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### INVESTIGATING THE INFLUENCE OF AIRBORNE PARTICULATE MATTER SOURCE

### AND SIZE ON HEALTH OUTCOMES

By

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> Dissertation presented in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Toxicology The University of Montana Missoula, MT October 2015

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### INVESTIGATING THE INFLUENCE OF AIRBORNE PARTICULATE MATTER SOURCE AND SIZE ON HEALTH OUTCOMES

### ABSTRACT

Air pollution is an environmental health concern throughout the world, and is responsible for an estimated 1 out of 8 deaths. As a common component of air pollution, particulate matter (PM) is one of six criteria air pollutants regulated by the Environmental Protection Agency (EPA), with existing ambient standards for both  $PM_{2.5}$  and  $PM_{10}$ . Currently there are no health-based regulations for the size fraction between 2.5 and 10 µm, commonly known as the coarse fraction (PM<sub>c</sub>). Previous studies show that  $PM_c$  can induce health effects such as increased inflammation, and may be more detrimental to susceptible populations when directly compared to  $PM_{2.5}$ . These differences in adverse health effects following exposures are not only influenced by the sizes of the particles, but may also be influenced by other factors including PM composition (chemical and biogenic), source and season.

Globally, biomass burning is a significant source of air pollution. Wood smoke is generated from residential home heating (wood stoves) during the winter months, from burning biomass fuels for cooking (cook stoves), from forest fires during the summer months, and from prescribed/controlled burns during the fall and spring. Wood smoke emissions lead to exposures in both the indoor and ambient environments. Further, regional forest fires can present significant PM exposures to firefighting personnel during occupational activities. Previous wood smoke exposure studies suggest both acute and chronic detriments, both in the lung, and systemically. However, there is still much to be learned about how wood smoke impacts human health, including investigating relationships between different emission sources, acute and chronic exposures, and resultant health effects.

Through three Aims, this project evaluated how different types of PM influenced inflammation and other health measures through both cell studies and a human exposure study. In Aim 1, significant effects were observed in cell studies where PM<sub>c</sub> was more pro-inflammatory compared to PM<sub>2.5</sub>. As determined in Aim 2, wood smoke source also played a role in the pro-inflammatory outcome. Results from Aim 2 revealed that wildfire smoke induced significantly more pro-inflammation compared to residential wood stove emissions. Finally, the Aim 3 human exposure study was designed to replicate wood smoke exposures to wildland firefighters during occupational activities. Through a 10-person crossover exposure trial, we evaluated the potential effects of increasing wood smoke exposure on pulmonary and oxidative stress markers in both plasma and exhaled breath condensate. Dose-response relationships in some of the measured markers were observed, including significant changes in pH, 8-isoprostane, and pentraxin-3 (PTX3). The PTX3 findings suggest that it may be a particularly sensitive acute phase protein to be investigated in future controlled and field studies related to smoke exposures. The development of this wood smoke inhalational facility will further research into acute and chronic health impacts of wood smoke exposure, and in the future provide a platform to address unique research questions related to wood smoke exposures and associated adverse health effects.

### ACKNOWLEDGEMENTS

I'd like to thank the Department of Biomedical and Pharmaceutical Sciences, for affording me this position as a student and teaching assistant. Moreover, I must convey my special gratitude to the Center for Environmental Health Sciences (CEHS) and their focused commitment for all their students, staff, and faculty. I cannot begin to describe how Paulette Jones does what she does, but she does it, and she does it with a passion for order and accountability. Thanks Paulette for helping keep my head out of the weeds. Also, Patrick Dye for always keeping his door open and is always willing to help (of which he did a lot of, on my behalf), thank you. I would like to thank Dr. Tony Ward, my main advisor and mentor, for his patience, his instruction, and most of all for his ongoing effort and willingness to bring me through the refining fire of this PhD program. Thank you for allowing me the freedom to experience the things I needed, both the difficult and the pleasant. My gratitude for Dr. Andrij Holian stems from a long list of learned skills and philosophies. The knowledge I have gained concerning pulmonary toxicology and the causal pathways of many diseases is invaluable. He has helped me form a solid foundation allowing me to critically evaluate toxicology studies, and has imparted useful tools for writing manuscripts and grant proposals, and much more.

Thanks to Dr. Curtis Noonan, for the many useful meetings and valuable feedback, as well as helping provide a wonderful laboratory environment. Also, I would like to acknowledge Dr. Chris Migliaccio for his kind guidance in helping me design and properly perform *in vitro* studies. My external advisory committee member, Dr. Chris Palmer, has provided some useful tools and feedback in many situations. I am fortunate to have had such a terrific committee.

I'd be remiss if I didn't acknowledge Gini Kay for her kind hands and patience in teaching me laboratory techniques. The same goes with Ray Hamilton and his ongoing efforts to keep our labs fully functional and his willingness to provide a helping hand. Also, thanks to Dr. Chuck Dumke for his collaborative help and support of our *in vivo* study. I'd also like to thank Leon Washut and his generous support of the Washut Endowment for Graduate Student Support in Biomedical Sciences. Also, my high school science teacher, Mr. Andy Allen, who taught all my science classes at Mt. Whitney High School (1994-1998), and for helping my love of science swell. I appreciate the Montana state entities, Department of Environmental Quality, and Missoula City-County Health Department (specifically, Nancy Davis, Hoby Rash, Dave Simonson, Doug Kuenzli, and Ben Schmidt) for help in acquiring data. Also, thank you to my fellow graduate students and the many fun adventures we have enjoyed. The help, advice, and friendship of my former lab mates, including Luke Montrose for his stout support, help, and encouragement; Marcy Ballman for the constant brainstorming and amusing discussions; Carolyn Hester, for her wisdom and delightful presence; Emily Weiler, for her fun spirit, intellect, and friendship; and Desirae Ware, for her support, as well as providing opportunities to volunteer and assist in scientific symposiums. Also, a big thank you to Mary French and Britt Postma for their unwavering help in sustaining some of the most difficult aspects of this study.

Finally, I must thank God for helping my family and I maintain a level of comfort and happiness throughout my time at the University of Montana. To that end, I must also thank my wife Patty Ferguson, for her ongoing support and love, for mourning together, and celebrating together. She is my life's anchor, without which I would be lost. Also, for our children William, Benjamin, Patrick, and Juliette, and for the love they shower upon me.

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### Layout of Dissertation

This dissertation presents the results of multiple research studies in which *in vitro* and *in vivo* models were used to investigate how exposure to airborne particulate matter (PM) impacts human health. There are five parts of this dissertation. In the introduction section (Chapter 1), background information related to PM and impacts to human health are provided. In Chapters 2-4, the three Aims of my project are presented in the format of journal manuscripts. As presented in the Chapter 5 conclusion section, I summarize the overall findings of my research projects, and some recommended next steps. Finally, an Appendix section is included that contains additional information on study protocols and standard operating procedures (SOPs).

#### **CHAPTER 1: Introduction**

**History of air pollution**. Since the days of ancient Rome, air pollution has historically been a major environmental health concern throughout the world. However, two major air pollution events occurred that led to public outcry and government intervention in curtailing air pollution emissions. In 1948, a pollution event occurred in Donora, Pennsylvania that killed 20 people and hospitalized 7,000 (Schrenk et al., 1949). The Donora Smog Disaster, as it was called, was attributed to stagnant air resulting from a temperature inversion, which allowed pollution from a number of sources including steel mills and smelting plants to accumulate until concentrations became deadly. Four years later under similar conditions, another pollution event called The Great Smog of 1952 occurred in London, England, killing between 4,000 and 12,000 people (Logan, 1953). These seminal air pollution events led to the future development and implementation of environmental regulations designed to control air pollution and protect human health.

**Air Pollution Regulatory Standards**. Air quality science was in its infancy during the mid-20<sup>th</sup> century as knowledge about air pollution around the world was poorly understood. Beginning in 1973, the World Health Organization (WHO) established an international urban air quality monitoring pilot project. Then, in 1976, and in joint collaboration between the WHO and the United Nations Environment Programme, it evolved into the Global Environment Monitoring System (USEPA, 1996). Their first report, which summarized measurements of specific air pollutants within large cities around the world from 1973-1980, revealed that global concentrations of sulfur dioxide (SO<sub>2</sub>), Total Suspended Particulates (TSP), and smoke mass concentrations averaged 45 μg/m<sup>3</sup>, 89 μg/m<sup>3</sup>, and 36 μg/m<sup>3</sup>, respectively (Bennett et al., 1985).

Partly in response to the Donora and London Fog events, the US government enacted the first air quality legislation in 1955 known as the Air Pollution Control Act. This act provided funding for research, but did not give legal authority to the federal government to implement air quality regulation. Eventually, the US Congress passed the Clean Air Act of 1963, officially signed into law by President Lyndon B. Johnson. This law gave legal authority to the US federal government to research techniques for air quality monitoring and control. The 1970 Amendments to the Clean Air Act further required regulations for pollution sources, with additional amendments passed in 1977 and 1990 (USEPA, 2013). In the 1990 Amendment, 189 toxic pollutants were added to the emission standards list, enforcement authorities were created, and chemicals that are known to deplete the ozone layer were proposed to be phased out.

**National Ambient Air Quality Standards.** As part of the 1990 amendments to the Clean Air Act, National Ambient Air Quality Standards (NAAQS) were implemented that required the Environmental Protection Agency (EPA) to set standards for six criteria pollutants considered harmful to public health and the environment. These regulated air pollutants include nitric oxides (NO<sub>x</sub>), ozone (O<sub>3</sub>), lead (Pb), carbon monoxide (CO), sulfur oxides (SO<sub>x</sub>), and particulate matter (PM). For PM, there are two size classes that are regulated, including  $PM_{2.5}$  (PM with a mean aerodynamic diameter of 2.5 µm and less) and  $PM_{10}$  (PM with a mean aerodynamic diameter of 10 µm and smaller).

Adverse health effects linked with air pollution exposure. According to WHO, air pollution contributed to 1 out of 8 deaths (around 7 million people) worldwide in 2012 (WHO, 2014), with the majority of these estimated mortalities from pulmonary and cardiovascular diseases. As summarized in **Table 1.1.**, outdoor air pollution exposures resulted in greater incidences of ischemic heart disease and stroke than indoor

exposures. Indoor exposures, on the other hand, resulted in more frequent chronic obstructive pulmonary disease (COPD) and acute lower respiratory infections in children. Importantly, these deaths were attributable to air pollution, but ultimately were the result of the listed diseases.

Additional studies confirm similar adverse health associations with ambient air pollution, including increased rates of emergency room visits due to exacerbated COPD following exposures (Anderson et al., 1997) as well as an increased propensity for COPD development (Lalande et al., 2011). Other comparable outcomes were reported and corroborate significant effects of ambient air pollution on asthma (Lavigne et al., 2012; Sumita et al., 2013) and cardiovascular effects (Brook and Kousha, 2015; Laden et al., 2013; Lavigne et al., 2014; Rodopoulou et al., 2014). Research studies have also shown that indoor exposures can lead to increased rates of COPD (Eric et al., 2015), asthma (Bentayeb et al., 2013), and cardiovascular effects (Bruno et al., 2014; Lin et al., 2013).

**Table 1.1.** Estimated percentage air pollution-caused deaths in a worldwide study

 investigating causative pathways of pulmonary and cardiovascular disease from air

 pollution exposures.

outable to At bient air oosures	ttributable to indoor air exposures
40%	26%
40%	34%
11%	22%
6%	6%
3%	12%
4	10% 10% 11% 6% 3%

(Modified from WHO, 2014)

**Airborne particulate matter.** As noted above, PM (including  $PM_{2.5}$  and  $PM_{10}$ ) is one of the six criteria pollutants regulated by the EPA through NAAQS. Another size class that is gaining more attention is the coarse fraction of PM, or  $PM_c$  (PM with a mean aerodynamic diameter between 2.5 µm and 10 µm). **Figure 1.1.** illustrates the three size fractions of PM. There are currently no NAAQS for the  $PM_c$  size fraction.

**General sources of PM**<sub>2.5</sub> and PM<sub>10</sub>. According to the USEPA (Figure 1.2.), about 60% of total ambient PM<sub>2.5</sub> mass across the United States is emitted from fires, dust, and agriculture activity (USEPA, 2015c). Fuel combustion contributed to total PM<sub>2.5</sub> emissions at levels similar to agriculture sources. For PM<sub>10</sub>, more than half of the total emissions come from dust (including road dust/street sand). Agriculture sources also contribute about 21% of total PM<sub>10</sub> mass (**see Figure 1.3**.).



**Figure 1.1.** Diagram showing the three PM sizes:  $PM_{2.5}$ ,  $PM_{10}$ , and  $PM_c$  (Modified from USEPA, 2013).



National PM2.5 Emissions by Source Sector (NEI 2011 v2 GPR)

Figure 1.2. The contribution of source on  $\mathsf{PM}_{2.5}$  total mass across the US (USEPA,

2015c).



**Figure 1.3.** The contribution of source on  $PM_{10}$  total mass across the US (USEPA, 2015c).

**Chemistry of PM**<sub>2.5</sub> and PM<sub>10</sub>. As illustrated in Figures 1.2 and 1.3, there are numerous sources that generate PM. Importantly, the type of source influences the chemistry of the PM emissions (Cheung et al., 2009; Schins et al., 2004b; Stephanie et al., 2011; Vega et al., 2007). In general, PM is a highly complex material made up of many different chemical compounds. However, there are specific classes of chemicals that are commonly found in all PM, including Organic Carbon/Elemental Carbon (OC/EC), anions and cations, and elements.

Carbon components of PM typically include elemental carbon (EC) and organic carbon (OC) fractions. Briefly, the EC component of PM is largely emitted from the burning of fossil fuels resulting in products that are purely carbon based such as graphite or graphene (Pöschl, 2003). Common chemicals composing the OC fraction in ambient PM consist of volatile and semi-volatile organic carbons (e.g., CO<sub>2</sub>, acetone, formaldehyde and some polycyclic aromatic hydrocarbons, ethanol and other alcohols, etc.), resin acids (e.g., abietic acids), carbohydrates (e.g., mannose, glucose, etc.), guaiacols (e.g., vanillin), and many other organic molecules (Fine et al., 2004; Graham et al., 2004; Ward and Noonan, 2008; Ward et al., 2006).

**Anions and cations.** Secondary reactions, e.g. photochemical reactions, leading to increased sulfates and nitrates in the ambient environment can also contribute significantly to overall PM masses. In previous studies investigating source contributions to ambient PM<sub>2.5</sub> in western Montana and Fairbanks, Alaska, concentrations of sulfate, nitrate, and ammonium were measured in elevated concentrations from nylon and quartz filters (Ward et al., 2012; Ward and Lange, 2010). Sources of sulfates largely come from power plants, industrial facilities, and residential fuel oil combustion, whereas nitrates largely come from mobile sources (USEPA, 2003).

**Elements.** Metals are also considered important components of PM. Mercury can absorb onto carbon particles (Seigneur et al., 1998), though it is a small component of PM compared to the crustal elements (Fe, Al, Si, etc.). As observed throughout many highly urban cities throughout the world (including India, China, Brazil, and the US), heavy metals such as chromium, zinc, and lead are also commonly measured in ambient PM (Deng et al., 2006; Quiterio et al., 2004; Shridhar et al., 2010; Song and Gao, 2011; Samara and Voutsa, 2005).

**Coarse fraction (PMc).** There are currently no NAAQS for the size fraction PM<sub>c</sub>. Epidemiological studies suggest negative outcomes following acute exposures (Brunekreef and Forsberg, 2005; USEPA, 2006) illustrating the importance of understanding PM<sub>c</sub> exposure outcomes. While PM<sub>2.5</sub> has been extensively characterized, unknown constituents of PM<sub>c</sub> may account for 10 – 50% of its mass (USEPA, 2009a). In 2009, the Clean Air Scientific Advisory Committee (CASAC; from the Office of the Administrator Science Advisory Board) sent a charge memo report to the former EPA administrator, Lisa P. Jackson, listing important questions that should be addressed regarding PM<sub>c</sub>. This included a candidate list of species to investigate, including biological materials, as well as distribution, sources, and health effects following exposure to PM<sub>c</sub> in the ambient environment (USEPA, 2009b). This facilitated the EPA to launch the National Core (NCore) monitoring network, which began measuring ambient PM<sub>c</sub> mass concentrations (as well as criteria pollutants and meteorological parameters) on January 1, 2011 (USEPA, 2015d).

**General chemistry and sources of PM**<sub>c</sub>. Significant sources of PM<sub>c</sub> include soil and/or road dust, not only in large mega cities (Wang et al., 2008) but also in other urban and

rural areas throughout the world (Manoli et al., 2002). Similar to  $PM_{2.5}$ , the coarse PM fraction is largely composed of OC/EC, ions, and crustal elements/minerals. In Thessaloniki, Greece, minerals (from soil/crustal elements) were found to compose roughly half of PM<sub>c</sub> mass at all locations where PM<sub>c</sub> was collected (Grigoratos et al., 2014). Also, winter months had higher burdens of OC than summer months, while mineral content of PM<sub>c</sub> was higher in the summer. Different chemical characterizations were observed in Lisbon where sea salt contributed 19% to 47% to overall PM<sub>c</sub> mass (Almeida et al., 2006).

The biogenic fraction is a large component of  $PM_c$  compared to the  $PM_{2.5}$  fraction, with a significant portion consisting of endotoxin (from the cell wall of gram-negative bacteria), mold spores, and pollen (Menetrez et al., 2007a). Endotoxin exposure has long been recognized as a causative agent in both immediate and sustained airflow obstruction in asthmatics (Liu, 2004) and infant wheezing (Ryan et al., 2009), and poses a significant risk factor for increased asthma prevalence (Thorne et al., 2009). A spatial-temporal analysis detected ambient endotoxin in the coarse fraction at levels 10 times higher compared to  $PM_{2.5}$  (Heinrich, 2003). This relationship has been observed with endotoxin and other biogenics (including  $\beta$ -glucan and total protein) in additional indoor and outdoor environments (Menetrez, 2009a). Another study reported up to two times more biological content in  $PM_c$  than  $PM_{2.5}$ , measured as a function of total protein content (Menetrez, 2007a).

The time of year also influences the makeup of the  $PM_c$  fraction. As the seasons change from winter to spring, ambient  $PM_c$  can begin to contain higher levels of biogenic material such as pollen and fungi. Summer months have been reported to contain up to four times more total protein content compared to winter months (Menetrez et al.,

2007a). This general seasonal trend was also observed in an eastern Mediterranean study measuring biomarkers of fungi in coarse PM (Burshtein et al., 2011). Specifically, measured concentrations of the biomarker arabitol (a sugar alcohol metabolic by-product in fungi) went from being the highest to the lowest concentration in the following order: autumn, spring, summer, and winter. Other studies have shown similar correlations between endotoxin content and temperature as well as its influence on fungi levels in the ambient air (Degobbi et al., 2011).

**Trends in Ambient PM**<sub>2.5</sub>, **PM**<sub>10</sub>, **and PM**<sub>c</sub> **mass concentrations across US.** Ambient measurements of PM mass are obtained across a national monitoring network overseen by the EPA (see **Figure 1.4.**). Monitoring at these sites is typically conducted by state environmental agencies, such as the Montana Department of Environmental Quality (MTDEQ), or local health departments (e.g., Missoula City-County Health Department).



