggregate analysis with all participants, expression of 258 proteins across all participants was significantly altered ($p < 0.05$) following WS exposure (Figure 3A). All significantly differentially expressed proteins, with the exception of 1, were decreased. Significantly differentially expressed proteins with the greatest magnitude of change following WS exposure included C-X-C motif chemokine ligand 6 (CXCL6), macrophage receptor with collagenous structure (MARCO), and GTP Cyclohydrolase I Feedback Regulator (GFRP), all of which were decreased in expression.

We also compared protein expression between males and females in the pre samples to capture baseline sex differences in the central airway proteome (Figure S3). 25 proteins were significantly differentially expressed between males and females at baseline, with 23 proteins

increased and 2 proteins decreased in males in comparison with females (Figure S3). The two proteins with lower expression in males than females were serpin family B member 10 (SPB10) and high mobility group nucleosomal binding domain 2 (HMGN2). Proteins with significantly higher expression in males included phosphatidylethanolamine binding protein 4 (PEBP4) and mucin 5B (MUC5B).

In females (Figure 3B), 368 proteins were significantly decreased. In males (Figure 3C), 11 proteins were decreased and 16 proteins were increased following WS exposure. In females, significantly differentially expressed proteins with the greatest magnitude of change following WS exposure included MARCO, GFRP, CXCL6, soluble inorganic pyrophosphatase 5 (PPA5), fatty acid-binding protein 4 (FABP4), and glutathione S-transferase omega 1 (GSTO1). In males, significantly differentially expressed proteins with the greatest magnitude of change following WS exposure included mucin 2 (MUC2), proteoglycan 2 (PRG2), repetitive proline-rich cell wall protein 1 (PRP1), cathepsin G (CATG), lactoperoxidase (PERL), and golgi apparatus protein 1 (GSLG1). Next, we identified the significantly altered proteins that overlapped between each group (Figure 3D). Of the 258 significantly altered proteins across all participants combined, 62 proteins were significantly differentially expressed uniquely in the aggregate analysis (no overlap with stratified male or female analyses), while 192 proteins overlapped with the female stratified analysis, and 4 proteins (DOPD, GALT6, NNRE, RCN1) overlapped with the male stratified analysis. The 192 overlapping proteins in the female stratified analysis included many proteins with high magnitudes of change, such as MARCO, GFRP, PPA5, CXCL6, FABP4 and GSTO1, suggesting aggregate data are primarily driven by females in the study. There was no overlap of significantly altered proteins between the female and male stratified analyses. Complete proteomic differential expression analysis results are available in Supplemental Table S4, and overlapping significantly differentially expressed proteins between analyses are available in Supplemental Table S5.

Proteomic Pathway Analysis

We used IPA to identify cellular pathways associated with differentially expressed proteins based on t-test results (Supplemental Tables S6-S8). Consistent with the overall decrease in protein levels, most canonical pathways were predicted to be inhibited (Figure 4). For all pathways depicted in Figure 4, the aggregate predicted activity appears to be driven by female proteomic responses. Neutrophil degranulation was the most significantly affected pathway within the all participant and female stratified analysis (highest magnitude z-score). Females demonstrated strong inhibition of this pathway while males showed minimal inhibition. These findings are consistent with sputum cellularity and cytokine data (Figure 2), where females had significantly lower concentrations of IL1β, IL-6, and IL-8 than males at 24 hours post WS exposure. For several of these pathways there were differences in directionality of z-scores between males and females (Figure 4). For example, females primarily showed inhibition and decreased protein measurements across the leukocyte extravasation pathway (Figure S4A), whereas males showed relatively greater activation in endpoints such as cell mobility, actin cytoskeleton contraction and increased measures of integrins (ITGAM, ITGL, ITGB2) that promote inflammation (Figure S4B), which also aligns with an increase in IL-8 (inflammatory

cytokine and chemoattractant) in males when comparing pre and post exposure (Figure 2). Females and males also exhibited large differences in the phagosome formation pathway, with females showing stronger inhibition throughout the phagocytosis pathway relative to males (Figures S5 and S6). Further, based on identification of the importance of reactive oxygen species (ROS) in response to WS in the literature previously (Barregard et al. 2008), we were also interested in the enrichment of the production of nitric oxides (NO) and reactive oxygen species in macrophages pathway. NO and ROS activity were predicted to be in opposite directions in females (inhibited) and males (activated) (Figure S7). Proteins of particular relevance in this pathway with opposite levels in males in females included nitric oxide synthase 2 (NOS2), signal regulatory protein alpha (SIRPA), and cytochrome b-245 alpha chain (CYBA).

Integration of Proteomic Results and Cell Differential Data

To further interrogate the pathway analysis results related to NO and ROS production in macrophages, we used sputum cell differentials, stained for macrophage subtype markers, as NO and ROS can be modified by macrophage subtype (Chen et al. 2020; Tan et al. 2016; Wang et al. 2014). Based on pathway analysis results, we hypothesized that males would have a greater percentage of iNOS+ macrophages post WS exposure, as NO and ROS production was predicted to be activated in males. Conversely, we hypothesized that females would have a greater percentage of CD301+ macrophages post WS, as NO and ROS production was predicted to be inhibited in females. We observed a trend of males having a higher percentage of iNOS+ macrophages post exposure, though this did not reach statistical significance (Figure 5). For CD301+ macrophages, sex had a significant main effect, with a trend of higher percentage of CD301+ macrophage in females (Figure 5). Overall, although not statistically significant at specific time points, these macrophage phenotype data generally support the sex-specific inhibition/activation of NO and ROS production found in the proteomic pathway analyses. Cell differential data also supported the activation of the IL-8 signaling pathway in males, with males having increased IL-8 concentration at 24 hours post exposure (Figure 2).

DISCUSSION

The purpose of this study was to determine how a controlled inhalational exposure to WS affected the human respiratory proteome in healthy adult human volunteers and specifically to explore whether sex significantly influenced proteomic changes. We found that across all 27 participants, the vast majority of significantly altered proteins from WS exposure showed a decrease in abundance. Next, we stratified our dataset by sex to study the sex-specific effects of WS exposure. We found sex-specific differences in both the number of significantly affected proteins as well as the magnitude of inhibition of canonical pathways such as neutrophil degranulation, leukocyte extravasation signaling, phagosome formation, and production of NO and ROS in macrophages. Given that a majority of the significantly changed proteins across all participants overlapped with those of the female stratified analysis, females appeared to be the primary driver of the downregulated WS response in the aggregate analyses. Sex stratification analyses further demonstrated that males had a minimal downregulated response in the sputum proteome by 24hr post WS exposure where only 27 proteins were significantly altered, whereas females had a strong downregulated response to WS by 24hr post where 368 proteins significantly changed.

Further comparisons revealed that many enriched canonical pathways were affected more in one sex than the other. Females demonstrated strong inhibition of leukocyte extravasation signaling and phagosome formation, which align with the unchanged levels of proinflammatory cytokines IL-6 and IL-8 observed in females from pre to 24 hours post WS. Interestingly, we measured a significant increase in sputum percent PMNs 24 hours post WS in females. We hypothesize that females induce a neutrophilic response to the airways upon WS exposure, but do not degranulate as suggested by inhibition of the neutrophil degranulation pathway. Another important pathway where we observed a sex difference was the production of NO and ROS in macrophages, a characteristic of pro-inflammatory iNOS+ macrophages. We found this pathway was predicted to be strongly inhibited in females yet activated in males. These pathway results were further supported by the decreased percentages of iNOS+ and greater percentage of CD301+ sputum macrophages reported in females relative to males, respectively. Males presented a mix of inhibition and activation signals across all four pathways mentioned, seeming to be a more mixed response to exposure. Males also produced significantly higher levels of IL-6 and IL-8 by 24 hours post exposure, as well as higher percentages of proinflammatory iNOS+ macrophages compared to females, suggesting a sustained inflammatory response in males. These pathway and sputum differential analyses demonstrate sex-specific inflammatory responses to WS. However, despite elevated levels of cytokines in males, we did not observe an increase in neutrophils; conversely, we observed an increase in neutrophil proportion in females but did not observe elevated cytokine levels. We hypothesize that these observations may be due to response kinetics between sexes. For example, cytokines may have already peaked in female participants at the time of sample collection, while cytokines were at their peak in males at the time of sample collection, with the potential for cellular composition changes in males at a later timepoint than sampled in this study. Interestingly, the sputum PMN response observed in our data is consistent with observed neutrophil responses in other inhalation exposures. For example, human controlled exposure studies have shown sputum PMN responses ranging from no change to over 20% absolute increases after ozone and endotoxin exposures (Burbank et al. 2018; Burbank et al. 2020; Burbank et al. 2019; Hernandez et al. 2010), and in a previous study using samples that