**Figure 1.4.** Locations of municipal air quality monitoring sites across the US for use in continual monitoring and reporting of criteria air pollutants (USEPA, 2015a).

To determine regulatory compliance for criteria pollutants, measured PM concentrations are compared with the EPA NAAQS. The standard for annual and 24-hour PM<sub>2.5</sub> concentrations is 12  $\mu$ g/m<sup>3</sup> and 35  $\mu$ g/m<sup>3</sup>, respectively. There is only one standard for PM<sub>10</sub> and occurs when 24-hour concentrations exceed 150  $\mu$ g/m<sup>3</sup> for more than one day a year. As mentioned previously, there are no standards for the coarse fraction. If an airshed exceeds PM<sub>2.5</sub> and PM<sub>10</sub> standards, the community is classified as "nonattainment". **Figure 1.5.** shows non-attainment areas for each of the six criteria pollutants in the US, with the color denoting the number of NAAQS exceeded.



Figure 1.5. Areas classified as non-attainment for all NAAQS, nation-wide (USEPA,

2015b).

As expected, **Figure 1.5.** shows that non-attainment designations are common in densely populated areas throughout the US, including Houston, Los Angeles, Salt Lake City, and New York City. Even with a state-wide population of approximately a million people, the state of Montana has historically had several counties in non-attainment due to exceedances of NAAQS for PM<sub>10</sub> and PM<sub>2.5</sub>.

Across the US, there are subtle differences between 14-year annual average PM concentrations and region (USEPA, 2014). For example, the average  $PM_{2.5}$  and  $PM_{10}$  concentrations for the western US during 2013 was 10 µg/m<sup>3</sup> and 75 µg/m<sup>3</sup>, respectively. Using the equation  $[PM_{10}] - [PM_{2.5}] = [PM_c]$ , average annual PM<sub>c</sub> mass concentrations were 65 µg/m<sup>3</sup>, or 87% of PM<sub>10</sub>. Similar 2013 concentrations were observed for the South, Central, Northwest, Northern Rockies and Plains regions. The upper Midwest, Northeast, and Southeast had average annual PM<sub>2.5</sub> and PM<sub>10</sub> concentrations of 8 µg/m<sup>3</sup> and 45 µg/m<sup>3</sup>, with less measured ambient PM<sub>10</sub>. Notably, PM<sub>2.5</sub> and PM<sub>10</sub> concentrations in the Southwest region averaged 8 µg/m<sup>3</sup> and 110 µg/m<sup>3</sup>, respectively, with PM<sub>c</sub> constituting 93% of the PM<sub>10</sub> fraction. This is likely the result of the arid environment of the southwest that contributes to increased dust and crustal particles (including the coarse fraction) in the ambient air (Gertler et al., 1995). Overall, regardless of region, average annual ambient PM<sub>c</sub> mass concentrations were much higher compared to PM<sub>2.5</sub> concentrations throughout the continental US.

**Trends in Ambient PM**<sub>2.5</sub>, **PM**<sub>10</sub>, and **PM**<sub>c</sub> mass concentrations in Missoula: In the city of Missoula, the PM monitoring stations are located at Boyd Park and on the roof of the Missoula City-County Health Department. At each of these sites,  $PM_{2.5}$  and  $PM_{10}$  are routinely measured using Beta Attenuated Monitors (BAMs; MetOne Instruments, Grants

Pass, OR). Using data collected at these monitoring sites, **Table 1.2** presents the ambient concentrations of PM<sub>c</sub>, PM<sub>2.5</sub>, and PM<sub>10</sub> ( $\mu$ g/m<sup>3</sup>) ± standard deviations measured in Missoula from January 2010 to July 2013. Overall, seasonal PM<sub>c</sub> concentrations were consistently higher than PM<sub>2.5</sub> with the exception of winter, when there are more combustion-related particles (from residential wood stove emissions) contributing to the PM<sub>2.5</sub> fraction (Ward and Lange, 2010). Unlike PM<sub>2.5</sub>, PM<sub>c</sub> concentrations were fairly consistent throughout the year, suggesting that this size fraction is a pollutant of concern to regional residents annually.

When compared to other areas of the US, results show that Missoula experiences similar annual  $PM_{2.5}$  concentrations (about 8-11 µg/m<sup>3</sup>) compared to other regions. However, annual  $PM_c$  and  $PM_{10}$  concentrations (ranging from 9-11 µg/m<sup>3</sup> and 15-20 µg/m<sup>3</sup>, respectively) are lower compared to national averages.

**Table 1.2.** Ambient concentrations of  $PM_c$ ,  $PM_{2.5}$ , and  $PM_{10}$  ( $\mu$ g/m<sup>3</sup>) ± standard deviations in Missoula, MT, January 2010 – July 2013.

	Winter	Spring	Summer	Autumn
	(Nov-Feb)	(Mar-May)	(Jun-Aug)	(Sep-Oct)
PMc	<b>8.91</b> (8.59)	<b>10.80</b> (7.27)	<b>10.14</b> (5.01)	<b>11.09</b> (6.51)
PM <sub>2.5</sub>	<b>11.73</b> (7.19)	<b>4.40</b> (2.68)	<b>5.49</b> (3.19)	<b>8.08</b> (5.91)
PM <sub>10</sub>	<b>20.38</b> (10.49)	<b>15.18</b> (9.18)	<b>15.42</b> (6.98)	<b>19.15</b> (11.05)

**Global and regional significance of biomass burning sources.** Throughout the world, a significant source of PM is biomass combustion. This includes a variety of sources including cookstoves, residential wood stoves, wildland fires, prescribed fires, and agricultural burning. The types of burning can also be different, including flaming and smoldering phases. There are also indoor and outdoor sources (as well as outdoor influences on indoor environments), further enhancing the intricacies of biomass burning exposure/health research.

*Global cookstove use:* It is estimated that about half of the world's households burn solid fuels for heating and cooking, the majority of which consists of wood fuel or the burning of agricultural residues (Smith et al., 2004). The variations of fuels used are largely a function of economic circumstances (Lim et al., 2012; Naeher et al., 2007). Fuel used for home heating and cooking around the world can range from the burning of animal waste in open-pits (Kim et al., 2011) to the burning of cured wood in an efficient clean-burning stove.

*Residential wood stoves:* Residential wood stove usage has been shown to significantly impact ambient PM levels in areas throughout the world. In Europe, wood combustion byproducts during winter months have been shown to contribute to the organic fraction of PM mass by about 68% in Aviero, Portugal, and 47% in Puszta, Hungary (Puxbaum et al., 2007). Subarctic areas can also experience high PM<sub>2.5</sub> levels resulting from residential wood combustion. For example, in Fairbanks, AK, wood stoves are a known major source of PM<sub>2.5</sub>, with about 9,000 heating/wood-burning devices being used for home heating (Carlson et al., 2010), contributing from 40-80% of ambient PM<sub>2.5</sub> mass during the winter months (Ward et al., 2012). In northern Rocky Mountain communities, residential wood combustion has also been shown to be a significant source of ambient

PM<sub>2.5</sub> (Ward et al., 2006; Ward and Smith, 2005), contributing from 56-77% of ambient wintertime PM<sub>2.5</sub> mass (Ward and Lange, 2010).

Due to the significant impacts of residential wood combustion on ambient air quality, community-wide wood stove changeout programs have been promoted by the EPA and promulgated by local municipalities as a way of reducing wintertime ambient PM<sub>2.5</sub>. This year alone, 12 US states (AK, CA, CO, ID, ME, MD, NV, NH, NY, OR, VT, and WA) and four Canadian territories (British Columbia, New Brunswick, Northwest Territories, and Nova Scotia) have or are currently participating in this or similar programs (HPBA, 2015). Efforts to change out wood stoves largely involve trading in an older, less-efficient model for a certified, more efficient wood stove. Rebates and incentives (e.g., \$500-\$5,000 rebate towards new stove) help to increase recruitment into the program.

Uncertified/older wood stove models release 15-30 grams of smoke per hour whereas EPA-certified wood stove emissions are below 4.5 grams of smoke per hour (USEPA, 2015e). Studies have demonstrated the efficacy of low emission stove technology on improving ambient air quality as community-wide changeout programs have resulted in significant reductions in ambient  $PM_{2.5}$  during the winter months (Ward et al., 2010).

Prior to a wood stove changeout program in Libby, MT, consistent exceedances of both 24-hour and annual  $PM_{2.5}$  NAAQS were experienced, largely the result of residential wood burning during the winter months. A Chemical Mass Balance (CMB) source apportionment study conducted during the winter of 2003/2004 showed that residential wood combustion contributed up to 82% of the total  $PM_{2.5}$  mass (Ward et al., 2006). In an effort to reduce wintertime  $PM_{2.5}$ , a community-wide wood stove changeout program was performed where 1,200 old stoves were replaced with more efficient stove models

between the years 2005 to 2007. CMB modeling conducted after the woodstove changeout showed a 25.6% decrease in overall  $PM_{2.5}$  (28.2 µg/m<sup>3</sup> before, and 20.1 µg/m<sup>3</sup> after changeout) and a 28% decrease in residential wood combustion contributions to overall  $PM_{2.5}$  (22.8 µg/m<sup>3</sup> before, and 16.4 µg/m<sup>3</sup> after changeout). Reductions were also observed for OC, EC, and levoglucosan (a chemical marker of wood smoke). Interestingly, consistent *increases* were observed for resin acids such as dehydroabietic acid and abietic acid (Ward et al., 2011).

Residential wood stoves can also be significant sources of elevated indoor  $PM_{2.5}$  concentrations (Noonan et al., 2011). In studies that have measured indoor  $PM_{2.5}$  in wood burning homes, mean  $PM_{2.5}$  concentrations have been reported from 12.8 to 54.0 µg/m (Allen et al., 2009; Ward et al., 2011; Noonan et al., 2012; Semmens et al., 2015; Noonan et al.; 2012). Many of the 24-hour measurements exceeded the  $PM_{2.5}$  24-hour NAAQS of 35 µg/m<sup>3</sup>. It is important to note that there are currently no indoor  $PM_{2.5}$  standards in the US. However, these findings are of concern since most people spend the majority of their time indoors.

Much less is known about the impact of woodstove changeouts on indoor environments, with only a handful of studies investigating this issue (Allen et al., 2009; Ward et al., 2011; Noonan et al., 2012; Semmens et al., 2015; Noonan et al., 2012). Results from these studies have been variable, with 33 to 45% of homes demonstrating no reduction in indoor PM<sub>2.5</sub> following the replacement of old stoves with EPA-certified wood stoves. Similar to ambient concentrations of abietic acid throughout the Libby wood stove changeout program, concentrations of some resin acids curiously increased following wood stove changeouts within homes (although dehydroabietic acid levels decreased nearly 100%). These resin acids are important, as they have been shown to be inhibitors

of inflammation (Kang et al., 2008; Takahashi et al., 2003). This change in  $PM_{2.5}$  chemical composition, which likely results from different burn conditions in modern vs. older wood stoves, is a significant observation since it implies that there may be subtle health impacts from wood stove changeouts that have not been previously investigated.

*Wildland forest fires:* Another major source of biomass smoke includes wildland fires. Wildfires can emit smoke plumes that not only impact air quality in areas directly near the fire, but also in distances far downwind. For example, wildland fires occurring in Russia and other Eastern European countries were shown to negatively impact air quality more than 1,000 km away (Niemi et al., 2005). These smoke plumes are not only composed of PM, as well as additional types of air pollutants. A wildfire smoke field study performed by Slaughter et al. (Slaughter et al., 2004) during wildland firefighting activities measured elevated concentrations of acrolein (0.002-0.018 ppm), formaldehyde (0.008-0.085 ppm), and CO (2.10-10.48 ppm). This point is further illustrated in **Table 1.3**, where a summary of wildfire smoke-related air pollutants is provided. **Table 1.3.** The mass of emissions released (in grams) as a function of burned fuel

Emissions	Mass	%
Carbon Dioxide	1564.8	71.44
Carbon Monoxide	120.9	5.52
Organic Carbon	5.2	0.24
Elemental Carbon	0.4	0.02
Particulate matter <i>d</i> < 2.5µ	10.3	0.47
Particulate matter $2.5\mu < d < 10\mu$	1.9	0.09
Particulate Matter <i>d</i> > 10µ	3.8	0.17
Nitric Oxide	8.5	0.39
Methane	5.9	0.27
Non-methane Hydrocarbon	4.3	0.20
Volatile Organic Compounds	5.2	0.24
Water	459.2	20.97

(kilograms) during wildfires (Liu et al., 2014; NRC, 2004).

Fuel burned during wildland fires is largely a function of region. For example, wildfires burning through the Everglades in Florida can slowly burn through large amounts of peat (Smith et al., 2001), whereas wildfires in the Northern Rockies burn mostly timber, including mixed conifer, and dead/downed woody material (NWCG, 2013). The fuel further dictates the chemistry of emitted pollutants, with varying amounts of VOC and SVOC components (including PM).

**2015 Western Montana Wildfire Season**. Through the end of August 2015, about 8 million acres burned as a result of an estimated 42,000 different fires throughout the western United States (Center, 2015a). This amount of burned acreage is about 2 million acres more than the national 10-year average. As seen in **Figure 1.6.**, wildfires burned throughout Idaho and Montana, with satellite images showing that smoke frequently impacted Missoula (**Figure 1.7.**).



**Figure 1.6.** MODIS satellite showing locations of fires occurring throughout 2015 in northern Idaho and western Montana (yellow). Areas that are red or orange indicate fires that occurred within 24 hours of the date the map was published, August 27<sup>th</sup>, 2015 (USFS, 2015).



**Figure 1.7.** Satellite imagery of northern Idaho and west Montana taken August 23<sup>rd</sup>, 2015 from NASA's Earth-Observing Aqua satellite. Red areas are active fires (Space, 2015).
In an effort to protect the health of regional residents, the Montana DEQ measures and reports ambient PM<sub>2.5</sub> concentrations and related health index values (**Table 1.4**) throughout the year. During the summer 2015 wildfire season, continuous PM<sub>2.5</sub> measures from the Boyd Park site provided information on wildfire smoke impacts to the city of Missoula. On the same day **Figure 1.7.** was obtained via satellite (August 23), DEQ reported PM<sub>2.5</sub> concentrations ranging from "good" to "unhealthy for sensitive groups" (**Figure 1.8.**).

Health Effect Categories	Visibility (miles)	24-Hour BAM (ug/m <sup>3</sup> )	8-Hour BAM (ug/m <sup>3</sup> )	1-Hour BAM (ug/m <sup>3</sup> )
Hazardous	< 1.3	>135.4	> 193.4	> 338.5
Very Unhealthy	2.1 - 1.3	80.5 - 135.4	115.0 - 193.4	201.1 - 338.5
Unhealthy	5.0 - 2.2	35.5 - 80.4	50.7 - 114.9	88.6 - 201.0
Unhealthy for Sensitive Groups	8.7 - 5.1	20.5 - 35.4	29.2- 50.6	51.1 - 88.5
Moderate	13.3 - 8.8	13.5 - 20.4	19.2 - 29.1	33.6 - 51.0
Good	> 13.4 +	0.0 - 13.4	0.0 - 19.1	0.0 - 33.5

 Table 1.4. EPA and Montana DEQ health index values (modified from: MTDEQ, 2015).



**Figure 1.8.** Reported hourly average  $PM_{2.5}$  concentrations and health index categories during the days of August 23<sup>rd</sup> through August 31<sup>st</sup> in Missoula, MT (MTDEQ, 2015b).

During August 28-31, PM<sub>2.5</sub> monitors (DustTrak, TSI, Model 8530, Shoreview, MN) were placed inside Skaggs room 158 and also on the roof of the same building in an effort to simultaneously measure indoor and outdoor PM<sub>2.5</sub> concentrations during the "unhealthy" days shown (August 28-31) in **Figures 1.8.** The DustTrak values presented in **Figure 1.9.** were modified to BAM values using the McNamara correction factor (Mcnamara et al., 2011) as MTDEQ's health index values were based on BAM technologies (MTDEQ, 2015). **Figure 1.9.** shows elevated PM<sub>2.5</sub> concentrations from mid-afternoon August 28<sup>th</sup> through mid-afternoon August 30<sup>st</sup>. The daily average PM<sub>2.5</sub> readings obtained on the roof of the Skaggs Building during August 28<sup>th</sup>, 29<sup>th</sup>, 30<sup>th</sup>, and 31<sup>st</sup> were, 320 µg/m<sup>3</sup>, 298 µg/m<sup>3</sup>, 46 µg/m<sup>3</sup>, and 12 µg/m<sup>3</sup>, respectively. Indoor levels on these same days were 231 µg/m<sup>3</sup>, 211 µg/m<sup>3</sup>, 46 µg/m<sup>3</sup>, and 7 µg/m<sup>3</sup>, respectively.

According to measured ambient PM<sub>2.5</sub> concentrations (from both the Boyd Park and University of Montana sites) during the days of August 28<sup>th</sup> and 29<sup>th</sup>, ambient PM<sub>2.5</sub> concentrations in Missoula were in the "hazardous" range. Following rain showers near the end of the 29<sup>th</sup>, a resulting decrease to "unhealthy" was observed during August 30th. On the 31<sup>st</sup>, air quality returned to "good." Importantly, average indoor PM<sub>2.5</sub> levels fell into the same health category when compared to the values in **Table 1.4**.

Consistent with our findings, other studies focused on wildland fires and prescribed burning events have measured levels of indoor PM similar to those observed outside during smoke events(Barn et al., 2008; Henderson et al., 2005; Phuleria et al., 2005; Sapkota et al., 2005). Henderson et al. (2005) determined that about 77% of the PM levels observed outside were measured inside (average of the reported indoor levels =  $14.8 \pm 8.1 \ \mu g/m^3$ ; average of the reported outdoor levels =  $19.2 \pm 14.0 \ \mu g/m^3$ ) during wildfire and prescribed burning events. Performing the same analysis with the data in

**Figure 1.9.** resulted in a similar observation where 75% of  $PM_{2.5}$  measured on the roof of the Skaggs Building was observed inside our laboratory (located inside the Skaggs Building). Even though the average  $PM_{2.5}$  levels observed in this comparison (average indoor levels =  $123.8 \pm 27.3 \ \mu g/m^3$  and average outdoor levels =  $169.1 \pm 162.7 \ \mu g/m^3$ ) was at least an order of magnitude greater than the Henderson et al. (2005) study, infiltration efficiency still remained about the same. Taken together, these data demonstrate that not only do regional wildfires create elevated wood smoke  $PM_{2.5}$  concentrations in the outside air, but concentrations indoors may be hazardous as well.

**Wildland firefighter exposures:** Occupational exposures to wood smoke are a significant concern to wildland firefighters. According to the US Bureau of Labor Statistics, an estimated 307,000 firefighters were fully employed in 2012, with an expected 7% increase of employed firefighters between 2012-2022 (Statistics, 2014). Field studies including wildland firefighting and prescribed burning activities have shown that firefighters are exposed to average concentrations ranging from 500-600  $\mu$ g/m<sup>3</sup> in a single work-day (Reinhardt and Ottmar, 2004) with spikes around 3,000  $\mu$ g/m<sup>3</sup> (Adetona et al., 2013). A field study performed by Slaughter et al. (Slaughter et al., 2004) during wildland firefighting activities reported that firefighters are exposed to average daily PM of 882  $\mu$ g/m<sup>3</sup> (ranging 235  $\mu$ g/m<sup>3</sup> to 1317  $\mu$ g/m<sup>3</sup>). As fires become more common in future summers (as a result of climate change, extended periods of drought, and a buildup of fuels in our regional forests, Westerling et al. [2014]), it will become increasingly more important to understand the health impacts related to wood smoke exposures to this occupational group.





96-hour period in Missoula, MT during the 2015 wildland fires.

Adverse health effects linked with PM<sub>10</sub> and PM<sub>2.5</sub> exposures. As previously described, there are multiple health impacts following exposures to air pollution (WHO, 2014). However many of these observed air pollution exposure outcomes likely result from inhalation of airborne PM. The Global Burden of Disease Study estimated that there were 3.5 million deaths and 4.5% of disability-adjusted life-years worldwide in 2010 attributed to exposure to indoor or household air pollution from the burning of solid fuels (Lim et al., 2012). This study also estimated 3.2 million deaths and 3.1% of global disability-adjusted life-years from ambient  $PM_{2.5}$  exposures (this includes PM from indoor burning of solid fuels). Epidemiological studies further suggest that exposure to  $PM_{2.5}$  can significantly increase the risk of allergic respiratory diseases and bronchial asthma, as well as exacerbate type 2 diabetes (Pereira Filho et al., 2008) and cardiovascular disease (Park et al., 2010). In looking at causative components in urban (Shanghai, China)  $PM_{2.5}$  mass, Qiao et al. (Qiao et al., 2014) determined that the OC and EC constituents from the combustion of fossil fuels have considerable impact on exposure outcomes.

Wilson et al. (1997) suggested that ambient  $PM_{2.5}$  outcomes are often correlated with  $PM_{10}$  (Wilson and Suh, 1997), resulting in epidemiological effects that are not easily distinguishable from the effects of  $PM_{2.5}$  alone. A full meta-analysis (using data from US, Canada, Europe, China, Japan, and New Zealand) of exposures to  $PM_{2.5}$  and  $PM_{10}$  was performed on lung cancer risk (Hamra et al., 2014). The result was similar relative-risk values of 1.09 and 1.08, for  $PM_{2.5}$  and  $PM_{10}$ , respectively, further illustrating the difficulty in distinguishing between the two PM size classes. A study focusing on  $PM_{10}$  alone showed an increase in mortality following exposures, with a 10 µg/m<sup>3</sup> increase resulting in mortality increases of 0.22% (Bangalore), 0.85% (Hyderabad), and 0.2% (Mumbai) in India (Dholakia et al., 2014). These results illustrate the many different health outcomes

related to  $PM_{2.5}$  and  $PM_{10}$  exposures, and further support the importance of future investigations into exposure outcomes.

Adverse health effects linked with PM<sub>c</sub> exposure. PM<sub>c</sub> is unique in that unlike PM<sub>10</sub>, it does not always correlate with PM<sub>2.5</sub> (Tager et al., 2010). For example, when comparing the effect of a 10  $\mu$ g/m<sup>3</sup> incremental increase of PM<sub>c</sub>, average daily mortality increased 4.07%, whereas PM<sub>2.5</sub> resulted in a 1.48% increase in mean daily mortality (Castillejos, 2000). Also observed in this study were increases in respiratory disease and cardiovascular disease with increased PM<sub>c</sub>. Another study measured the impact of PM<sub>c</sub> on health outcomes by compiling previously reported PM<sub>2.5</sub> and PM<sub>10</sub> epidemiological studies (Brunekreef and Forsberg, 2005). PM<sub>c</sub> caused similar and often more cases of increased COPD, asthma, and respiratory hospital admissions compared to PM<sub>2.5</sub>. Evaluating the impact of PM<sub>10</sub> and PM<sub>c</sub> on mortality rates in Rome, Italy showed a 9.55% increase and a 9.73% increase, respectively, during periods of increased wind-blown dust in the ambient air (Mallone et al., 2011). The causes of mortality were natural, cardiac, cerebrovascular, and respiratory diseases.

 $PM_c$  has also been shown to cause a more significant pulmonary inflammatory response than  $PM_{2.5}$  in animal models (Schins et al., 2004b; Tong et al., 2010), as well as increased inflammatory potential in multiple cell lines, including macrophages (Becker et al., 2005; Gualtieri et al., 2010). Becker et al. (2005) reported that the coarse fraction composing  $PM_{10}$  can account for more than 90% of the inflammatory response. This is likely explained by the biological composition of  $PM_c$ , as the coarse fraction can contain up to twice the levels of endotoxin (Steerenberg et al., 2004), and up to 10 times the amount of biological content when compared to  $PM_{2.5}$  (Heinrich et al., 2003). This

biological content is composed of danger associated molecular patterns (DAMPS), including endotoxin, fungi, and other proteins (such as those found in indoor environments, e.g. dust mite allergens (Ryu et al., 2013)). This biological composition has been shown to increase inflammasome output and productivity and initiates the NF- $\kappa$ B pathway and downstream pro-IL-1 $\beta$  production via TLR4 (Bauernfeind et al., 2009; Schins et al.; Tschopp and Schroder, 2010; Yazdi et al., 2010). **Figure 1.10.** illustrates this point in a macrophage cell. However, as hypothesized through this dissertation research, smoke particles may be causing the lysosomal damage (rather than cholesterol crystals as shown in **Figure 1.10**), whereas endotoxin represents the TLR4 ligand (note: in **Figure 1.10.** another ligand for TLR4, modified LDL, is represented).

These observations suggest a  $PM_c$ -specific effect in PM-induced adverse health outcomes. There have been very few studies (population based or laboratory based) that have investigated the health effects related to  $PM_c$  exposures, especially when directly compared to other well-studied PM fractions such as  $PM_{2.5}$  and  $PM_{10}$ .



TRENDS in Immunology

**Figure 1.10.** Canonical pathway of inflammasome activation and subsequent extracellular release of IL-1 $\beta$  (De Nardo and Latz, 2011).

Adverse health effects linked with wood smoke exposure. Indoor cooking has been shown to contribute to indoor exposures, and has been shown to significantly reduce birth rate (Boy et al., 2002). Studies have shown that simply adding a chimney to an open burn pit can significantly reduce PM exposures indoors, as well as reduce wood smoke PM induced effects, such as an observed decrease in high blood pressure occurrences (Mccracken et al., 2007). In other related studies, reduced indoor exposures to wood smoke has been shown to decrease reported wheezing, respiratory infections, colds, bronchitis, influenza, and throat infections in children (Noonan et al., 2012).

Individuals exposed to wood smoke during traditional temazcal (sweat lodge) usage showed increased levels of urinary mutagenic potency (Long et al., 2014). Mutagenicity (including DNA damage) and other downstream effects (e.g. cancer) are important outcomes that follow excessive oxidative stress in the biological environment (Ames et al., 1995). These outcomes suggest that oxidative stress plays a significant role in the various observed adverse effects following wood smoke exposures. Further investigations demonstrated that humans exposed to wood smoke resulted in general increases in psychological/neurological effects, increased inflammatory responses, as well as a general increase in irritated mucosa (Riddervold et al., 2011).

Other wood smoke studies have been performed in occupational settings and include measures of PM<sub>2.5</sub> exposure levels and health impacts (e.g., lung function). Field studies investigating the influence of entire seasons of wildland firefighter activity on lung function show significant declines in lung function. Lui et al. (1997) reported significant declines in mean FVC and FEV<sub>1</sub> values post-season (0.09 and 0.15 L/s, respectively). Similar results were observed by Betchley et al. (Betchley et al., 1997).

Exposure to wildland forest fire smoke has also been shown to induce significant medical costs. For example, an extended period (~14 days) of unhealthy air pollution caused by wildfire smoke was estimated to cost \$5 million to \$15 million in health expenses alone during a 1997 haze in Singapore (Hon, 2006). Finally, wildfires have been estimated to contribute up to 339,000 deaths each year (Johnston et al., 2012). This is alarming, as future wildfires occurrences, as previously discussed, are predicted to increase in frequency and scale due to rising temperatures and shrinking snow pack (Westerling et al., 2014).

**Controlled human exposure smoke studies.** There are many challenges when conducting comprehensive field studies assessing the impact of wood smoke exposures on human health, including logistical, safety, and monetary issues. Because of these reasons, controlled human exposure studies involving wood smoke have been performed in only a few facilities, including three in Europe (1. Swedish National Testing and Research Institute, 2. Umeå University (Sweden), and 3. Aarhus University (Denmark)) and one in the US (EPA's human study facility at the University of North Carolina).

A comprehensive listing of the controlled wood smoke / human exposure studies conducted to date is presented in **Table 1.5.** These studies have reported various levels of exposures, durations of exposures, and potential inclusions of physical exertions. Results from these studies are also variable. A study performed by the Environmental Public Health Division of the EPA reported pulmonary and systemic inflammation, and decreases in maximal heart rate following exposures to  $PM_{2.5}$  concentrations of 485 µg/m<sup>3</sup> (Ghio et al., 2012). Other studies similar to this one observed varying significance

on inflammation, cardiovascular stress, reactive oxygen species (ROS), and other measures.

Having an exercise component in the studies appeared to influence findings. Only two of the studies (in **Table 1.5.**) that did not include exercise resulted in significant measures of effect. These included increased clara cell protein 16 (in blood) and significant measures of psychological stress, including "irritated body perceptions" and irritated mucosa (Riddervold et al., 2011; Stockfelt et al., 2012). One study did not observe significant changes in effect, even though it included an exercise component (Hunter et al., 2014). Four other studies that did not include exercise as part of their exposure methodologies also reported no significant measures of effect (Bønløkke et al., 2014; Forchhammer et al., 2012; Riddervold et al., 2012; Stockfelt et al., 2013). In the other studies that included exercise, significant effects on reactive oxygen species (ROS) biomarkers (Barregard et al., 2008; Barregard et al., 2006; Danielsen et al., 2008; Sehlstedt et al., 2010), cardiovascular impacts including arterial stiffness and heart rate effects (Unosson et al., 2013), and further effects on inflammation (Barregard et al., 2008; Barregard et al., 2006; Sällsten et al., 2006) were observed.

## **Table 1.5.** A summary of previous controlled wood smoke human exposure studies.

References	Ν	Exercise	Exposure duration	Wood smoke exposure concentration(s)	Studied measurements and/or media	Effects observed
Hunter et al. 2014	16 adult males	bike every 15 minutes	1-hour	filtered-air and ~1 mg/m3	Blood and vascular measures	no significant effects observed on vascular vasomotor or fibrinolytic function, or increased thrombus formation
Bønløkke et al. 2014	24 adult males and females	at rest	3.5 hours	filtered-air (13), 222, and 385 $\mu\text{g/m3}$	Blood, urine, and heart rate	• no significant effects on clara cell protein 16 and surfactant protein (both SP-D and -A) levels
Unosson et al. 2013	14 adult males and females	bike every 15 minutes	3 hours	filtered-air and 214 $\mu g/m3$	Vascular measures	Arterial stiffness and heart rate increased, as compared to filtered-air, no changes in blood pressure
Stockfelt et al. 2013	16 adult males and females	at rest	3 hours	filtered-air, 146, and 295 $\mu\text{g/m3}$	Blood and urine	$\bullet$ No significant increases in inflammation observed, urinary $F_{2}\mbox{-}isoprostane$ levels decreased after
Ghio et al. 2012	10 healthy individuals <sup>a</sup>	bike every 15 minutes	2 hours	filtered-air and 485 $\mu\text{g/m3}$	Spirometry, blood and BALF	<ul> <li>No changes in pulmonary function, but 20h-post results indicate inflammatory potential (immune cell differentials)</li> </ul>
Stockfelt et al. 2012	16 adult males and females	at rest	3 hours	filtered-air, 146, and 295 $\mu\text{g/m3}$	EBC, blood, and urine	Significant increases in CC16 in serum and fraction of exhaled nitric oxide in EBC
Forchhammer et al. 2012	20 adult males and females	at rest	3 hours	filtered-air (14), 220, and 354 $\mu\text{g/m3}$	Blood	No significant effects on markers of microvascular function, oxidative stress (including DNA damage), adhesion molecules, and pro-inflammatory cytokines
Riddervold et al. 2012	20 adult males and females	at rest	3.5 hours	filtered-air, 200, and 400 $\mu\text{g/m3}$	Spirometry, EBC, nasal lavage	• No significant differences in effect, some signs of inflammation neared significance in EBC
Riddervold et al. 2011	20 adult males and females	at rest	3.5 hours	filtered-air, 200, and 400 $\mu\text{g/m3}$	Symptomatic questionaires and irritated mucosa assessement	Significant effects on sensory perceptions (e.g., "irritative body perceptions", "psychological/neurological effects") and general irritated mucosa
Sehlstedt et al. 2010	19 adult males and females	bike every 15 minutes	3 hours	filtered-air and 224 $\mu\text{g/m3}$	Spirometry, exhaled NO, and BALF	Significant release of GSH into BALF, no noticable signs of inflammation or changes in lung function
Danielsen et al. 2008	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 $\mu g/m2$	Blood and urine	<ul> <li>Systemic effects were observed, but no major DNA damage, likely explained by enhanced repair mechanisms and the time of sample collection. For example, increased mRNA levels of hOGG1 significantly increased</li> </ul>
Barregard et al. 2008	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 $\mu\text{g/m3}$	EBC and blood	Increased alveolar NO, malondialdehyde, and serum clara cell protein 16
Sällsten et al. 2006	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 250 µg/m3	EBC, blood, and urine	• Results in Barregard et al. 2006. This paper is mostly a methods and characterization manuscript
Barregard et al. 2006	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 µg/m3	EBC, blood, and urine	Serum levels of amyloid A and factor VIII, including the factor VIII/von Willebrand factor ratio, as well as urinary levels of ROS, were increased

"Gender details of participating individuals not outlined in manuscript

The differences in methodology between these controlled human wood smoke exposure studies likely explain the dissimilar outcomes. Those that focused on cardiovascular endpoints found inconsistent results that might be explained by the differences in exercise activity, exposure lengths, and exposure concentrations among the studies. For example, exposing individuals for hours with intermittent exercise resulted in changes in heart rate, as compared to filtered-air controls (Ghio et al., 2012; Unosson et al., 2013). Yet, in another study, changes in heart rate, were not observed (Bønløkke et al., 2014). Also, Hunter et al. (Hunter et al., 2014) exposed individuals for 1-hour with 15-minute rotations of light bike exercise, and did not observe significant cardiovascular effects. It is possible that that the "no exercise" component in the Bønløkke et al. (2014) study, or the lower duration of exposure in the Hunter et al. (Hunter et al., 2014) study, partly explains these differences in cardiovascular outcomes. There is much to be learned in future studies investigating the causes of these variable outcomes among similar studies.

**Overall Research Rationale**. Worldwide, PM exposures are ubiquitously occurring with resulting significant adverse human health outcomes. Even though a large number of research studies have focused on PM, there are still many unanswered questions related to exposure and associated adverse health effects. Few studies have investigated the significance of season on PM exposure outcomes, or have focused on the PM<sub>c</sub> size fraction (compared to PM<sub>2.5</sub> and PM<sub>10</sub>), despite epidemiological data supporting its potential adverse impacts on human health (Brunekreef and Forsberg, 2005). In addition, links between the various sources of wood smoke in the Rocky Mountain region and health outcomes following exposures needs to be investigated and understood.

The research described in the three Aims below begin to address these specific issues. Our results will also provide additional results towards informing regulatory policy, with the goal of protecting human health not only across the US, but globally as well. This especially relates to population exposures to the coarse fraction of PM (currently not regulated by NAAQS) and wood smoke exposures, including occupational wood smoke exposures to wildland firefighters.

**Research Aims**. Research Aims are proposed that will advance the current literature and basic scientific knowledge regarding PM exposures and resultant adverse health effects. Through three Aims, my overall hypothesis is that compared to other particle types, wildfire smoke and ambient PM<sub>c</sub> induce significantly greater pro-inflammatory responses, and that wood smoke levels of exposure commonly experienced by wildland firefighters results in systemic and pulmonary inflammation in a dose-response manner.

Specific Aim 1 (Chapter 2): Evaluate how the size of PM (PM<sub>2.5</sub> vs. PM<sub>c</sub>) influences the pro-inflammatory response. Harvest ambient  $PM_{2.5}$  and  $PM_c$  with a particle concentrator during the winter and summer seasons. Using macrophages (mouse bonemarrow derived cells), assess the influence of seasonal  $PM_{2.5}$  and  $PM_c$ , respectively, on inflammation (IL-1 $\beta$  and TNF- $\alpha$ ) and cytotoxicity (LDH). *Hypothesis: By comparing seasonal ambient PM<sub>c</sub> to PM<sub>2.5</sub>, and also evaluating the influence of increased PM<sub>c</sub> burden in PM*<sub>10</sub>, *PM<sub>c</sub> will be more bioactive (i.e. pro-inflammatory) than PM<sub>2.5</sub> in the Rocky Mountain region, serving as an important predictor of health outcome.* 

Manuscript status: This study has been published.

– Ferguson MD, Migliaccio C, Ward T. 2013. Comparison of how ambient PM<sub>c</sub> and PM<sub>2.5</sub> influence the pro-inflammatory potential. *Inhalation Toxicology*. 25(14):766-773

Specific Aim 2 (Chapter 3): Evaluate how the source of wood smoke influences health effects. Using a particle concentrator, harvest  $PM_{2.5}$  from an older model wood stove, EPA-certified low emission stove, and during a summer wildfire event (ambient wildfire  $PM_{2.5}$ ). Using THP-1 cells, assess the influence of biomass smoke source on inflammation (IL-1 $\beta$ ) and cell viability (MTS assay). *Hypothesis: Exposures to ambient wildfire smoke will induce a greater pro-inflammatory response when compared to residential wood stove smoke; and that the older model woodstoves will induce a greater inflammatory response when compared to an EPA-certified low emission stove.* 

*Manuscript status:* This manuscript has been submitted to the Journal of Environmental and Public Health, and is currently under review.

– Ferguson M, Migliaccio C, and Ward T. 2015. Impact of wood smoke source on proinflammatory responses in an *in vitro* human macrophage cell model. *Journal of Environmental and Public Health*, in review.

Specific Aim 3 (Chapter 4): As a translational Aim, human pulmonary and systemic responses to occupationally-relevant wood smoke exposures will be evaluated.

Aim 3a. Modify the Inhalational and Pulmonary Physiology Core facility to conduct human exposure studies focused on biomass smoke and health effects. Aim 3b. Using exhaled breath condensate, plasma, and spirometry data from 10 human subjects, assess the significance of increased wood smoke exposures on inflammatory and oxidative stress, as well as changes in lung function. *Hypothesis: Short-term exposure to wood smoke at 250 µg/m<sup>3</sup> and 500 µg/m<sup>3</sup> concentrations will result in significant* 

increases in pulmonary and systemic inflammation and oxidative stress when compared to clean air exposures.

Manuscript status: The Aim 3a manuscript was submitted to the Journal of Occupational and Environmental Hygiene and is under review.