partially overlapped with this study, similar trends in cell differentials and cytokine concentrations were observed (Cobos-Uribe et al. 2025). In both studies, we found that that macrophages per mg and macrophage percentage in sputum concurrently decrease, suggesting that previously observed neutrophilia (indicated by percentage of neutrophils) may be a result of decreased total numbers of macrophages rather than increased total numbers of neutrophils.

Our proteomics data support several potential explanations for the sex differences observed. First, at the 24hr post timepoint, females may have transitioned from the pro- inflammatory phase to a resolving phase, as evidenced by a large number of downregulated proteins, inhibition of inflammatory canonical pathways, and upregulation of CD301+ macrophages. Second, it could suggest a sexually dimorphic response to wood smoke exposure, where in females, respiratory immune activity is generally suppressed or less reactive compared to males. The literature has shown in certain cases that females appear to have a protective advantage to deleterious effects of exogenous exposures. For example, in the context of influenza and coronavirus infections, higher levels of estrogen are associated with improved outcomes (Abramenko et al. 2021; Robinson et al. 2014). The difference in inflammatory resolution between the sexes may also be partially attributed to differences in neutrophil function. Gupta and colleagues measured greater type I IFN pathway activation in female derived neutrophils, greater ROS production, and NET formation than males (Gupta et al. 2020). They also found that males had more immature neutrophil markers including significantly higher mitochondrial DNA and a transcriptomic profile of immature neutrophil phenotypes (Gupta et al. 2020). Blazkova and colleagues made similar conclusions, where females exhibited greater levels of surface maturation markers and in a more activated state at baseline (Blazkova et al. 2017).

Other potential mechanisms that may explain our observed sex-based difference in inflammatory response relate to the ability of estrogen to downregulate the NF-kB signaling pathway and decrease IFN signaling to decrease inflammation (Pelekanou et al. 2016; Xing et al. 2012). This specific pathway has also been studied in the context of macrophages. One group found that macrophages treated with estradiol had reduced NF-kB transcriptional activity and accelerated IL-10 production in response to inflammatory stimuli (Villa et al. 2015). These data suggest that estrogens promote more protective mechanisms in response to inflammation in females. Other studies report that estradiol shifts macrophages toward an M2/alternatively activated phenotype that is enhanced by IL-4 activity, a key activator of M2 macrophages and promotes M2 migration(Harding and Heaton 2022; Keselman et al. 2017; Routley and Ashcroft 2009). In these studies, E2 also reduced NF-kB and NO production associated with pro-inflammatory macrophages, the latter finding also observed in this study(Harding and Heaton 2022; Nebert and Vasiliou 2004). Our data support these studies, as females had higher percentages of sputum anti-inflammatory M2 macrophages and less induction of pro-inflammatory cytokines (IL-6 and IL-8), although the specific role of WS and estradiol in mediating these differences requires further investigation.

Previous studies investigating sex differences following exposure to WS have similarly suggested suppression of immune responses in females. For example, Rebuli and colleagues found that after controlled WS exposure paired with a live attenuated influenza virus exposure, males showed increased expression of inflammatory genes while females downregulated genes

critical for host defense responses, which contrasted with robust LAIV response in the female filtered air exposure (Rebuli et al. 2019). Another study that exposed human nasal epithelial cells (hNECs) to WS particles also found greater downregulation of gene expression in female derived cells than male derived cells and that WS particle exposure may dampen the female antiviral response (Brocke et al. 2022). Lastly, Black and colleagues analyzed the response of peripheral blood mononuclear cells (PBMCs) from a cohort of rhesus macaques that were exposed as infants to ambient wildfire smoke to LPS or flagellin and found that female derived PBMCs had significantly reduced synthesis of IL-8 compared to PBMCs from males (Black et al. 2017). Together, these data support our findings that WS likely differentially affects male and female respiratory immune responses and results in immune suppression in females. An alternative hypothesis is that both males and females have a pro-inflammatory response to WS exposure but that the male response is more prolonged. However, additional research is needed to test this hypothesis and to understand whether female specific immune suppression is beneficial or detrimental in terms of development of long-term respiratory disease due to exposure.