- Ferguson MD, Semmens, EO, Weiler E, Dimitrovich, J, Migliaccio C, Palmer C, Dumke C, and Ward T. 2015. Lung function measures following a wildland firefighter controlled exposure study. *Journal of Occupational and Environmental Hygiene,* in review.
- *Manuscript status:* The Aim 3b manuscript was submitted to the *Journal of Occupational and Environmental Medicine* and is under review.
- Ferguson MD, Semmens EO, Dumke C, Quindry J, and Ward T. 2015. Measures pulmonary and systemic markers of inflammation and oxidative stress following wildland firefighting simulations. *Journal of Occupational and Environmental Medicine*, in review.

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# Chapter 2: Comparison of how $PM_c$ and $PM_{2.5}$ influence the inflammatory potential

#### Abstract

Airborne particulate matter (PM) is one of six criteria air pollutants currently regulated by the U.S. Environmental Protection Agency (EPA), with existing ambient standards for  $PM_{2.5}$  and  $PM_{10}$ . Currently there are no health-based regulations for the size fraction between 2.5 and 10 µm, commonly known as the coarse fraction (PM<sub>c</sub>). The present study investigates current gaps in knowledge for PM<sub>c</sub> including exposure toxicity and PM ratios (PM<sub>c</sub>:PM<sub>2.5</sub>) in PM<sub>10</sub>. Throughout the world, all three PM size fractions have been shown to be associated with adverse impacts. Recent studies have shown that PM<sub>c</sub> can be more detrimental to susceptible populations when directly compared to PM<sub>2.5</sub>, and that the PM<sub>c</sub> fraction in PM<sub>10</sub> can account for the majority of the inflammatory response from PM<sub>10</sub> exposure.

In our studies we utilized a bone marrow-derived mouse macrophage *in vitro* system to compare the inflammatory potential of  $PM_c$ ,  $PM_{2.5}$ , and mixtures of the two. The result was a linear increase in interleukin(IL) -1 $\beta$  with increasing levels of exposure to winter and summer  $PM_c$ , as compared to  $PM_{2.5}$ , which exhibited logarithmic growth. Also, exposure to  $PM_{10}$  as a function of  $PM_{2.5}$  and  $PM_c$  mass ratios showed that IL-1 $\beta$  and TNF- $\alpha$  levels increased synergistically with a greater burden of  $PM_c$ . Endotoxin content in the PM did not correlate with these results, suggesting that other activators in  $PM_c$  are likely responsible for activating the NF- $\kappa$ B pathway and the inflammasome.

#### Introduction

Airborne particulate matter (PM) is a known causative agent in inducing or exacerbating a variety of respiratory and cardiovascular diseases (Pelucchi et al. 2009, Brook et al. 2010, Dvonch et al. 2009, Samet et al. 2009, Weinmayr et al. 2010, Zanobetti et al. 2010, Xu et al. 2011). PM is also one of six criteria air pollutants currently regulated by the United States Environmental Protection Agency (EPA), with existing ambient standards for both  $PM_{2.5}$  and  $PM_{10}$  (i.e. particulate matter (PM) with mean aerodynamic diameters <2.5 and <10 micrometers ( $\mu$ m), respectively). Currently there are no healthbased regulations for the size fraction between 2.5 and 10  $\mu$ m, commonly known as the coarse fraction (PM<sub>c</sub>). Compared to ambient PM<sub>2.5</sub> and PM<sub>10</sub> (which are routinely measured across the US), much less is known about PM<sub>c</sub> seasonal concentrations and health effects. In multiple research studies conducted throughout the world, all three PM size fractions have been shown to be associated with adverse impacts (Gilmour et al. 2007, Gordian et al. 1996, Host et al. 2008, Mar et al. 2004, Monn and Becker 1999, Ostro et al. 2000, Pelucchi et al. 2009 2007).

 $PM_c$  has been shown to cause a more significant pulmonary inflammatory response than  $PM_{2.5}$  in animal models (Tong et al. 2010, Wegesser and Last 2009), as well as increased inflammatory potential in multiple respiratory cell lines (Gualtieri et al. 2010). Becker et al. (Becker et al. 2005) reported that compared to  $PM_{2.5}$ ,  $PM_c$  induced higher levels of the inflammation markers IL-6 and Cox-2 mRNA in alveolar macrophages, and higher levels of IL-8 and Cox-2 mRNA in normal human bronchial epithelial cells. These studies suggest that the coarse fraction in  $PM_{10}$  accounts for 90-95% of the resulting inflammatory response (Becker et al. 2005). These findings may be explained by the biological composition of  $PM_c$ , as the coarse fraction can contain up to 10 times the amount of biological content compared to  $PM_{2.5}$  (Heinrich et al. 2003). This biological

content includes pathogen associated molecular patterns (PAMPS), such as endotoxin, which prime the activation of the inflammasome (Tschopp and Schroder 2010, Yazdi et al. 2010). The inflammasome is a protein complex that once formed can stimulate maturation of pro-IL-1 $\beta$  to its active form, IL-1 $\beta$ . However, a pool of pro-IL-1 $\beta$  will need to be maintained allowing the inflammasome to continue its function. Endotoxin has also been shown to trigger the NF- $\kappa$ B pathway and downstream pro-IL-1 $\beta$  production via TLR4 (Schins et al. , Bauernfeind et al. 2009), suggesting that ambient PM-induced adverse health outcomes may be heavily influenced by the PM<sub>c</sub> fraction.

A tenet of toxicology is that exposure to environmental toxicants will likely occur as a mixture, with the outcome being a function of the joint effects of this mixture. A measure of how a mixture can maintain, lower, or increase a toxic outcome is termed additivity. For example, laboratory studies exposing humans to both carbon black particles and  $O_3$  resulted in greater lung inflammation and suppression of alveolar macrophage phagocytosis than would be predicted using the sum of both toxicities alone (Jakab and Hemenway 1994). These results suggest a *synergism* in the lung, or a *greater than additive* toxicity between carbon black and  $O_3$ .

Toxic consequences from mixture exposures have two other outcomes other than synergism. An inverse relationship to synergism may also be observed where *antagonism* takes place, i.e. a decrease in toxic potential, *less than additive*. Finally, when two toxicants are introduced in a mixture and if one toxic does not affect the other, and visa versa, the mixture effect is termed *additive*.

Although multiple studies have been conducted linking either  $PM_{2.5}$  or  $PM_{10}$  to respiratory disease throughout the world, little research has been conducted investigating the role of these two PM size fractions in mixture, specifically how the  $PM_c$ 

size class influences overall  $PM_{10}$  inducible toxicity. In the real world, exposure to  $PM_{10}$  occurs as a function of  $PM_c$  and  $PM_{2.5}$  mass ratios. These mixtures can change as a function of geography, temporality, and seasonal conditions (e.g. wildfires, winter inversions, etc.). In this manuscript, we describe the results of studies that evaluated the additive effect of ambient  $PM_{2.5}$  and  $PM_c$  mixtures. Following the harvesting of ambient PM using a particle concentrator, an *in vitro* model of inflammation was used to determine the contributions of  $PM_c$  within a mixture. The overall goal of these studies was to not only investigate the inflammation potential of  $PM_c$  compared to  $PM_{2.5}$ , but also to elucidate the relative contributions of these two size fractions to observed health effects.

#### Methods

#### **Collection of Ambient Particles**

Seasonal PM was collected in Missoula, Montana, a valley airshed located in westcentral Montana. With a population of 70 000, Missoula is the 2nd largest city in the state of Montana. Particles were collected using a versatile aerosol concentration enrichment system particle concentrator (VACES-PC, University of Southern California). The concentrator has three parallel sampling lines that simultaneously collect PM<sub>2.5</sub> and PM<sub>c</sub>, respectively, at a set flow rate of 110 L/min (Kim et al. 2001a, Kim et al. 2001b). Following each sample run, the product (i.e. particles in ultrapure water) was stored at -20° and later lyophilized to concentrate particles for further studies. The particle concentrator is housed on the roof of a four story building on the University of Montana campus. Particles were harvested during the winter (February 2012), spring (May 2012), and summer months (July and August 2012). These collection periods yielded from 100 µg to about 1 mg of each PM type, depending on conditions.

#### Ambient PM Levels in Missoula

Although  $PM_c$  sampling is not currently conducted in Missoula, continuous  $PM_{2.5}$  and  $PM_{10}$  are routinely measured as part of Missoula's EPA compliance monitoring program. This program uses these continuous PM data to calculate 24-hour ambient PM averages, which are then reported. Using these continuous  $PM_{2.5}$  and  $PM_{10}$  data (collected with a MetOne BAM at the Missoula Boyd Park site), we were able to calculate, by difference, the seasonal ambient  $PM_c$  mass concentrations during 2012 ( $[PM_{10}] - [PM_{2.5}] = [PM_c]$ ). We also calculated the 24-hour average mass concentrations

on days when PM was collected with the particle concentrator and used in this work. The unique seasonal patterns in Missoula, MT are accounted for in this study, as high latitudes and high elevations stimulate longer cold seasons. For the purpose of this manuscript, winter months are categorized as the months of November, December, January, and February; spring are the months of March, April, and May; summer months are the months of June, July, and August; and autumn is September and October.

#### Mice and Bone Marrow-Derived Macrophages

A major component of respiratory immunity is the pulmonary macrophage, which plays a key role in the regulation of inflammation as well as subsequent adaptive responses. Pulmonary macrophages are perpetual residents of the lung and act as sentinels or first responders for particle uptake. These macrophages have the necessary receptors for particle uptake/phagocytosis (Hirayama et al. 2011, Geiser 2010, Goodridge et al. 2012, Underhill and Goodridge 2012). Our laboratory has published multiple *in vivo* particulate exposure studies using the Balb/c mouse model (Migliaccio et al. 2005, Migliaccio et al. 2010, Migliaccio et al. 2013). In addition to these studies, our laboratory has also utilized an *in vitro* macrophage system using bone marrow-derived macrophages (BMdMs) to evaluate the effects of particles on cell functions (Migliaccio et al. 2005, Migliaccio et al. 2008). Therefore, by exposing mouse BMdMs to PM, an inflammatory response can be measured.

A variety of studies have described the key role of the pulmonary macrophage in particle clearance (Geiser 2010), and its link between the innate and adaptive immune systems

(Gordon 2003, Mosser 2003). Bone marrow-derived macrophages utilized in this study to evaluate the inflammatory response of relevant doses of different PM fractions (PM<sub>2.5</sub>, PM<sub>10</sub>, and PM<sub>c</sub>). Methods for BMdM cultures are previously described (Pfau et al. 2004, Migliaccio et al. 2008). Briefly, hind legs of euthanized Balb/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were flushed with media (RPMI 1640 with 10% FBS) to release the marrow. After overnight stromal elimination, cells were stimulated with M-CSF for an additional 7-10 days of culturing. On day 10, cells were aliquoted (10<sup>5</sup> cells/well) in 96-well plates for culturing with particles. All animal procedures were approved by the University of Montana Institutional Animal Care and Use Committee (Missoula, MT, USA). Method of euthanasia was by lethal dose of sodium pentobarbital via intraperitoneal injection.

### PM<sub>c</sub> vs. PM<sub>2.5</sub> Comparison Studies

During the summer (July, 2011) and winter (February, 2012) months,  $PM_{2.5}$  and  $PM_c$ were simultaneously harvested in Missoula using our VACES-PC. Stock solutions of PM were made by diluting pre-determined masses of PM into a small volume of PBS (< 0.5 mL) followed by sonication. The volume of PBS necessary is dependent on the amount of PM harvested. Cells were incubated with particles for 1 hour (mixing) in 1.5 mL eppendorf tubes, then aliquoted to 96-well plates and cultured overnight. Final cultures contained 150 µL per well of media, with each condition in triplicate exposures at 100,000 cells/well. A media negative control was also used that contained no particles. Particle exposure levels included varying masses of  $PM_{2.5}$  or  $PM_c$  at 5 µg, 20 µg, and 40 µg/well. Collection of supernatants occurred after 24 hours.
#### PM Mixture Studies

PM<sub>2.5</sub> and PM<sub>c</sub> used in the mixture exposures were harvested during May 2012. BMdMs were exposed in a similar fashion as the comparison studies described above, but at one level of exposure at 20 µg/well. To represent the varying conditions of PM<sub>10</sub>, 20 µg mixtures of PM<sub>2.5</sub> and PM<sub>c</sub> varied from 0%, 20%, 40%, 60%, 80%, and 100% PM<sub>c</sub> by mass. For these studies, 20 µg was used, as it was the lowest mass of exposure that induced the most significant differences between PM size and season with both IL-1β and TNF- $\alpha$ . In similar studies using *in vivo* models, particulate exposures ranged from 5-50 µg of particles per instillation (Porter et al. 2013, Stoeger et al. 2006). Considering that there are roughly 200-300 thousand alveolar macrophages in the mouse lung, if 50 µg is distributed evenly, each cell will theoretically receive approximately 0.17-0.25 ng of particles. In our model using 20 µg and 100 000 cells, if particles were distributed evenly, each cell would receive roughly 0.2 ng of particles. Therefore, the levels of particulates used in this research are representative of *in vivo* studies.

Collection of supernatants occurred after 24 hours of incubation. The resulting inflammatory cytokine levels were used to determine the additivity potential of the mixtures. To ensure that these particles were undergoing phagocytosis, bright field illumination microscopy of BMdMs following 24-hours of exposure to PM<sub>10</sub>, was performed (Nikon Eclipse E800, Tokyo, Japan; Nuance Multispectral Imaging System, Hopkinton, Massachusetts).

#### Endotoxin Analysis

Levels of endotoxin were evaluated using methods adapted by Thorne (Thorne 2000). Briefly, the harvested PM samples were analyzed for endotoxin levels using a kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay (Charles River Endosafe® -Endochrome-K <sup>™</sup>, Charles River Laboratories, Charleston, SC, USA), where a 12-point standard curve ranged from 50 to 0.005 EU/ml. The absorbance was measured at 405 nm every 30 seconds for 90 minutes.

#### Mixture Effect Calculation

Additivity was modeled using equation 1, a modified version of the toxic unit model (PapeLindstrom and Lydy 1997). Modification was necessary, as endpoints were not based on concentration/dose response thresholds, but were based on the resulting levels of the expressed cytokines from 20  $\mu$ g of PM exposure. Where additivity is > 1, = 1, or < 1 the effect of the mixture is deemed synergistic, additive, or antagonistic, respectively. The fraction of PM<sub>2.5</sub> or PM<sub>c</sub> in PM<sub>10</sub> is represented with *f* and can range anywhere from 0 to 1. For example, if PM<sub>10</sub> contained 25% PM<sub>2.5</sub> and 75% PM<sub>c</sub>, *f* would equal 0.25 and 0.75, respectively.

$$f_{PM_{2.5}} \frac{\left[PM_{10}\right]}{\left[PM_{2.5}\right]} + f_{PM_c} \frac{\left[PM_{10}\right]}{\left[PM_c\right]} = additivity \qquad \text{equation 1}$$

As the ratios of  $PM_{2.5}$  and  $PM_c$  change within total  $PM_{10}$ , a departure from an effect level of 1 explains whether a larger burden of one PM size fraction is either affecting a synergistic (additivity >1) or an antagonistic (additivity <1) effect. One other alternative is that the mixture will not cause a departure from 1, and that the increased presence of one or another PM type will result in an additive response.

# Cytokine and Statistical Analysis

Collection of pro-inflammatory cytokines was completed following the 24-hour exposure. The collected media/supernatant was then stored at -20° C prior to analysis via enzymelinked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN, USA). Each well's concentration was measured in duplicate. Levels of IL-1 $\beta$  and TNF- $\alpha$  released from BMdMs were then plotted using Prism, version 5.0a. Student t-tests were also performed in Prism to determine significant differences between two PM types within each mass of exposure (5 µg, 20 µg, and 40 µg).

## Results

## Ambient air data in Missoula, MT

Table 2.1 summarizes the average concentrations of  $PM_{2.5}$ ,  $PM_c$ , and  $PM_{10}$  measured throughout 2012, illustrating that  $PM_c$  is not only a substantial component of  $PM_{10}$  throughout the year, but also is present in elevated concentrations (compared to  $PM_{2.5}$ ) throughout all four seasons in this northern Rocky Mountain airshed. The data presented in Table 2.1 indicate that ambient PM concentrations ( $PM_{2.5}$ ,  $PM_c$ , and  $PM_{10}$ ) vary as a function of season. Throughout the year, seasonal  $PM_c$  concentrations measured in Missoula, MT were consistently higher than  $PM_{2.5}$ , even in winter when there were more combustion-related particles in the Missoula airshed due to residential wood stove use (Ward and Lange 2010). Unlike  $PM_{2.5}$ ,  $PM_c$  average concentrations were fairly consistent through winter and spring (9.3-9.6  $\mu$ g/m<sup>3</sup>), with a marked increase in PM<sub>c</sub> from summer to fall.  $PM_{2.5}$  concentrations in fall were at least twice as high as all other seasons.

	Winter	Spring	Summer	Fall
	Nov-Feb	Mar-May	June-Aug	Sept-Oct
PMc	9.3±7.7	9.6±7.7	13.8±10.7	25.0±30.4
PM <sub>2.5</sub>	9.1±4.9	5.9±2.6	7.8±6.4	18.1±18.5
<b>PM</b> <sub>10</sub>	18.4±10.0	15.5±9.8	21.6±12.4	43.1±37.0

**Table 2.1.** Average and  $\pm$  standard deviation of ambient PM concentrations ( $\mu$ g/m<sup>3</sup>) in 2012, Missoula, MT.

PM concentrations on days that ambient particles were harvested for the controlled laboratory exposure studies ( $PM_c vs. PM_{2.5}$  comparison and mixture studies) are presented in Table 2.2. During the days that PM was harvested during the winter months, the PM<sub>c</sub> concentrations were more than twice as high compared to the winterlong average presented in Table 1, though PM<sub>2.5</sub> concentrations were similar. The authors are unsure why PM<sub>c</sub> was elevated on these sample days. When comparing PM<sub>c</sub> concentrations measured during harvest days compared to overall summer averages, concentrations are comparable. The same is true for collected Spring PM. Also during these collection days (Table 2.2), PM<sub>2.5</sub> concentrations were comparable to the winter, spring, and summer averages presented in Table 2.1.

**Table 2.2.** Average and  $\pm$  standard deviation concentrations ( $\mu$ g/m<sup>3</sup>) during winter and summer days when ambient particles were harvested.

	Winter	Spring	Summer
	Feb, 2012	May, 2012	July, 2011
PMc	22.6±9.2	7.4±1.9	9.7±4.4
PM <sub>2.5</sub>	12.3±3.0	5.3±1.3	7.4±2.1
<b>PM</b> <sub>10</sub>	34.9±8.5	12.7±3.2	17.1±6.5

#### PM non-mixture exposures, in vitro

In side-by-side controlled exposures within the laboratory, winter  $PM_c$  induced the highest levels of IL-1 $\beta$  and TNF- $\alpha$  at all three doses (Figure 2.1.), supporting our hypothesis that  $PM_c$  is more biologically active compared to  $PM_{2.5}$ . Summer  $PM_c$  was the second most bioactive PM, followed by summer  $PM_{2.5}$  and winter  $PM_{2.5}$ . In each season (winter and summer), the most bioactive size fraction of PM was  $PM_c$ , with winter  $PM_c$  inducing more than twice the levels of IL-1 $\beta$  than summer  $PM_c$ . Similar results were observed for TNF- $\alpha$ . IL-1 $\beta$  increased in a linear fashion in response to both winter and summer  $PM_c$ . However, IL-1 $\beta$  from winter and summer  $PM_{2.5}$  increased logarithmically, and leveled out at around 25 pg/mL. This logarithmic growth was not observed for TNF- $\alpha$ , as all four PM types generated linear increases in cytokine expression.



**Figure 2.1.** Levels of cytokine production by mass of PM added per well (containing 100 000 BMdMs). The left and right graphs are IL-1 $\beta$  and TNF-  $\alpha$  levels, respectively, in response to PM and controls.

The data on the significant differences between  $PM_c$  and  $PM_{2.5}$  are listed in Table 2.3. Two-tailed, Student *t*-tests were used to determine if the two PM size fractions induced significantly different responses at a single dose. Both summer and winter  $PM_c$  induced significantly higher IL-1 $\beta$  levels compared to their  $PM_{2.5}$  counterparts (at 20 µg, IL-1 $\beta$ between summer  $PM_c v$ .  $PM_{2.5} p=0.02$ , and winter  $PM_c v$ .  $PM_{2.5} p=0.03$ ). TNF- $\alpha$  levels were only significantly different between  $PM_c$  and  $PM_{2.5}$  for winter, but not for summer (at 20 µg, TNF- $\alpha$  between summer  $PM_c v$ .  $PM_{2.5} p=0.09$ , and winter  $PM_c v$ .  $PM_{2.5} p=0.0002$ ). IL-1 $\beta$  levels from winter  $PM_c$  were triple that of summer  $PM_c$ . Differences in IL-1 $\beta$  levels were mostly non-significant when comparing low doses of PM exposure, except between both summer and winter  $PM_{2.5}$  where there was no significant differences. These results suggest that  $PM_c$  is an important mediator in inducing an inflammatory response.

**Table 2.3.** P-values comparing cytokine production of IL-1 $\beta$  or TNF- $\alpha$  between two PM exposure types at 5, 20, or 40 µg.

	Winter PM <sub>2.5</sub>	Winter PM <sub>c</sub>	Summer PM <sub>2.5</sub>	Summer PM <sub>c</sub>
Winter PM <sub>2.5</sub>		*, 0.03, 0.01	*, 0.27, 0.16	*, <0.01, 0.01
Winter PM <sub>c</sub>	<0.01, <0.01, <0.01		0.11, 0.04, 0.01	0.16, 0.06, 0.02
Summer PM <sub>2.5</sub>	<0.01, <0.01, 0.17	<0.01, <0.01, <0.01		0.10, 0.02, 0.01
Summer PM <sub>c</sub>	<0.01, <0.01, 0.06	0.01, 0.01, 0.02	0.06, 0.09, 0.29	

Each of the grouped (three) reported p-values from left to right are from 5, 20, and 40  $\mu$ g exposures, respectively. *Italicized* = TNF- $\alpha$ , normal type = IL-1 $\beta$ , \* = cytokines not detected, cannot compare. Null hypothesis = Cytokines levels are the same between exposure treatments.

## Cellular uptake of ambient PM<sub>10</sub>

Most of the particles used in these exposures were phagocytosed by the BMdMs, as the majority of the particles were intracellular (Figure 2.2). Using BFI microscopy, we determined that the cells exposed to  $PM_{10}$  had phagocytosed the majority of the particles in solution. In other images where  $PM_c$  and  $PM_{2.5}$  burden in  $PM_{10}$  varied, similar uptake was observed (images not shown). Particle size (both  $PM_{2.5}$  and  $PM_c$ ) did not seem to play a major role in the uptake of these particles. Small particles and larger sized particulates, such as the fungi, *Torula herbarum* (Figure 2.2), with diameters of approximately 3-4 µm, appear to be equally phagocytosed. This confirms that both  $PM_c$  and  $PM_{2.5}$  are being taken up by the BMdMs.



**Figure 2.2.** Cellular uptake of ambient PM<sub>10</sub>. (A) 40X magnification of BMdMs containing PM<sub>10</sub>, (B) Oil immersion 100X magnification showing fungi (spp. *Torula herbarum*) inside macrophage.

# PM endotoxin burden

Endotoxin levels were determined as a function of PM mass (Figure 2.3). Results show that overall endotoxin concentrations were highest during the summer months compared to the winter months for both fractions. As predicted, there was more endotoxin in the winter  $PM_C$  compared to the winter  $PM_{2.5}$ . Endotoxin concentrations during the summer were comparable between the two size fractions. These results suggest that endotoxin concentrations composing ambient PM can vary as a function of PM size and season, and not primarily found in the coarse fraction (compared to the fine fraction).

# Endotoxin content in collected PM



Figure 2.3. PM Endotoxin content from winter and summer collected ambient particles.

### Mixture effects

As noted earlier,  $PM_{2.5}$  and  $PM_c$  used in the mixture exposures were harvested during May 2012. Results from the mixture studies show that varying the combinations of  $PM_{2.5}$ and  $PM_c$  in  $PM_{10}$  influence cytokine output, with Figure 2.4. illustrating the effect of  $PM_c$ in a  $PM_{10}$  mixture. All cytokine concentrations (i.e. [IL-1β] and [TNF-α]) varied as a function of exposure to 20 µg of each PM type ( $PM_{10}$ ,  $PM_{2.5}$ , or  $PM_c$ ). Shown in Figure 2.4., as  $PM_{10}$  gains a greater proportion of  $PM_c$ , a synergistic release of IL-1β and TNF-α occurs. Conversely, as the fraction of  $PM_{10}$  contains more  $PM_{2.5}$  than  $PM_c$ , antagonistic effects are observed. These results support our hypothesis that the  $PM_c$  fraction is an important mediator in inducing an inflammatory response with  $PM_{10}$  exposure.



**Figure 2.4.** Additivity values from different ratios of spring  $PM_{2.5}$  and  $PM_c$  mixtures. The left and right graphs are additivity values calculated from IL- 1  $\beta$  and TNF-  $\alpha$  levels, respectively.

### Discussion

Understanding the mass ratio mixtures of ambient  $PM_{2.5}$  and  $PM_c$  composing  $PM_{10}$  can help determine additivity of the inflammatory response. As shown in our controlled laboratory studies,  $PM_c$  was constantly more bioactive than  $PM_{2.5}$  throughout each sideby-side exposure in comparing both summer and winter months, and with its increased burden in  $PM_{10}$ . We were successful in showing that there are certainly additive effects from these different mass ratio exposures. Furthermore, our results suggest that the outcome from exposure to  $PM_{10}$  can be partly understood as a function of  $PM_{2.5}$  and  $PM_c$ burden in  $PM_{10}$ .

A source apportionment study has yet to be completed for  $PM_c$  in Missoula, MT. However,  $PM_{2.5}$  source apportionment modeling has shown that the largest  $PM_{2.5}$  source throughout the year is wood combustion, contributing an average of 41% to the fine fraction. This includes residential wood combustion during the winter, slash and prescribed burns from the surrounding areas during the spring and fall, and smoke from wildfires during the summer months. Other sources of  $PM_{2.5}$  throughout the year include diesel exhaust (19%), secondary ammonium nitrate (17%), local industry (20%), and street sand (5%) (Ward and Smith 2005, Ward and Lange 2010). Though these studies did not focus specifically on apportioning the sources of  $PM_c$ , they do provide an idea of the types of exposure conditions that exist throughout the year in Missoula, MT.

The present study is the first attempt at determining ambient concentrations of  $PM_c$  in the state of Montana within the last 15 years. As illustrated in Table 2.1., ambient concentrations of  $PM_c$  were consistently greater than  $PM_{2.5}$  throughout the year, with the exception of the winter months where the average  $PM_c$  and  $PM_{2.5}$  concentrations were not significantly different. It should be noted, however, that harvested particles used in our laboratory studies were collected on days when the average  $PM_c$  mass

concentrations were about 2 - 10  $\mu$ g/m<sup>3</sup> greater than PM<sub>2.5</sub>. In addition, particles were harvested during sample days in which there was higher winter PM<sub>c</sub> concentrations (22.6±9.2  $\mu$ g/m<sup>3</sup>) compared to the winter 2012 average PM<sub>c</sub> concentrations presented in Table 1 (9.3±7.7  $\mu$ g/m<sup>3</sup>). PM<sub>2.5</sub> concentrations (both throughout the winter of 2012 and during the dates of sample collection) were consistent (9.1±4.9  $\mu$ g/m<sup>3</sup> vs. 12.3±3.0  $\mu$ g/m<sup>3</sup>). During the *PM<sub>c</sub>* vs. *PM<sub>2.5</sub> Comparison studies*, particle exposure levels (PM<sub>2.5</sub> and PM<sub>c</sub>) were normalized at 5  $\mu$ g, 20  $\mu$ g, and 40  $\mu$ g/well in the 96-well plates. Therefore, it is unknown how the overall ambient concentrations during the days of particle harvesting influenced the results of the comparison studies (if at all). Additional studies within the laboratory across multiple seasons/years will be able to address this issue.

As evidenced in Figure 2.3., the differences in endotoxin content on the PM cannot explain the differences in the resulting IL-1 $\beta$  expression. Something other than endotoxin (e.g., mold) is potentially stimulating the production of pro-IL-1 $\beta$  upon exposure to PM<sub>c</sub>, as the levels of IL-1 $\beta$  do not seem to be limited with an increase in PM<sub>c</sub> exposure. In addition, increasing concentrations of PM<sub>2.5</sub> did not induce a linear increase in IL-1 $\beta$  expression (Figure 2.1). Further studies can evaluate the levels of pro-IL-1 $\beta$  in cells, via western blot, to determine if a decreased IL-1 $\beta$  release is due to a lowered level of expression of the pro form of IL-1 $\beta$  or if the inflammasome is simply not active.

Figure 2.1. supports the hypothesis that ambient  $PM_c$  is more bioactive than  $PM_{2.5}$ . However, these exposures were done on a side-by-side comparison using controlled laboratory conditions, and did not take into account that real-world exposures to these two size fractions occur concomitantly. The mixture exposures take into account possible mixing conditions of  $PM_{2.5}$  and  $PM_c$  in  $PM_{10}$ . The results of the *in vitro* mixture exposures showed that  $PM_{2.5}$  is not as potent an activator of the inflammasome as  $PM_c$ ,

even in the presence of another PM type. In order to stimulate these levels of IL-1 $\beta$ production, the inflammasome complex must be activated by a receptor (e.g. binding of receptor to PAMP) or lysosomal stress (e.g. the release of Cathepsin B) (Tschopp 2008, Yazdi et al. 2010). Potentially, there are components in  $PM_c$  that are responsible for priming the formation of the inflammasome, such as endotoxin, an activator of the NF- $\kappa$ B pathway via TLR4, and another component affecting lysosomal membrane integrity. NFκB activation is a precursor to transcription induction of pro-IL-1β (Yazdi et al. 2010, De Nardo and Latz 2011). Activation of the TLR or priming of the NF- $\kappa$ B pathway has been shown to stimulate the production of pro-IL-1 $\beta$  and further production of NLRP3 inflammasome precursors (De Nardo and Latz 2011). It is likely that not only is the inflammasome being formed, but also more pro-IL-1 $\beta$  is being produced following PM<sub>c</sub> exposure. Also, reactive oxygen species (ROS) may play a significant role in inflammation as generation of ROS from particulates has been shown to exacerbate the inflammatory response (Fubini and Hubbard 2003). Furthermore, exposure to biogenic substances, such as pollen, largely found in PM<sub>c</sub>, can increase ROS in the lung (Boldogh et al. 2005).

### Conclusion

In this study, we utilized harvested ambient particles and biological *in vitro* models to investigate potential adverse health effects following exposures to different size fractions found in the Rocky Mountains in North America. The results of these studies conducted under controlled laboratory conditions suggest that there are properties (chemical, physical, or biological) of PM<sub>c</sub> that are responsible for elevated levels of inflammation activity compared to ambient PM<sub>2.5</sub>. An important step to further this research would be to investigate PM<sub>c</sub> harvested throughout the year. For example, winter PM<sub>c</sub> contained about two-thirds the endotoxin burden as compared to summer PM<sub>c</sub>, yet winter PM<sub>c</sub> induced more than twice the levels of IL-1  $\beta$  than summer PM<sub>c</sub>. Other methods of *in vitro* exposure could also be employed, such as an air-liquid interface model. Also, an alternative method of collecting particles that does not include the removal of water (via lyophilization) could be explored to minimize the loss of volatile and semi-volatile compounds. Such methods could include employing the use of a cyclone or another dry method of harvesting ambient air particles.

The mixture results presented here suggest that if the percentage of PM<sub>c</sub> is known, the relative bioactivity of the ambient PM<sub>10</sub> might be predicted. This study supports our hypothesis that PM<sub>c</sub> adversely affects macrophage activity with increasing ratios of PM<sub>c</sub>:PM<sub>2.5</sub> in PM<sub>10</sub>. It is also important to note that there are currently no National Ambient Air Quality Standards (NAAQS) for the coarse fraction, though our results suggest that a new PM<sub>c</sub> ambient standard should perhaps be considered in addition to the existing daily and annual PM<sub>2.5</sub> NAAQS. Including additional chemical and biogenic characterizations will facilitate our future source apportionment studies for different PM fractions. Also, evaluation of reactive oxygen species activity in both PM<sub>c</sub> and PM<sub>2.5</sub> should be considered in future research. Further seasonal and dose-response

relationship studies will aid in determining real-world mixture effects, as well as provide an understanding of the causal health effects from exposure to PM<sub>c</sub>.

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# Chapter 3: Impact of wood smoke source on pro-inflammatory responses in an

*in vitro* human macrophage cell model

#### Abstract

Throughout the world, biomass burning is a significant source of air pollution and can result in adverse health effects. In the Northern Rocky Mountain region of the US, wood smoke sources contribute to inhalable particulate matter (PM), and is predominantly emitted from residential home heating (wood stoves) during the winter months and wildland fires during the summer. Many uncertainties remain regarding how these different sources impact health. Particles were collected from a high emission residential wood stove, an EPA-certified low emission residential wood stove, and during a summer wildfire event (ambient wildfire PM<sub>2.5</sub>). The influences of wood smoke source on inflammation (IL-1 $\beta$ ) and cell viability (MTS assay) were assessed *in vitro*. In a doseresponse manner, harvested wildfire smoke PM<sub>2.5</sub> stimulated significantly more IL-1β release than either wood stove sources and urban particulate matter. PM<sub>2.5</sub> from both wood stoves similarly induced the highest levels of cell toxicity compared to wildfire smoke PM<sub>2.5</sub>, with overall findings following similar trends in the measured endotoxin content of the particle types. These findings suggest that exposure to different sources of wood smoke PM<sub>2.5</sub> may result in varying adverse health effects, and additional studies are needed to further understand these relationships and mechanisms.

## Introduction

Particulate matter (PM) is a ubiquitous component of air pollution, with exposures resulting in increased rates of premature mortality and morbidity (Anderson et al., 2012; Anenberg et al., 2010; Chambliss et al., 2014; Cheng et al., 2013; Nel, 2005; Pope and Dockery, 2006; Punger and West, 2013; Salma et al., 2002). In the United States, PM is one of six criteria air pollutants currently regulated by the Environmental Protection Agency (EPA), with National Ambient Air Quality Standards (NAAQS) set for two specific PM sizes: PM<sub>2.5</sub> and PM<sub>10</sub> (PM with mean aerodynamic diameters of 2.5 µm and 10 µm and less, respectively). These standards were selected primarily due to the ability of particles of these general size fractions to penetrate deep into the lungs. The resulting effects once in the lung, however, are based largely on the properties (e.g., physical, chemical, etc.) of these particles. Emerging studies indicate that not only PM size, but also PM source, plays an important role in the causative pathway of PM-induced adverse health effects (Barregard et al., 2006; Bølling et al., 2012; Kocbach, 2008a; Sussan et al., 2014; Uski et al., 2012).

Globally, biomass burning is a large source of both indoor and ambient  $PM_{2.5}$  (Smith et al., 2004), not only in rural communities, but in urban cities as well (Larson et al., 2004; Naeher et al., 2007; Ward et al., 2004; Ward and Lange, 2010). This is especially true in the northern Rocky Mountain region of the US where wood smoke emissions are one of the largest sources of  $PM_{2.5}$  annually. This includes forest fire smoke during the summer months (EPA, 2013), and residential wood combustion (wood stoves) during the winter months (Larson et al., 2004; Naeher et al., 2007; Ward et al., 2004; Ward and Lange, 2010).

Epidemiological studies have shown that biomass exposures can result in adverse health outcomes similar to those observed in populations exposed to industrial or urban particulate matter (Boman et al., 2003; Fairley, 1999; Hales et al., 2000; Lipsett et al., 1997; Mcgowan et al., 2002; Sanhueza et al., 2009; Schwartz et al., 1993; Noonan et al., 2012). This includes general increases in daily mortality due to cardiovascular and respiratory disease, as well as significant increases in morbidity affecting those with chronic pulmonary diseases (Boman et al.), with sensitive populations particularly susceptible to wood smoke induced adverse health effects (Naeher et al., 2007). Healthy individuals are also at risk as wood smoke inhalation can cause an inflammatory response and oxidative stress both in the lungs (Barregard et al., 2008) and systemically (Swiston et al., 2008).

A common theme in particle exposure research involves determining the proinflammatory influence of particles (e.g., nanoparticles, traffic-induced PM, wood smoke PM, etc.). Our laboratory has performed particle exposures using macrophage-like transformed THP-1 cells in a number of settings, including evaluations of the proinflammatory response (Hamilton et al., 2014; Hamilton et al., 2013a; Hamilton et al., 2013b; Wu et al., 2014). Although wood smoke effects on the cardiovascular system are largely unknown, outcomes such as pulmonary inflammation following wood smoke exposures may be an important contributor to cardiovascular effects (Nel, 2005; Sharkey et al., 1997; Utell et al., 2002).

Given that exposure to PM is among the world's leading contributor to premature death (WHO, 2014), further investigations are needed on how different sources of PM influence resultant health effects. In the Northern Rocky Mountain region, studies investigating different types of wood smoke PM are especially important, as wood smoke

is a major annual source. To this end, we harvested PM<sub>2.5</sub> from an EPA-certified lowemission wood stove, an older-model high-emission wood stove, and ambient wood smoke during a wildfire event. The objective of this study was to compare the toxicities (i.e., cell viability), the resulting pro-inflammatory response, and endotoxin content among these common sources of wood smoke found in the western US.

## METHODS

### Ambient PM<sub>2.5</sub> Data and a Regional Wildfire

A versatile aerosol concentration enrichment system particle concentrator (VACES-PC) was used to collect ambient wood smoke PM<sub>2.5</sub> during a wildfire event located approximately 10 miles west of Missoula, MT during the days of August 20<sup>th</sup>, 21<sup>st</sup>, 22<sup>nd</sup>, and 23<sup>rd</sup> in the year 2013. Following the methods outlined in Ferguson et al. (Ferguson et al., 2013), the VACES-PC collected ambient air via three parallel tubes at a combined rate of 110 L/min. PM<sub>2.5</sub> particles were collected in biosamplers and then lyophilized to harvest PM (Sioutas et al., 1999). The particle concentrator was located on the roof of the Skaggs Building on the University of Montana's campus during this wildfire event. Continuous monitoring of ambient PM<sub>2.5</sub> was conducted in Missoula, MT over this same time period using a Beta Attenuation Monitor (BAM, Met One Instruments, Inc., Grants Pass, OR). For comparison, hourly mass concentrations were provided by the Montana Department of Environmental Quality, with 24-hour average PM<sub>2.5</sub> was being harvested with the particle concentrator.