One important limitation to this study is that our sample size for the proteomics analysis was relatively small (n = 27 total, n=14 males, n=13 females) and thus was underpowered for detecting statistically significant changes when adjusting for multiple hypothesis testing. Although these results may therefore be considered hypothesis-generating, they are the first to suggest sex specific responses in the human respiratory proteome in response to WS exposure. For the exposure, this study was limited to use of one type of biomass (kiln-dried red oak), and future studies are needed that include other regional biomass types to evaluate potential for differential effects by local ecology and to better simulate real-world burns, which include a variety of fuel sources. We also only conducted proteomics analysis on the pre- and 24 hours post-exposure timepoints; additional earlier or later timepoints would have allowed for improved understanding of the temporal response to WS. Additionally, the use of induced sputum has limitations, including 1) capturing only extracellular proteins, and 2) the potential for contamination via the oral cavity during sampling, which we addressed through paired analyses. Because we were unable to assess cellularity or protein biomarkers from participants with insufficient sample, it is not possible to say whether these participants responded differently to woodsmoke than participants who did produce sufficient sample. Furthermore, while we completed sex-specific analyses, we were unable to control for estrogen levels or hormonal cycle as this was not included in the original study design. To evaluate the potential for sex hormones to drive observed differences, future studies would be needed. Finally, assays assessing immune cell function (e.g,,phagocytosis, ROS, NETosis, additional cell surface markers) are needed to validate the proteomic findings that suggest a more immune-suppressed phenotype in females and assess macrophage phenotypes with more granularity than was possible with staining for only iNOS and CD301.

To our knowledge, this study is the first to demonstrate that WS exposure in healthy adults induces sex specific airway proteomic responses. Males exhibited a mix of increased and decreased protein levels as well as proteins associated with activation of leukocyte extravasation signaling, NO and ROS in macrophages, and phagosome formation. Moreover, sputum from males showed higher percentages of PMNs and pro-inflammatory iNOS+ macrophages compared to females across multiple timepoints. Conversely, females exhibited distinct decreased protein levels and showed inhibition of proteins associated with leukocyte

extravasation signaling, NO and ROS in macrophages, and phagosome formation. Sputum collected from females showed lower PMN percentages and higher levels of anti-inflammatory CD301+ macrophages across multiple timepoints compared to the males. While further work is needed to understand the mechanisms of sex specific response and confirm changes in cellular function, these results further define sex-specific inflammatory responses to air pollution exposure and may aid future research in sex-specific inflammatory mitigation strategies. These results also suggest that male and female susceptibility to WS and adverse effects induced by exposure may differ substantially, highlighting that future research and regulatory assessments should consider sex when evaluating adverse health effects of WS.

DATA LINKS

Analysis script and files are available via Github (https://github.com/UNC-CEMALB/Exploratory-analysis-of-the-impact-of-sex-on-sputum-proteomic-response-to-inhaled-wood-smoke-in-huma). Raw data, processed data, supplemental figures, and supplemental tables are available via Dataverse (Nalesnik et al. 2025) (private url for peer review: https://dataverse.unc.edu/previewurl.xhtml?token=33ff1142-cbc3-4b62-9e71-7808b209fc8b).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

FUNDING

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AUTHOR CONTRIBUTIONS

IJ, NEA, and DBP conceived of and designed the study. MA, NEA, IJ, and DP contributed to sample acquisition. MN, EH, LH, ALM, ACM, AP, JER, IJ and MER contributed to sample and data analysis. MN, NEA, DBP, and MER contributed to data interpretation. MN, EH, NEA, and MER created the initial manuscript draft. All authors reviewed the manuscript critically for important intellectual content and approved of the final version. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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TABLES

Table 1. Demographic data stratified by sex for the cohort of participants who were recruited, produced sufficient sputum for sputum cell analysis, and whose samples were used for proteomic analysis.

	Recruited		Sufficient Sputum		Proteomic Analysis	
	Female (N=50)	**Male (N=29)**	**Female (N=27)**	**Male (N=22)**	**Female (N=14)**	**Male (N=13)**
Asthmatic						
No	40 (80.0%)	24 (82.8%)	18 (66.7%)	17 (77.3%)	11 (78.6%)	10 (76.9%)
Yes	10 (20.0%)	5 (17.2%)	9 (33.3%)	5 (22.7%)	3 (21.4%)	3 (23.1%)
Race						
Asian	3 (6.0%)	2 (6.9%)	0 (0%)	2 (9.1%)	0 (0%)	2 (15.4%)
Black	6 (12.0%)	4 (13.8%)	5 (18.5%)	3 (13.6%)	1 (7.1%)	3 (23.1%)
Other	2 (4.0%)	2 (6.9%)	1 (3.7%)	2 (9.1%)	0 (0%)	1 (7.7%)
White	39 (78.0%)	20 (69.0%)	21 (77.8%)	15 (68.2%)	13 (92.9%)	7 (53.8%)
American Indian/Native Alaskan	0 (0%)	1 (3.4%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Hispanic or Latino						
No	44 (88.0%)	25 (86.2%)	23 (85.2%)	19 (86.4%)	13 (92.9%)	13 (100%)
Yes	5 (10.0%)	4 (13.8%)	4 (14.8%)	3 (13.6%)	1 (7.1%)	0 (0%)
Missing	1 (2.0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Age at Consent						
Mean (SD)	27 (± 6.1)	28 (± 6.0)	27 (± 6.3)	28 (± 6.4)	26 (± 4.7)	28 (± 7.3)
BMI						
Mean (SD)	25 (± 5.1)	25 (± 4.0)	26 (± 5.3)	26 (± 4.4)	24 (± 4.5)	24 (± 3.5)

FIGURE LEGENDS

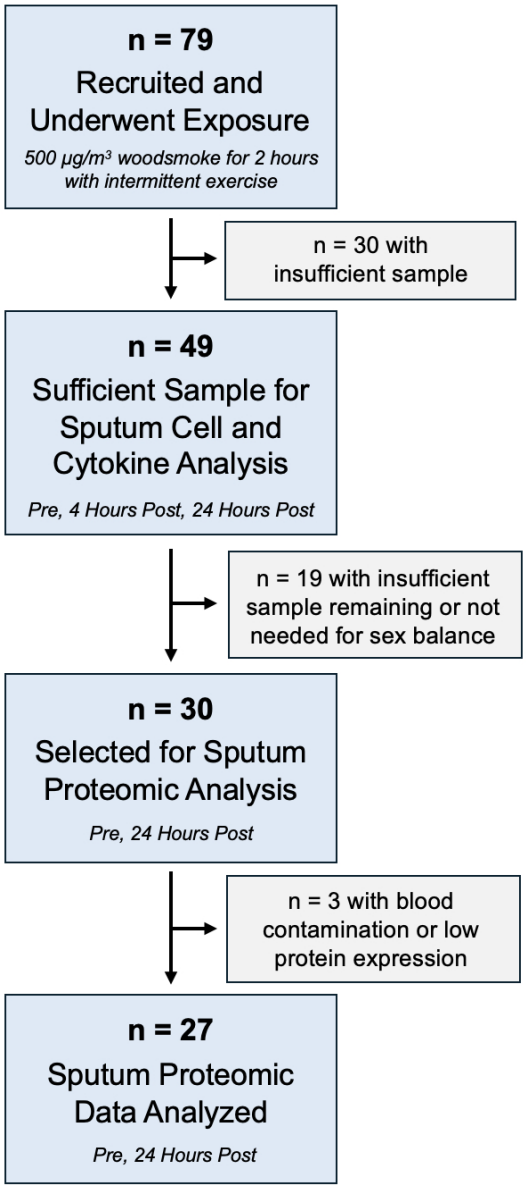
Figure 1. Study design. 79 healthy adults from 18-40 years of age were recruited for this study. Each person was exposed to 500 ug/m^3 woodsmoke (WS) for 2 hours with intermittent exercise. Induced sputum was collected pre, 6 hours post, and 24 hours post WS exposure. 49 participants produced sufficient sample for sputum cell and cytokine analysis. Samples from 30 participants were selected for sputum proteomic analysis. 3 of these samples were contaminated with blood or had low protein expression; thus, sputum proteomic data were analyzed for 27 participants.