PM<sub>2.5</sub> emissions from residential wood stoves were collected utilizing both an oldermodel wood stove (Englander, England Stove Works, Inc., Monroe, VA) and an EPAcertified wood stove (2100 Millennium, Quadra-Fire, Colville, WA) within the Center for Environmental Health Sciences' Inhalational and Pulmonary Physiology Core at the University of Montana. Smoke generated by the wood stoves was first routed through a port about 0.5 meters up from the bottom of each chimney, then passed through a 6' heated/insulated tube (Teague Enterprises, Woodland, CA) before entering a 2' x 6' x 2' aluminum chamber (Teague Enterprises, Woodland, CA). This dilution chamber allowed

the wood smoke PM to cool prior to collection using the VACES-PC at flow rates of 110 L/min.

Particles were ultimately deposited in biosamplers, with the PM/condensate samples stored at -20° C before being lyophilized using a Labconco freeze dryer (Kansas City, MO). Immediately following lyophilization, harvested particles were again stored at -20° C until analyses. To prepare the particles for use in our *in vitro* exposures, predetermined masses of PM<sub>2.5</sub> were diluted in sterile PBS using 1.5 mL Eppendorf (eppi) tubes with stock PM<sub>2.5</sub> solutions at 5 mg/L. Particle suspensions were then sonicated for 1 minute using a Misonix Inc. Sonicator (Farmingdale, NY) immediately prior to cell exposure.

## Cell Culture

THP-1 cells obtained from American Type Culture Collection (ATCC, Manassas, VA) were grown in 0.2  $\mu$ m filtered HEPES-buffered RPMI 1640 supplemented with L-glutamate,  $\beta$ -mercaptoethanol, 10% heat-inactivated fetal bovine serum, and sodium pyruvate (all supplements; Mediatech, Manassas, VA). Cell cultures were maintained at 37° C, and 5% CO<sub>2</sub>, in a water-jacketed incubator (ThermoForma, Houston, TX). Cells were differentiated into macrophage-like cells by adding (150 nM) 1,25-dihydroxy vitamin D<sub>3</sub> overnight prior to particle exposure.

## Cell Exposure and Analysis

Just prior to exposure, cells were co-stimulated with a small amount of phorbol myristate acetate (PMA; 5 nM) and endotoxin (10 ng/mL or 0.2 ng/well). These two additives stimulate cellular PM interactions and are common additives in *in vitro* studies. Cells were exposed in triplicate using 96-well plates to wildfire smoke PM<sub>2.5</sub>, two types of

wood stove smoke  $PM_{2.5}$  (older-model wood stove smoke  $PM_{2.5}$  and EPA-certified wood stove smoke  $PM_{2.5}$ ), and urban particulate matter (UPM) SRM 1648 from NIST (Gaithersburg, MD). Acid-washed crystalline SiO<sub>2</sub> was used as a positive control. "Noparticle" exposures were also performed as a negative control. All particle exposures occurred at 0.25, 0.5, 1, 5, and 20 µg/well, except for SiO<sub>2</sub> (at 20 and 100 µg/well). Each well contained 100,000 cells with a total volume of 200 µL. These exposure levels are similar to other peer-reviewed research studies where collected wood smoke or ambient PM was used in similar cell models (Becker et al., 2005; Bølling et al., 2012; Danielsen et al., 2011; Jalava et al., 2012).

After 24-hours of exposure, plates were centrifuged for 5 minutes at 1,500 rpm to remove particles and cells from supernatant. The supernatant was then transferred and stored at -20° C. As an indicator of pro-inflammatory influence, IL-1β was measured in all samples with enzyme linked immunosorbent assays (ELISA; Human IL-1β DuoSet, R&D Systems, Minneapolis, MN). A cell proliferation assay (CellTiter 96<sup>®</sup> AQueous One Solution, Promega, Madison, WI) was used to measure cell viability.

Utilizing a kinetic chromogenic *Limulus* amebocyte lysate (LAL) assay (Charles River Endosafe® - Endochrome-K <sup>™</sup>, Charles River Laboratories, Charleston, SC, USA), endotoxin content was measured for each of the four particle types. Briefly, each stock solution used in this study (i.e., wildfire smoke PM<sub>2.5</sub>, older-model wood stove PM<sub>2.5</sub>, EPA-certified wood stove PM<sub>2.5</sub>, and UPM) was analyzed for endotoxin content and compared with a 12-point standard curve (ranging 50 to 0.005 endotoxin units [EU]/mL). The absorbance was measured at 405 nm every 30 seconds for 90 minutes and reported as EU per mg of particles. Except for the MTS-assay, all data reported are from duplicate measurements from each well.

# Statistical Analyses

Cell viability and IL-1β measurements were plotted as a function of exposure type. Levels of IL-1β, cell viability, and endotoxin burden were plotted using Prism, version 5.0a. One-way ANOVA, Tukey's multiple comparison test, and student t-tests were also determined in Prism.
# Results

## Ambient PM<sub>2.5</sub> Levels

**Figure 1** shows the daily  $PM_{2.5}$  concentrations throughout the entire month of August 2013, with the highlighted portion the days that ambient wildfire smoke  $PM_{2.5}$  was collected. The graph shows a sharp increase in ambient  $PM_{2.5}$  levels following the day the wildfire was ignited (August 18, 2013) and continues elevated for approximately 10 days. The average ambient  $PM_{2.5}$  concentrations in Missoula, MT over the four days that wildfire smoke PM was collected (August 20<sup>th</sup>, 21<sup>st</sup>, 22<sup>nd</sup>, and 23<sup>rd</sup>) was 30.3 µg/m<sup>3</sup>, whereas the entire month of August 2013 had an average  $PM_{2.5}$  concentration of 13.9 µg/m<sup>3</sup>. "Typical" August  $PM_{2.5}$  concentrations range from 6-14 µg/m<sup>3</sup>.



Figure 3.1. Average 24-hour concentrations of ambient PM<sub>2.5</sub> throughout August 2013 in

Ambient PM<sub>2.5</sub> Levels Throughout August, 2013 in Missoula, MT

Missoula, MT. The days wildfire smoke was collected are highlighted in gray.

#### Source and the Pro-Inflammatory Outcome

The source of  $PM_{2.5}$  and the resulting pro-inflammatory response following THP-1 cell exposures is shown in **Figure 2**. The two graphs are showing the same results with the upper graph showing columns at each level of exposure, and the lower graph showing dose-response curves for each particle type. Post-hoc comparisons (Tukey's Multiple Comparisons Test) between source type and the pro-inflammatory outcome indicate that wood smoke from wildfires induces a significantly higher pro-inflammatory response when compared to wood smoke emissions from either the older-model (high emission) wood stove or the EPA-certified (low emission) wood stove. Wildfire smoke PM also induced significantly more IL-1 $\beta$  release compared to UPM.

As exposure to wildfire smoke PM increased a concomitant increase in the proinflammatory response was observed. There were no significant pro-inflammatory differences between SiO<sub>2</sub> and wildfire smoke PM<sub>2.5</sub> at 5  $\mu$ g/m<sup>3</sup> and 20  $\mu$ g/m<sup>3</sup>. UPM exposure outcomes followed a similar pattern as wildfire smoke PM<sub>2.5</sub> for the 0.25, 0.5, and 1.0  $\mu$ g/well points, but leveled off at the 5 and 20  $\mu$ g/well points. Overall, UPM induced IL-1 $\beta$  remained consistently lower than wildfire smoke PM<sub>2.5</sub> throughout each level of exposure. Smoke emissions harvested from both the older-model and EPAcertified wood stoves displayed an opposite effect on the pro-inflammatory outcome. As exposure to wood stove PM<sub>2.5</sub> concentrations increased (both the older-model wood stove and the EPA-certified wood stove), a resultant decrease in IL-1 $\beta$  followed. It should be noted that the observed elevated baseline of IL-1 $\beta$  (i.e. ~1000 pg/mL, levels observed in the no-particle control) are not uncommon in THP-1 cell exposure experiments. Studies with comparable methodologies measured similar levels of IL-1 $\beta$ in no-particle exposures using the same cell line (Hamilton et al., 2013a; Hamilton et al., 2014).



**Figure 3.2.** Source of  $PM_{2.5}$  and the resulting pro-inflammatory response following THP-1 cell exposures (n=3). A) Bar graph with Tukey's post-hoc comparisons (p<0.05) to no-particle control, \*. All columns were significantly different (p<0.05) from the 100 and 20 µg/well of SiO<sub>2</sub> except the 5 and 20 µg/well of wildfire PM. B) line graph.

One-way ANOVA comparing the effect of particle type showed significant differences, with the exception of the two residential wood stove particle types. P-values for the following comparisons were as follows: (a) older-model wood stove  $PM_{2.5}$  v. EPA-certified wood stove  $PM_{2.5}$ , 0.288; (b) older-model wood stove  $PM_{2.5}$  v. ambient wildfire smoke  $PM_{2.5}$ , 0.015; (c) older-model wood stove  $PM_{2.5}$  v. UPM, 0.017; (d) EPA-certified wood stove  $PM_{2.5}$  v. ambient wildfire smoke  $PM_{2.5}$  v. uPM, 0.016; and (f) ambient wildfire smoke  $PM_{2.5}$  v. UPM, 0.019, respectively. In summary, smoke emissions from residential wood stoves  $PM_{2.5}$  and UPM did.

## Cell Toxicity

**Figure 3** shows the effects of wood smoke source on THP-1 cell viability. The trends observed in cell viability outcomes partly explain the resulting pro-inflammatory response from both residential wood stove emission types observed in **Figure 2**. At the 20  $\mu$ g/well, wood stove PM<sub>2.5</sub> nears the same levels of toxicity as the SiO<sub>2</sub> controls. Importantly, at this same level, PM<sub>2.5</sub> from both wood stoves showed a negligible level of IL-1 $\beta$  released from the cells. At each of the concentrations, ambient wildfire smoke PM<sub>2.5</sub> and UPM resulted in cell viability that was significantly higher than SiO<sub>2</sub> controls.



Figure 3.3. Source of PM<sub>2.5</sub> and the resulting influence on THP-1 cell viability (n=3). A) Bar graph with Tukey's post-hoc comparisons, p<0.05 for both 20 and 100 µg/well SiO<sub>2</sub> (\*) and; p<0.05 for 100  $\mu$ g/well only (#). B) line graph.



Media only SiO2 Urban PM Wildfire PM New Stove PM

# Endotoxin Content

**Figure 4** shows the endotoxin concentrations measured from the four PM source types. As seen in **Figure 2**, the biggest inducer of the pro-inflammatory response, ambient wildfire smoke  $PM_{2.5}$ , also contained the highest concentrations of endotoxin at 208.2 EU/mg. This was followed by UPM at 95.77 EU/mg. The resulting endotoxin concentrations in  $PM_{2.5}$  harvested from both the old and EPA-certified wood stoves were 0.31 and 1.2 EU/mg, respectively. These findings suggest that endotoxin may have a role in the observed pro-inflammatory response.



**Figure 3.4.** Endotoxin concentrations measured as a function of PM<sub>2.5</sub> sources. Student \* = p < 0.01 compared to old wood stove t-te#s=wsr@perfeompsine@t0101.etw widowpstoseove comparisons, \*; and EPA-certified

wood stove comparisons, #.

#### DISCUSSION

Results from studies that Investigate how specific sources of inhalable PM induces variable health outcomes can lead to important findings that have the potential to influence future environmental regulations. In this study, we exposed macrophage differentiated THP-1 cells to four particle types: wood smoke PM<sub>2.5</sub> from three different sources (older-model high emission wood stove, EPA-certified low emission wood stove, and ambient wildfire smoke), as well as a NIST standard for UPM. Our findings showed that exposure to these different PM<sub>2.5</sub> sources resulted in various pro-inflammatory and cytotoxic effects. To our knowledge, this is the first wood smoke source investigation comparing different sources of wood smoke PM<sub>2.5</sub>, including ambient wildfire particles, and their impacts on the pro-inflammatory response in an *in vitro* macrophage cell model.

In a previous study, traffic-derived particles affected the greatest pro-inflammatory response, while residential wood stove PM emissions and diesel PM emissions induced little to no noticeable increases in inflammation in a similar THP-1 cell model (Kocbach, 2008). Our findings confirm that *in vitro* exposures to residential wood stove  $PM_{2.5}$  emissions do not induce a significant inflammatory response, as indicated by IL-1 $\beta$  levels. Furthermore, the type of wood stove (low emission v. high emission) did not make a difference in this outcome. However, wildfire smoke  $PM_{2.5}$  proved to be the most potent inducer of the pro-inflammatory response when compared to UPM and both wood stove emission sources.

In our study, both wildfire smoke  $PM_{2.5}$  and UPM had significantly higher endotoxin content than both wood stoves. The biological composition of inhaled PM is reported to be a significant causative agent in the resulting pro-inflammatory response (Kocbach,

2008; Monn and Becker, 1999; Soukup and Becker, 2001; Thorne, 2000). Biological content includes danger associated molecular patterns (DAMPS), such as bacteria or endotoxin, which increases inflammasome output and productivity (Tschopp and Schroder, 2010; Yazdi et al., 2010). This inflammasome is a protein complex that, once formed, triggers downstream cleavage of pro-IL-1 $\beta$  mediated by caspase 1. This results in the extracellular release of mature IL-1 $\beta$ , an active pro-inflammatory cytokine. Endotoxin has also been shown to trigger the NF-κB pathway and downstream pro-IL-1 $\beta$  production via TLR4 (Bauernfeind et al., 2009; Schins et al., 2004). Associative trends between endotoxin and the pro-inflammatory outcome observed in this study are very suggestive, but further investigations are needed to fully understand this relationship.

Cell viability was assessed via colorimetric method in which viable cells lead to increased catalyzation of a tetrazolium compound, MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt]) to the formazan product readily absorbed at 490 nm. The amount of this formazan product is proportional to the number of viable cells. This is likely due to a decreased formation of NADPH or NADH, which is theorized as a key component to this conversion (Berridge et al.; Berridge and Tan, 1993; Tan and Berridge, 2000). In a similar study, comparisons of wood stove technology on exposure outcomes using macrophages showed that residential wood stove PM induces significant increases in apoptosis (Jalava et al., 2012). It is unknown if the residential wood stove smoke  $PM_{2.5}$  exposures at 5 µg/well influenced the cells to undergo similar metabolic pathways. Understanding what is truly causing these effects will require deeper investigation in future studies.

#### Limitations of study

In this study, we investigated the influence of only one PM size fraction (PM<sub>2.5</sub>). Other important size fractions that could be collected and used in a comparative model include

PM<sub>1</sub> (ultrafines), PM<sub>10</sub>, and the size fraction between PM<sub>2.5</sub> and PM<sub>10</sub>, commonly referred to as the coarse fraction, or PM<sub>c</sub>. Ambient PM<sub>c</sub> levels can reach levels that are twice as high as PM<sub>2.5</sub> in summer months in western Montana, suggesting that this size fraction is an important particle size to consider when evaluating concomitant exposures to ambient PM<sub>2.5</sub> (Ferguson et al., 2013). These results were also determined in a single model of exposure. Other models include co-cultures where macrophages and epithelial tissues are exposed in a similar manner (Bølling et al, 2012). Finally, due to the lyophilization of the PM/condensate samples, it is unclear if highly-volatile chemicals were lost prior to cell exposure. Consideration of these additional factors would enhance future work.

#### Conclusion

This study evaluated the variable influence of wood smoke source on pro-inflammatory outcome and cell viability. Using macrophage-like differentiated THP-1 cells, we investigated the influence of three types of wood smoke emissions (wildfire smoke PM<sub>2.5</sub>, and PM<sub>2.5</sub> emissions harvested from both an older-model and an EPA-certified low emission stove) and UPM in side-by-side comparisons. The results of these exposures suggest that the source of wood smoke is an important predictor of exposure outcome. Our results further suggest that wildfire smoke PM<sub>2.5</sub> is a significant inducer of the pro-inflammatory response, and could have greater health impacts compared to exposures to residential wood smoke (depending on durations and magnitudes of exposure). Also, depending on susceptibility, exposures to ambient or indoor sources of wood smoke (especially during wildfire events) may cause increased inflammatory responses which can exacerbate a number of chronic health diseases (e.g., COPD and asthma).

Endotoxin content of the particles was measured from each PM type used in this study. There were associated trends between endotoxin content and the resulting proinflammatory response. Additional next-steps include utilization of multiplex assays, analyzing other DAMPS (e.g., chitin, ergosterol, etc.) as well as evaluating other chemical constituents of the particles (e.g., elemental and organic carbon, metals, PAHs, etc.) to investigate the corresponding impact of specific biogenic/chemical classes on pro-inflammatory outcomes and cell viability. Cell exposure models should also be explored for other sources of wood smoke, such as smoke emitted during a prescribed burn and agricultural waste burns (e.g. orchard waste); or even particle types that vary as a function of smoke age, or a smoldering fire versus a hot/flaming fire.

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# Chapter 4a: Lung function measures following a wildland firefighter

controlled exposure study

## ABSTRACT

Across the world, biomass smoke is a major source of air pollution, and is linked with a variety of adverse health effects. This is especially true in the western US, where wildland forest fires are becoming a more frequent and significant source of PM<sub>2.5</sub> during the summer months. Wildland firefighters are especially impacted, as they experience elevated PM<sub>2.5</sub> concentrations over extended periods of time. Many epidemiological studies have investigated how wood smoke impacts human health, and include investigating occupational field exposures experienced by wildland firefighters. As there are numerous challenges in carrying out these field studies, having the ability to research the potential health impacts to this occupational cohort in a controlled setting would provide important information that could be translated to the field setting.

To this end, we have carried out a controlled wildland firefighter exposure study in a wood smoke inhalation facility. Utilizing a randomized crossover trial design, we exposed 10 subjects once to clean filtered-air, 250 µg/m<sup>3</sup>, and 500 µg/m<sup>3</sup> wood smoke PM<sub>2.5</sub>. Subjects exercised on a treadmill at an absolute intensity designed to mimic wildland firefighting for 1.5 hours. In addition to measured PM<sub>2.5</sub> smoke concentrations, mean levels of CO<sub>2</sub>, CO, and % relative humidity for each exposure type were recorded, and were representative of occupational 'real-world' wildland firefighting exposures. Pulmonary function was measured at three time points: before, immediately after, and 1-hour post-exposure. Although there were some reductions in FVC, FEV<sub>1</sub>, and FVC:FEV<sub>1</sub> measures following exposures, results of the spirometry testing did not show significant changes in lung function. The development of this wood smoke inhalation facility will further research into acute and chronic health impacts of wood smoke exposure, and in the future provide a platform to address unique research questions related to wood smoke exposures and associated adverse health effects.

## INTRODUCTION

Air pollution has a major impact on human health throughout the world, and is a leading cause of premature mortality (Cohen et al., 2005; Pope et al., 2002; WHO, 2014). Both globally and throughout the US, urban areas with large populations often have elevated levels of air pollution, including airborne particulate matter (PM). This is also true for rural areas of the Northern Rocky Mountain region of the US, where biomass burning is a major source of elevated PM<sub>2.5</sub> concentrations throughout the year, leading to ambient concentrations comparable to those measured in more urban centers. This includes residential wood stove emissions during the winter months and wildland forest fires during the summer months.

Residential wood stoves are a common method for home heating. In the US, they are the most intensively used type of space heater, with over 11 million homes currently using wood as either a primary or secondary heating source (USDOE, 2009). Eighty percent (80%) of existing wood stoves are old and inefficient models, leading to significant impacts to ambient air quality throughout the winter months (AQMWG, 2005). Chemical Mass Balance PM<sub>2.5</sub> source apportionment modeling has suggested that residential wood stoves contribute between 56-77% of the ambient wintertime PM<sub>2.5</sub> in multiple communities throughout western Montana (Ward and Lange, 2010).

In addition to being a significant source of ambient  $PM_{2.5}$  (Larson et al., 2004; Naeher et al., 2007; Ward and Lange, 2010; Ward and Smith, 2005) wood stove use can result in elevated, sustained  $PM_{2.5}$  exposures within homes (Noonan et al., 2011; Ward et al., 2011; Ward and Noonan, 2008). Several studies have measured mean  $PM_{2.5}$  concentrations within wood burning homes ranging from 12.8 to 54.0 µg/m<sup>3</sup> (Allen et al., 2009; Noonan et al., 2012; Semmens et al., 2015; Ward et al., 2011). Many of these wood stove homes had 24-hour  $PM_{2.5}$  average concentrations that exceeded not only the World Health Organization 24-hour air quality guideline of 25 µg/m<sup>3</sup>, but also the current

Environmental Protection Agency (EPA)  $PM_{2.5}$  24-hour National Ambient Air Quality Standard (NAAQS) of 35 µg/m<sup>3</sup> (Noonan et al., 2012). Although there are currently no indoor  $PM_{2.5}$  standards in the US, these indoor exposures are of particular concern as people spend most of their time indoors (Jenkins et al., 1992).

Globally, smoke from wildland fires has been attributed to 339,000 deaths a year (Johnston et al., 2012). Due to a changing climate, wildland fires are predicted to continue or worsen in many regions throughout the world (Mckenzie et al., 2014). Each year, there are more than 100 thousand wildland fires in the US, burning between 3 million and 10 million acres of land depending on year. They are most common in the western US where heat, drought, and frequent thunderstorms lead to ideal wildland fire conditions (Westerling et al., 2014). During these wildland fire events, significant levels of wood smoke PM<sub>2.5</sub> can be emitted, impacting ambient air quality in communities thousands of kilometers downwind (Le et al., 2014). Forest fire smoke can also impact indoor air quality when PM infiltrates homes, resulting in PM concentrations similar to those observed outside (Barn et al., 2008; Henderson et al., 2005; Phuleria et al., 2005; Sapkota et al., 2005).

With predicted increases in forest fires, the number of wildland fire fighter crews deployed to fight these fires will also increase. Firefighters can experience smoke PM levels up to 2,930  $\mu$ g/m<sup>3</sup>, with average levels of exposure during wildland fire fighting activities ranging from 509-558  $\mu$ g/m<sup>3</sup>, and average CO levels of exposure ranging from 1.3-1.7 ppm (Adetona et al., 2013). When working on project fires or prescribed burns, firefighters can experience average concentrations ranging from 500-630  $\mu$ g/m<sup>3</sup> throughout an entire work shift (Reinhardt and Ottmar, 2004). Related health studies conducted in the field have found an overall general decrease in lung function following wildland fire fighting activities (Betchley et al., 1997; Liu et al., 1992; Rothman et al., 1991).

Given the many sources of biomass smoke exposures leading to a variety of exposure scenarios, there is a need to study the health effects following smoke exposures in a controlled facility. This is especially important to wildland firefighters, as they are exposed to elevated PM<sub>2.5</sub> wood smoke concentrations for extended periods of time. To this end, the objective of this study was to deliver wood smoke PM<sub>2.5</sub> exposures to 10 human subjects in an effort to investigate the potential respiratory impacts following wood smoke exposures typically experienced by wildland firefighters. Below we describe the design and methods utilized in carrying out the exposure trials, and present exposure and lung function results.

## METHODS

The Inhalation and Pulmonary Physiology Core within the Center for Environmental Health Sciences at the University of Montana was originally developed to conduct wood smoke exposure trials using mice, but was modified in this application to conduct human exposure trials. In the study described here, a 10-subject pilot project was conducted to simulate occupational wood smoke exposures encountered by wildland firefighters when fighting wildfires. Following recruitment into the study and initial entry level measurements (Day 1), 10 subjects participated in three experimental trials, each one occurring one week apart (i.e., Day 2, Day 3, and Day 4). Each subject was blinded to his exposure assignment and was exposed, while exercising, once to either clean filtered-air (0 µg/m<sup>3</sup>), 250 µg/m<sup>3</sup>, or 500 µg/m<sup>3</sup> wood smoke PM<sub>2.5</sub>, in random order for 1.5 hours. Throughout each exposure, PM<sub>2.5</sub>, CO, CO<sub>2</sub>, and % relative humidity levels were continuously monitored. Spirometry measures were collected prior to, immediately after, and 1-hour post each exposure.

# Inhalation Facility and Exposure Levels

Wood smoke for these exposure trials was generated using an older-model wood stove (Englander, England Stove Works, Inc., Monroe, VA) and routed through dilution chambers before ultimate delivery to the subject through a breathing mask. The wood used in this study was cured (~15% moisture content) western larch (*Larix occidentalis* Nutt.), which is a common species in western Montana. The technique for building and maintaining the fire was uniform throughout each exposure. Prior to each exposure trial, some ash from previous fires was removed to ensure a constant layer of ash (~0.5-1" deep). Each fire started with about 1 kg of wood as well as kindling (1-2 pages of newspaper). About 300 g of wood was then added every 15-20 minutes over a two-hour period, with each fire started 25-30 minutes prior to each exposure trial.

During each exposure trial, smoke pulled from the wood stove chimney was routed through dilution chambers where filtered air (Cambridge Absolute Filter, Cambridge Filter Corp., Syracuse NY) was introduced in an effort to dilute the smoke (Figure 4.1.). Wood smoke was then delivered from the dilution and mixing chambers to the subject via a modified mask respirator (Hans Rudolph, Inc., Shawnee, Kansas). The major pump pulling air from the wood stove chimney and through the dilution chambers was placed in line between the chambers and the mask. This allowed air to be 'pushed' through the mask at rates (~90-100 L/min) appropriate for an individual to comfortably breath while exercising on a treadmill.



Figure 4.1. A simplified schematic showing the path of wood smoke through the

inhalation system. Ambient air is pushed through the air filter prior to entering the dilution chamber.

En route to the mask and following the pump, wood smoke PM first passed through 2.5 feet of flex tubing before coming to a T-valve that directed the wood smoke PM to both the mask and to a fume hood where excess wood smoke PM was exhausted. Tubing to the mask included 108" of Clean-Bor tubing (VacuMed, Ventura, CA) made of ethylene vinyl acetate. The mask utilized was a Rudolph Nasal & Mouth Breathing Face Mask with a two-way non-rebreathing T-valve. Another hose exited the mask and was directed to the fume hood for exhaust.

The exposure room  $(11'10" \times 5'10" \times 8')$  contained a treadmill (Model Q65, Quinton Instrument Company, Bothell, WA) attached to a control station (Model Q4000, Quinton Instrument Company, Bothell, WA), and other items intended for subject comfort. This included a fan, which improved air circulation in the room. A stand within arm's reach, and at eye level, was also placed in front of the treadmill providing a platform for the subject to set a magazine, book, tablet, or phone. If desired, subjects were also allowed to listen to music throughout their exposure trial. For comfort, the mask and tubes were suspended from the ceiling by adjustable straps. This allowed the mask to be placed at an appropriate height, reducing the burden of mask and tubing weight on the subject's head, and allowed the mask to move and shift with the subject while they were exercising on the treadmill.

Two PM<sub>2.5</sub> monitors (DustTrak, TSI, Model 8530 and Model 8534, Shoreview, MN) were used during the exposures to measure continuous readings of real-time and average PM<sub>2.5</sub> concentrations directly routed to the mask (**see Figure 4.1.**). The first DustTrak (Model 8534) was used to adjust wood smoke PM<sub>2.5</sub> concentrations delivered through the dilution chambers. The second DustTrak (Model 8530) measured continuous PM<sub>2.5</sub> concentrations delivered to the mask just prior to inhalation. All PM<sub>2.5</sub> concentrations reported in this manuscript were obtained from this second DustTrak.

Carbon monoxide (CO), carbon dioxide (CO<sub>2</sub>), and % relative humidity at the mask were also monitored with a Q-Trak (TSI, Model 7565, Shoreview, MN) and collocated to the second DustTrak. This CO measurement was especially important to ensure low levels of CO during each exposure trial.

#### **Inclusion Criteria and Recruitment**

Subjects participating in this study included 10 healthy, non-smoking males, aged 18-40 years, with no pre-existing chronic lung diseases. Subjects did not have wood smoke exposures at home or work (via cigarettes or wood stoves), and had to complete a moderate physical exercise protocol three times during the study. Due to the small size of this pilot study, and to remove the potentially confounding impact of gender on findings, only males were included. Additional inclusion criteria described in more detail below under "Day 1" included answering 'No' to all questions on a Physical Activity Readiness Questionnaire (PARQ), as well as having a VO<sub>2</sub> max > 40 ml/kg/min.

Following study approval from the University of Montana's Institutional Review Board, subjects were recruited from the University of Montana student, faculty, and staff population. Flyers were posted throughout the campus. Upon the initial meeting with subjects, enrolled volunteers were administered oral and written informed consent, and then scheduled for Day 1 measures. Participants received a stipend upon completion of each of the three exposure trials (Days 2-4, respectively).

#### Day 1

Day 1 of the study was used to determine eligibility for the Days 2-4 exposure trials, with inclusion/exclusion criteria intended to reduce the risk of adverse response occurrences throughout each exposure. Participants were reminded to fast for three hours before presenting to reduce impact of meals on outcomes. They were then asked

to complete a personal information questionnaire and PARQ, and undergo a test to verify their maximum level of oxygen uptake (VO<sub>2</sub> max) was greater than 40 ml/kg/min. The percentage of body fat for each subject was also determined via an underwater weighing test. Personal information collected included age, height, weight, percentage of body fat, VO<sub>2</sub> max, and illnesses and medications taken during the study period. If subjects met all the inclusion criteria, scheduling was initiated for Days 2, 3, and 4. The entire process for Day 1 took approximately 1.25 hours/subject.

# Day 2-4 Exposure Trials

Following the Day 1 evaluations, subjects participated in three exposure trials, each one occurring one-week apart (i.e., Day 2, Day 3, and Day 4). During the study, each subject was exposed once to either clean filtered air,  $250 \ \mu g/m^3$ , or  $500 \ \mu g/m^3$  wood smoke PM<sub>2.5</sub> in a double-blind randomized crossover design. During smoke exposure, subjects were asked to walk on a treadmill at a set rate and incline (3.5 mph and 5.7% grade) for 1.5 hours to simulate working on a fireline, with a short (e.g., 20-30 seconds) break every 15 minutes to evaluate perceived stress and drink a predetermined amount of water. A researcher was constantly monitoring both the CO and PM<sub>2.5</sub> concentrations to the mask and signs of subject discomfort at all times during the exposure trials. Each of the three experimental trials took approximately 3 hours.

# **Pulmonary Function**

Spirometry is the most widely used assessment of pulmonary function for diagnosis and prognosis of pulmonary status and disease, including chronic obstructive pulmonary disease (COPD) and other restrictive diseases (Buffels et al., 2004; Celli, 2000; Halbert et al., 2006; Nowak et al., 1979). Evaluated spirometry measures used in this study include the volume of exhaled breath during the first second of forced expiratory air

following maximum inhalation (FEV<sub>1</sub>), the vital capacity (FVC; the maximum volume of air forced out of the lungs following maximum inhalation), and the ratio FEV<sub>1</sub>/FVC (also known as the Tiffeneau-Pinelli index). For each of the three trials, spirometry measurements were collected from the subjects before, immediately post-exposure, and 1-hour after each exposure. Each assessment was conducted by having the participant blow air rapidly and forcefully into the mouthpiece of a Koko Legend Spirometer (Ferraris Respiratory, Louisville, CO). To ensure accurate and reliable results during the pulmonary function test, a strict protocol was followed that included both specific subject instructions as well as quality control measures.

# **Quality Assurance / Quality Control**

The DustTrak was zero calibrated prior to each exposure trial. The tubes connecting the flex tubing to the mask were replaced following each exposure trial, and the mask was thoroughly cleansed after each exposure. This was done by disassembling the mask, with each part thoroughly washed in warm water with mild detergent. This ensured that subjects were equipped with a clean mask at the start of each exposure. The building of each fire was consistent using standardized procedures (e.g., starting mass, stoking mass, etc.). To further reduce source variability, the same people conducted the fire loading and stoking throughout the entire study.

#### **Data Analysis**

Each subject was randomly assigned an identification number at the start of the study, with all samples, questionnaire responses, physiological measurements, and other data collection forms labeled with this number. We define exposure as 1) filtered-air, 2) 250  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>2.5</sub>, or 3) 500  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>2.5</sub>. Due to the small sample size for this pilot project, we utilized Friedman's test, the nonparametric

analog to a repeated measures ANOVA, to evaluate if observed pre- to post-exposure changes in lung function differed significantly by wood smoke exposure condition. Comparisons were also made using Dunnett's multiple comparison tests. These tests were performed using Excel and Prism (GraphPad, v.5.0a).

## RESULTS

Environmental conditions and spirometry results are reported below. Also, due to fatigue in one subject and another subject dropping out before their final exposure, two subjects did not complete all spirometry measures.

## Day 1 Measures

Participating subjects had an average age of 26.4 ( $\pm$  3.7). Average height (in inches) of all subjects was 70.13 ( $\pm$  3.1). The body weight (kg) and percent body fat outcomes were 79.03 ( $\pm$  12.2) and 14.16 ( $\pm$  2.6), respectively. All subjects showed acceptable VO<sub>2</sub> max levels (ml/kg/min) following Day 1 test measures at 53.53 ( $\pm$  7.2). No illnesses or medications were reported prior to Day 1 measures.

#### **Exposure Concentrations**

We were able to successfully deliver consistent, reproducible exposures in the wood smoke inhalation facility. **Figure 4.2.** presents an example of a subject's delivered smoke PM<sub>2.5</sub> concentrations at the mask throughout the 250  $\mu$ g/m<sup>3</sup> and 500  $\mu$ g/m<sup>3</sup> exposure trials. Across all trials, the average measured concentrations of PM<sub>2.5</sub> from filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> exposures were 5.2 (±4.9)  $\mu$ g/m<sup>3</sup>, 253.9 (± 5.8)  $\mu$ g/m<sup>3</sup>, and 506.2 (± 4.8)  $\mu$ g/m<sup>3</sup>, respectively. Greater than 99% of PM<sub>2.5</sub> mass measured in the dilution chamber was in the PM<sub>1</sub> fraction (as measured by the DustTrak Model 8534, data not shown). The average levels of CO from all filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> exposures were 0.003 (± 0.007) ppm, 0.87 (± 0.28) ppm, and 1.87 (± 0.65) ppm, respectively. Mean levels of CO<sub>2</sub> from all filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> exposures were 443 (± 22) ppm, 464 (± 28) ppm, and 482 (± 21) ppm, respectively. From all filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> exposures, relative humidity was 14.1 (± 8.4) %, 12.1 (± 5.3) %, and 13.1 (± 2.7) %, respectively.



**Figure 4.2.** Continuous  $PM_{2.5}$  concentrations and averages plotted for a 250 µg/m<sup>3</sup> (top) and 500 µg/m<sup>3</sup> (bottom).

# Lung Function

As presented in **Table 4.1.**, calculated "change from pre-exposure values" included normalizing each subject's post- and 1-hour post-exposure spirometry values (FVC, FEV<sub>1</sub>, and FVC:FEV<sub>1</sub>) to their perspective pre-exposure levels, for each of the three exposures. This included subtracting pre-exposure values from post- and 1-hour postexposure, for each individual exposure. This normalization decreases within-subject dayto-day variation, as well as between-subject variation.

Overall, spirometry results showed no significant changes following wood smoke exposures **(Table 4.1.)**. The mean pre-exposure FVC results ranged from 5.41 (0.53) - 5.61 (0.93) liters. There was no impairment in lung function measured at the post-exposure time point, but there were slight reductions in FVC for the 250  $\mu$ g/m<sup>3</sup> and 500  $\mu$ g/m<sup>3</sup> exposures at the 1-hour post time point (-0.07 and -0.04 liters, respectively). This same trend is observed in the FEV<sub>1</sub> measures. Pre-exposure baseline FEV<sub>1</sub> measures ranged from 4.37 (0.34) - 4.55 (0.64) liters. Following the exposures, there was a post-exposure reduction at the 250  $\mu$ g/m<sup>3</sup> trial (-0.19 liters), and reductions of -0.27 liters (250  $\mu$ g/m<sup>3</sup>) and -0.05 liters (500  $\mu$ g/m<sup>3</sup>) measured in the 1-hour post exposure spirometry tests. Consistent with the FEV<sub>1</sub> measures, we saw insignificant reductions in the ratio of FVC:FEV<sub>1</sub> post-exposure at the 250  $\mu$ g/m<sup>3</sup> trial (-3.40), and insignificant reductions in 1-house post exposure measures for 250  $\mu$ g/m<sup>3</sup> (-3.02) and 500  $\mu$ g/m<sup>3</sup> (-0.29) exposure trials.

		Pr	e-exposi	ure (n=9	<u> </u>			Pos	st-expos	ure (n=	6			1-hr P	ost-exp	osure (n	=10)	
	Filter	ed-air	250 µ	g/m <sup>3</sup>	500 µ	ıg/m³	Filtere	d-air	250 µ	g/m <sup>3</sup>	500 p	g/m <sup>3</sup>	Filtere	d-air	250 µ	ıg/m <sup>3</sup>	500 µ	g/m <sup>3</sup>
Measurement category	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)	Mean	(ps)
FVC																		
Unadjusted values (liters)	5.41	(0.53)	5.58	(0.82)	5.61	(0.93)	5.37	(0.61)	5.62	(0.79)	5.69	(0.96)	5.37	(0.46)	5.51	(0.77)	5.57	(0.94)
change from pre-exposure levels <sup><math>A</math></sup>							-0.04	(0.30)	0.04	(0.21)	0.08	(0.28)	0.00	(0.33)	-0.07	(0.27)	-0.04	(0.21)
FEV,																		
Unadjusted values (liters)	4.37	(0.34)	4.55	(0.64)	4.44	(0.67)	4.41	(0.46)	4.35	(06.0)	4.64	(0.64)	4.42	(0.42)	4.27	(0.89)	4.39	(0.62)
change from pre-exposure levels <sup><math>A</math></sup>							0.04	(0.31)	-0.19	(1.00)	0.20	(0.36)	0.05	(0.48)	-0.27	(1.03)	-0.05	(0.33)
FVC:FEV <sub>1</sub>																		
Unadjusted values (ratio)	81.11	(6.34)	81.26	(6.44)	79.72	(8.10)	82.37	(6.97)	77.86	(14.0)	81.85	(7.29)	82.89	(06.7)	78.24	(15.3)	79.43	(8.54)
change from pre-exposure levels <sup><math>\Lambda</math></sup>							1.26	(2.39)	-3.40	(14.2)	2.13	(3.33)	1.78	(5.66)	-3.02	(15.5)	-0.29	(4.32)
<sup>A</sup> Estimated changes from pre-exposure to immediate	bost-exposni	e and chai	ige from pi	.e-exbosnu	e to 1-hou	r post-expc	sure.											