Figure 2. Sputum cellular differential counts pre, 6 hours post WS, and 24 hours post WS stratified by sex. A mixed repeated measures ANOVA was used to determine the effect of sex and timepoint on sputum cell differentials and cytokine concentrations. Paired pairwise t-tests with Benjamini-Hochberg correction were used to follow up on endpoints with overall $p < 0.05$ for either sex or timepoint. Data were also stratified by sex and compared between timepoints using pairwise paired t-test with Benjamini-Hochberg correction. Data were missing for some participants at one or more timepoints; thus, for each endpoint, only participants with complete data across all three timepoints were retained when testing for significant differences for that endpoint (see Supplemental Tables S1-S3 for detailed N and statistical results per endpoint and factor). All endpoints with the exception of percentage cell metrics were log2-transformed prior to analysis and are plotted with log2 transformation. Symbol indicates comparison being made. Number of symbols indicates significance. For example, # = $p < 0.05$, ## = $p < 0.01$, and ### = $p < 0.001$. PMN = polymorphonuclear leukocyte/neutrophil.

Figure 3. Differential expression of proteins pre vs. 24 hours post WS exposure in females and males. (A) Across all participants, 257 proteins were significantly decreased, and 1 protein was significantly increased. (B) In females, 368 proteins were significantly decreased. (C) In males, 11 proteins were significantly decreased, and 16 proteins were significantly increased. (D) Of the 258 significantly altered proteins across all participants combined, 62 proteins were uniquely differentially expressed, while 192 proteins overlapped with female stratified analysis and 4 proteins overlapped between male stratified analysis. Female stratified analysis had 176 uniquely altered proteins and male stratified analysis had 23 uniquely altered proteins. There was no overlap of significantly changed proteins between female stratified and male stratified data. Proteins were considered significantly differentially expressed with $p < 0.05$ via t-test. NS = not significant ($p \geq 0.05$). Annotated proteins in A-C are the proteins with the 10 highest absolute value of the $\log_2$ fold change within each analysis.

Figure 4. Canonical pathway enrichment of sputum proteome with WS Exposure. Z-scores for significantly enriched ($p < 0.05$) canonical pathways with biological relevance identified from the sputum proteome with WS exposure. Dotted lines indicate z-scores of -2 and 2. Pathways with z-score > 2 are considered significantly activated, while pathways with z-score < -2 are considered significantly inhibited.

Figure 5. Macrophage phenotypes pre, 6 hours post WS, and 24 hours post WS stratified by sex. Cytospin slides were stained for iNOS and CD301, and cell percentages were calculated based on a minimum of 100 macrophages counted per slide. A mixed repeated measures ANOVA was used to determine the effect of sex and timepoint on macrophage phenotypes. $p < 0.05$ was considered significant.



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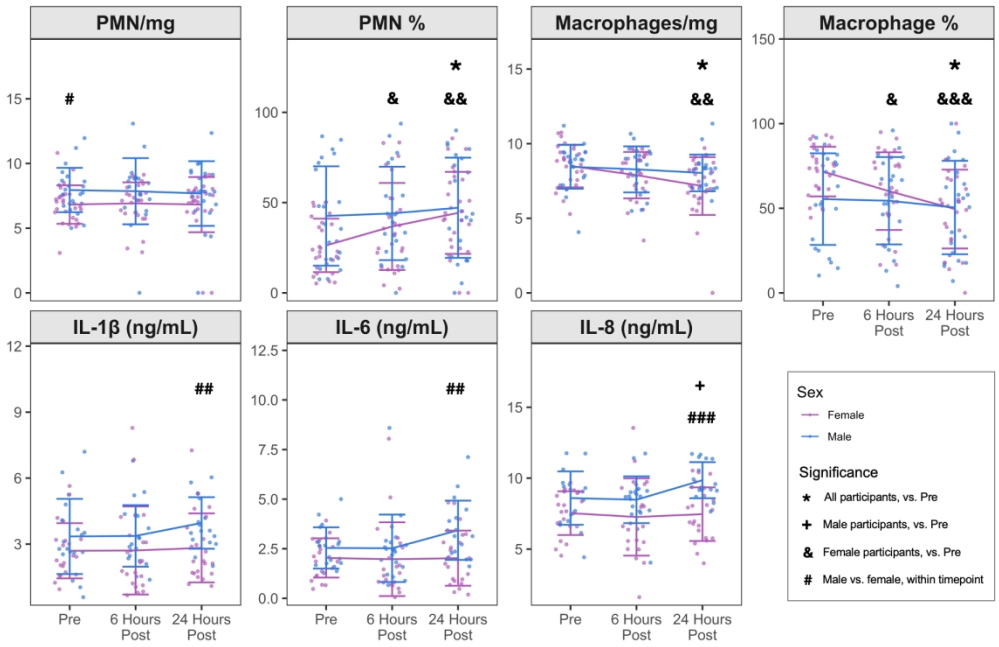

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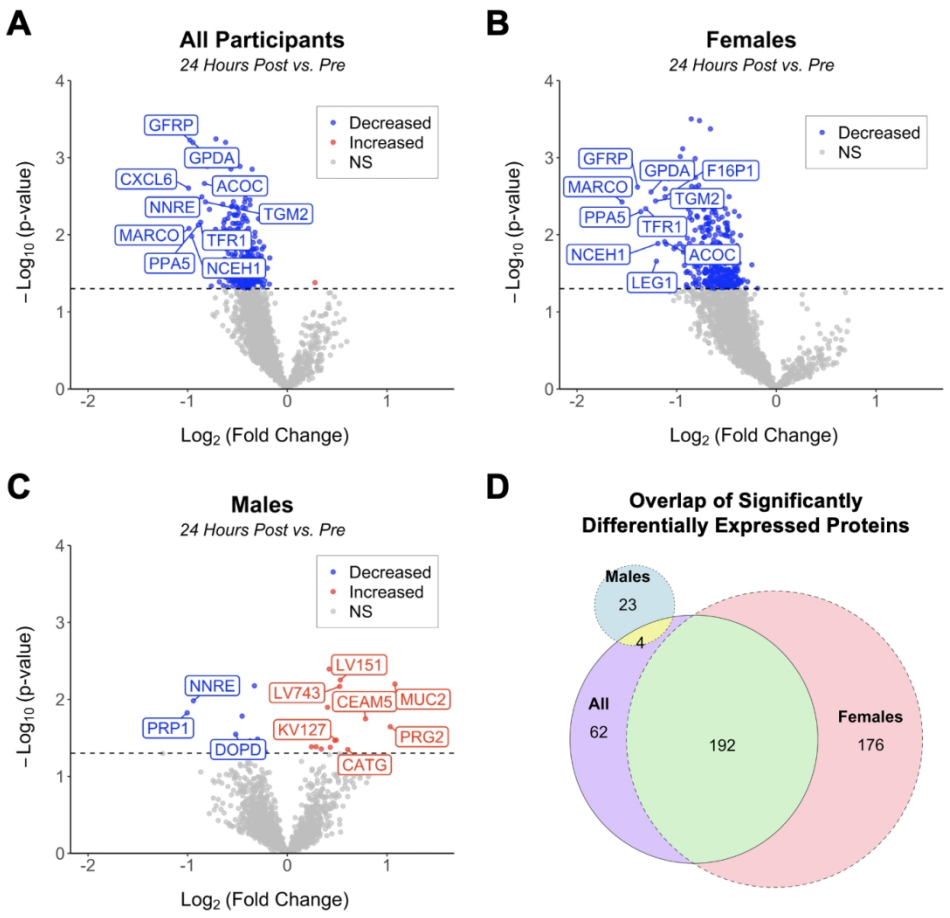

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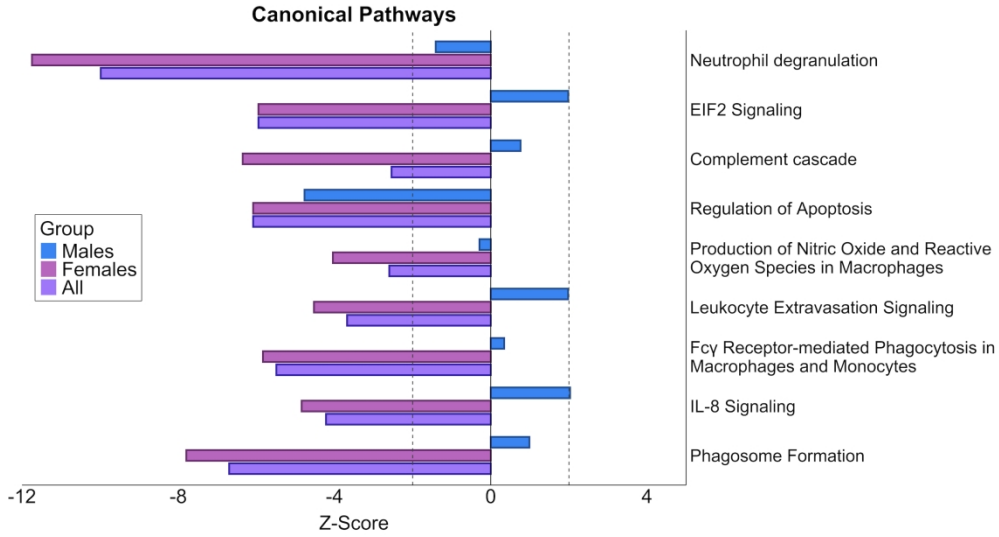

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511x278mm (144 x 144 DPI)

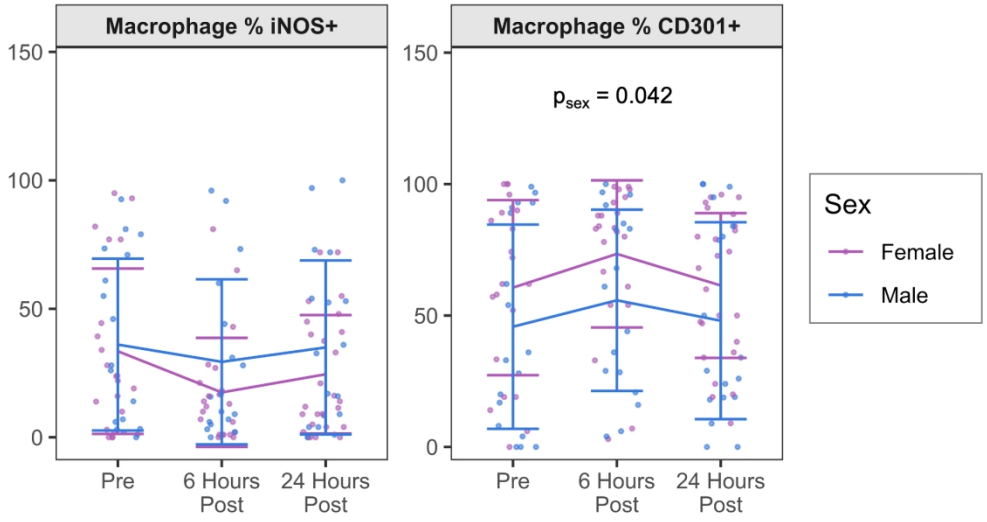

Figure 5. Macrophage phenotypes pre, 6 hours post WS, and 24 hours post WS stratified by sex. Cytospin slides were stained for iNOS and CD301, and cell percentages were calculated based on a minimum of 100 macrophages counted per slide. A mixed repeated measures ANOVA was used to determine the effect of sex and timepoint on macrophage phenotypes. $p < 0.05$ was considered significant.

343x186mm (144 x 144 DPI)