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pirometry (	
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Table 4.1. l	

## DISCUSSION

One goal of this pilot project was to deliver specific concentrations of PM<sub>2.5</sub> wood smoke that mimicked occupational exposures encountered by wildland firefighters. As demonstrated in Figure 4.2., the measured concentrations of PM<sub>2.5</sub> were consistent with (and representative of) field research studies where PM levels (and CO concentrations) were recorded during wildland firefighting activities (Adetona et al., 2013). Importantly, our exposure concentrations are also representative of exposures encountered in other settings, providing future opportunities to investigate other exposure scenarios. The lower concentration of wood smoke  $PM_{25}$  exposure in this study (250  $\mu$ g/m<sup>3</sup>) is comparable to concentrations recorded when biomass is burned for cooking or heating purposes in homes without ventilation (Dills et al., 2006), and consistent with concentrations used in other European human/biomass smoke exposure studies (Barregard et al., 2008; Barregard et al., 2006; Danielsen et al., 2008; Forchhammer et al., 2012; Riddervold et al., 2011; Sällsten et al., 2006; Sehlstedt et al., 2010; Stockfelt et al., 2012). The higher level of exposure (500  $\mu$ g/m<sup>3</sup>) is comparable to human exposure studies conducted by EPA's Ghio et al. (Ghio et al., 2012), where subjects were exposed to an average concentration of 485 µg/m<sup>3</sup> over a 2-hour period. Similarly, occupational studies have reported average wood smoke PM exposures (i.e., wildland fire firefighters) in the range of 500-800  $\mu$ g/m<sup>3</sup> (Adetona et al., 2013; Reinhardt and Ottmar, 2004; Slaughter et al., 2004). These same studies reported average CO and CO<sub>2</sub> levels ranging about 1-7 ppm and 400-500 ppm, respectively. While the source of smoke is stove-derived, it may be more appropriate for this model as wildland firefighters are exposed to fresh smoke, not aged smoke (with atmospheric and UV influences).

#### Health Effects Associated with Wood Smoke Exposure

Most of the current knowledge regarding the adverse health effects (both acute and chronic detriments) following wood smoke exposures have come from epidemiological studies. Ambient wildland fire PM levels exceeding 40 µg/m<sup>3</sup>, relative to concentrations less than 10 µg/m<sup>3</sup> are associated with more than a doubling of observed asthmatic presentations (Johnston et al., 2002). Other observations following similar events included increased risk of allergic respiratory disease, as well as bronchial asthma, exacerbation of type II diabetes (Filho et al., 2008) and cardiovascular disease (Mott et al.; Park et al., 2010). Significant decreases in lung function were reported in several studies following occupational exposures (e.g. wildland firefighting) to wood smoke PM (Betchley et al., 1997; Slaughter et al., 2004; Tepper et al., 1991). Additional studies have shown a general increase in emergency room and outpatient visits during and following smoke events (Chen et al., 2006; DuClos et al., 1990; Emmanuel, 2000; Kunzli et al., 2005; Mott et al., 2005).

#### Human Exposure Wood Smoke Studies

The limited number of studies involving human exposures to wood smoke PM in controlled environments show varying results. **Table 4.2.** presents a summary of human wood smoke studies that have been conducted in a variety of settings. Throughout the literature, PM levels in the human exposure studies ranged from around 150 to 1000  $\mu$ g/m<sup>3</sup>, with durations of exposures from 1 to 4 hours. Studies reported a varying degree of physical activity throughout the trials, from sedentary 3-hour exposures (Stockfelt et al.) to riding an exercise bike at light effort (~70 W) for two 25-minute periods during a 4-hour exposure [PM<sub>2.5</sub> concentrations 243-279  $\mu$ g/m<sup>3</sup> (Barregard et al., 2006)]. The majority of the parameters (e.g., PM concentrations, duration of exposure, etc.) used in the present pilot study were within the range of those outlined in **Table 4.2.** The route of

exposure (using a mask to deliver the exposure) and the exercise component (briskly walking on a treadmill at a set rate and incline (3.5 mph and 5.7% grade) for 1.5 hours) are notable differences between our study and those summarized in **Table 4.2**.

Spirometry results from our study are consistent with previous studies (Ghio et al., 2012; Riddervold et al., 2012; Sehlstedt et al., 2010) where, in healthy individuals, no significant changes in lung function were observed following controlled acute wood smoke exposures. Previous field studies investigating the influence of wildland fire smoke on wildland firefighter lung function, on the other hand, have shown significant effects from smoke inhalation (Betchley et al., 1997; Liu et al., 1992). Liu et al. (1992) gathered spirometry data from sixty-three "seasoned" firefighters before and after a full season of fighting wildland fires. Significant declines in mean FVC and FEV<sub>1</sub> values were observed post-season (0.09 and 0.15 L/s, respectively). In a comparable study, Betchley et al. (Betchley et al., 1997) observed similar declines in FVC and FEV<sub>1</sub> in a cohort of seventy-six volunteers following a full season of fighting wildland fires. Compared to our study conducted in a controlled environment, these field studies had much longer exposure durations and wood smoke concentrations, with subjects also having elevated ventilation throughout the entirety of exposure.
References	Ν	N Exercise		Wood smoke exposure concentration(s)
Hunter et al. 2014	16 adult males	bike every 15 minutes	1-hour	filtered-air and $\sim 1 \text{ mg/m}^3$
Bønløkke et al. 2014	24 adult males and females	at rest	3.5 hours	filtered-air (13), 222, and 385 $\mu$ g/m <sup>3</sup>
Unosson et al. 2013	14 adult males and females	bike every 15 minutes	3 hours	filtered-air and 214 $\mu g/m^3$
Stockfelt et al. 2013	16 adult males and females	at rest	3 hours	filtered-air, 146, and 295 $\mu$ g/m <sup>3</sup>
Ghio et al. 2012	10 healthy individuals <sup>4</sup>	bike every 15 minutes	2 hours	filtered-air and 485 $\mu$ g/m <sup>3</sup>
Stockfelt et al. 2012	16 adult males and females	at rest	3 hours	filtered-air, 146, and 295 $\mu$ g/m <sup>3</sup>
Forchhammer et al. 2012	20 adult males and females	at rest	3 hours	filtered-air (14), 220, and 354 $\mu$ g/m <sup>3</sup>
Riddervold et al. 2012	20 adult males and females	at rest	3.5 hours	filtered-air, 200, and 400 $\mu$ g/m <sup>3</sup>
Riddervold et al. 2011	20 adult males and females	at rest	3.5 hours	filtered-air, 200, and 400 $\mu$ g/m <sup>3</sup>
Sehlstedt et al. 2010	19 adult males and females	bike every 15 minutes	3 hours	filtered-air and 224 $\mu g/m^3$
Danielsen et al. 2008	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 $\mu$ g/m <sup>3</sup>
Barregard et al. 2008	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 $\mu$ g/m <sup>3</sup>
Sällsten et al. 2006	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 250 $\mu$ g/m <sup>3</sup>
Barregard et al. 2006	13 adult males and females	25 minute bike ride, 2x	4 hours	filtered-air and 243-279 µg/m <sup>3</sup>

Table 4.2.	Summary of Controlled Human Exposure Studies.

<sup>4</sup>Gender details of participating individuals not outlined in manuscript

#### Study Limitations and Next Steps

The results of both epidemiological studies and the controlled human studies presented in **Table 4.2.** have demonstrated that there are a variety of adverse health effects following exposure to wood smoke. Importantly, these studies have reported conflicting results and outcomes. Several of these studies found no significant proinflammatory responses (Forchhammer et al., 2012; Sehlstedt et al., 2010; Stockfelt et al., 2013), whereas increases were observed in others (Ghio et al., 2012; Riddervold et al., 2012; Sällsten et al., 2006). Differences such as exposure levels, durations of exposure, varying physical activities, even biological media type (e.g. blood, EBC, etc.) and time of sample collection can partly explain these disparities. Also, the different lung function outcomes between short duration controlled exposures versus those following chronic exposures (i.e. wildland firefighters) suggest a possible role in exposure durations and recurrences. These outcomes should be considered in subsequent research trials.

As we designed this study to mimic exposures and environmental conditions experienced by wildland firefighters, exercise during the exposure trials was an important component of this study. As presented in **Table 4.2.**, about half of the aforementioned controlled human wood smoke exposure studies did not participate in an activity that might increase breathing and heart rate. In the studies where exercise was included, it was generally intermittent and non-strenuous. Importantly, the majority of observed effects occurred in subjects that exercised intermittently during exposures. Also, due to the small size of this pilot study, only males were included. However, about 12%-16% of the wildland firefighter community is female (NWFF, 2010). We intend to incorporate both genders in future occupational studies.

#### CONCLUSION

Globally, it is estimated that about half of the world's households continue to cook with solid fuels. The burning of these fuels around the world are estimated to contribute up to 3.5 million deaths and 4.5% disability-adjusted life-years (Lim et al., 2012). Wood stoves are the largest source of ambient PM<sub>2.5</sub> during the winter months in many northern Rocky Mountain communities (Ward and Lange, 2010), and they can also be a major source of indoor PM<sub>2.5</sub> (Noonan et al., 2011). As rising temperatures and shrinking snow pack have both been impacted by climate change, it is hypothesized that the frequency, magnitude, and intensity of wildland fires will increase during future summers. In a 2008 commissioned report, it was concluded that "the most important research question with respect to wildland fire particle emissions is the relationship between emission, acute and chronic exposure, and health effects" (Austin, 2008). All of these factors point to more research needed for wildland firefighters who are exposed to wood smoke PM<sub>2.5</sub> during occupational activities, members of the public who are exposed to wildland and prescribed fire smoke in downwind populations, and the public who are exposed to wood smoke each winter during the common use of residential wood stoves.

Given the complexities (and dangers) of studying wood smoke exposures/health effects during actual wildland fire scenarios, our inhalational facility is novel in that it provides an opportunity to investigate human health effects following exposures to a range of relevant wood smoke PM concentrations during physical stress (including increased breathing rate) that mimics absolute intensity of a wildland firefighter. While focusing on firefighting activities, this study will also provide meaningful data on how wood smoke PM exposures might influence general and susceptible populations. In summary, this pilot study offers a unique method for delivering wood smoke PM at specific concentrations in a closed system. Controlling the physical exertion of our

subjects provides another innovative aspect of this study. The fire type, fuel type, mixing of filtered air with smoke effluent, and having direct control of those levels entering the mask provides a unique tool to answer important questions regarding human health impacts from wood smoke exposures. Future directions include evaluating systemic and pulmonary effects from these exposures, and investigating inflammatory outcomes and oxidative stress, including biomarkers of cardiovascular disease risk.

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# Chapter 4b: Measured pulmonary and systemic markers of inflammation and

oxidative stress following wildland firefighter simulations

# ABSTRACT

To quantify the impact of forest fire smoke on human health, studies performed in a wellcontrolled environment are needed. To this end, we recruited 10 healthy participants to be exposed to two different doses of wood smoke while exercising on a treadmill. Exercise workloads were 3.5 mph at 5.7% grade for 90 minutes for each of the exposure trials (filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>2.5</sub>). Exhaled breath condensate (EBC) and blood plasma samples were obtained pre-exposure, immediately post-exposure, and 1-hour post-exposure. Measures of 8-isoprostane, pH, and myeloperoxidase were measured in EBC while H<sub>2</sub>O<sub>2</sub>, surfactant protein D, and pentraxin-3 (PTX3) were measured in both EBC and plasma. Significant changes in pH, 8-isoprostane, and PTX3 were observed as a function of wood smoke exposures. Measures of PTX3 suggest a promising role as an acute phase protein that may be particularly sensitive to wood smoke inhalation.

#### INTRODUCTION

Exposures to fine particulate matter (PM<sub>2.5</sub>) are linked to increased morbidity and mortality in a dose-dependent fashion (Chambliss et al., 2014; Dockery et al., 1993; Lim et al., 2012). There are many anthropogenic sources of PM<sub>2.5</sub>, but a major natural (and sometimes human caused) source in the western half of the US is wildland forest fires (Niemi et al., 2005). Wildfire smoke can impact downwind populations in addition to impacting those that fight fires directly. In 2014, the US had 63,612 wildfires and 17,044 prescribed burns with a combined burned area of about 6 million acres, leading the US Department of Interior and the US Forest Service to spend approximately \$1.5 billion on suppression efforts (National Interagency Fire Center, 2014). As predicted, wildland fires in these regions have increased in both frequency and intensity in recent years (Bachelet et al., 2003; Westerling et al., 2006). In response, the US Bureau of Labor Statistics estimates that the firefighter employment numbers will continue to increase in the years to come (Bureau of Labor Statistics, 2014).

Average firefighter dose exposures during wildfire and prescribed burn activities typically range from 500-600 µg/m<sup>3</sup> in a single work-day (Adetona et al., 2013; Reinhardt and Ottmar, 2004). Importantly, acute smoke exposures can exceed 6,000 µg/m<sup>3</sup> (Reinhardt and Ottmar, 2004). While the medical impact of wood smoke exposure is poorly quantified to date, preliminary findings from foundational field studies reveal that these exposures are associated with diminished lung function performance (Adetona et al., 2011; Betchley et al., 1997; Liu et al., 1992; Slaughter et al., 2004), mucosa irritation (Rothman et al., 1991), pulmonary inflammation (Gaughan et al., 2008) and systemic inflammation (Dorman and Ritz 2014; Hejl et al., 2012; Swiston et al., 2008). Chronic exposure may contribute to an increased incidence of chronic obstructive pulmonary disease (COPD) and asthma exacerbations (Henderson and Johnston, 2012).

In an effort to limit occupational exposures, it is important to understand more precisely the PM<sub>2.5</sub> smoke exposures that wildland firefighters are being subjected to during actual wildfire events. One challenge is to identify biomarkers that are sensitive to acute smoke exposure, but could also be easily collected in future field studies without an extensive array of lab equipment and supplies. Accordingly, an alternative approach is to conduct occupational exposure studies in a controlled laboratory setting. Fundamental to these laboratory investigations is to use physiologically relevant doses of smoke while also identifying which biomarkers are of the highest utility in subsequent field tests.

Some studies have shown that wood smoke exposure induces systemic inflammation and oxidative stress (Sällsten et al., 2006). Inactive inflammatory cells reside in the pulmonary vasculature and are likely to be mobilized following a significant challenge of the air lung-interface. Accordingly, examination of respiratory-derived inflammatory markers would be essential in better quantifying the stress of wood smoke exposure. However, results from prior laboratory-based studies have yielded variable results related to inflammation and/or oxidative stress, (Barregard et al., 2008, Ghio et al., 2012).

One methodological approach to investigate inflammatory and oxidative stress biomarkers from biological samples, including exhaled breath condensate (EBC) and corresponding markers from the blood plasma. EBC levels of inflammation and oxidative stress that are sensitive to physiologic stressors (including smoke), include pH, nitrite, H<sub>2</sub>O<sub>2</sub>, 8-isoprostane, and a variety of cytokines (Formanek et al., 2002; Hunt et al., 2000; Jobsis et al., 1998; Riddervold et al., 2012; Shahid et al., 2002; Vaughan et al., 2003; Zanconato et al., 2004). Less understood is how some emerging markers in

plasma function, including pentraxin-3 (PTX3; a pattern recognition receptor in the pentraxin superfamily) or their modulation following inflammatory stimuli (Du Clos, 2013; Jaillon et al., 2014). In other studies, measures of 8-isoprostane and  $H_2O_2$  have been shown to change in EBC or plasma as a function of oxidative stress (Basu, 1998; Baynes, 1991; Horvath et al., 2005; Janssen, 2001).

The purpose of this study was to mimic, as closely as possible, occupational conditions encountered by wildland firefighters and to determine the effects of increasing wood smoke exposures. These include assessment of inflammatory and oxidative stress in both EBC and plasma. Key dependent biomarkers include 8-isoprostane, myeloperoxidase and pH from EBC samples, and a direct comparison of  $H_2O_2$ , PTX3, and surfactant protein D (SP-D) concentrations from both EBC and plasma samples.

## METHODS

The Center for Environmental Health Science's Inhalational and Pulmonary Physiology Core at the University of Montana was utilized for the controlled wood smoke human exposure studies. Ten healthy males were exposed to three different conditions, including (1) filtered-air, (2) 250 µg/m<sup>3</sup> wood smoke, and (3) 500 µg/m<sup>3</sup>. Each wood smoke exposure trial was 90 minutes, occurring every 7 days over three consecutive weeks. For each participant, the exposure trials were assigned using a randomized cross-over design. Participants exercised at an absolute intensity (3.5 mph at 5.7 %) designed to mimic wildland firefighting activities for the duration of exposure. Blood and EBC were collected pre-exposure, immediately post-exposure, and 1-hour postexposure for each of the 10 participants. Further details concerning experimental design and a full description of the inhalational facility is outlined in Ferguson et al. (2015a).

*Participants.* Inclusion criteria included a  $VO_2 \max \ge 40 \text{ mg/kg/min}$ , be a nonsmoker, and have no current wood smoke exposure within their homes (e.g., wood stoves). Prior to data collection this project was approved by the University of Montana's Institutional Review Board (IRB, #22-14) and informed consent was obtained prior to participant testing.

*Exposure facility.* Wood smoke was generated in a wood stove (Englander, England Stove Works, Inc., Monroe, VA). The wood species used in this study was western larch (*Larix occidentalis* Nutt.) with a moisture content of 15%. During each exposure trial, smoke was delivered through a mixing and dilution chamber prior to reaching a nasal and mouth breathing facemask (Hans Rudolph, Inc., 8900 Series, Shawnee, Kansas) worn by the participant and equipped with a two-way non-rebreathing T-valve (Hans

Rudolph, Inc., T-Shape<sup>™</sup> Valve, Shawnee, Kansas). All exposures occurred while exercising (3.5 mph at 5.7%) on a treadmill (Model Q65, Quinton Instrument Company, Bothell, WA). PM<sub>2.5</sub> concentrations were measured at the mask using a continuous PM<sub>2.5</sub> monitor (DustTrak, TSI, Model 8530, Shoreview, MN). Another continuous PM<sub>2.5</sub> monitor (DustTrak TSI, Model 8533, Shoreview, MN), located at the dilution chamber, simultaneously measured PM<sub>2.5</sub> and PM<sub>1</sub>. Throughout exposures, carbon monoxide (CO) was also continuously monitored and recorded at the mask using a Q-Trak (TSI, Model 7565, Shoreview, MN).

*Biological sampling*. Blood samples and EBC were collected before the exposure trials (pre-exposure), immediate post-exposure, and 1-hour post-exposure. EBC was collected using an R-tube breath condensate collector (Respiratory Research Inc., Charlottesville, VA) consisting of a pre-cooled aluminum sleeve placed over the outside of a disposable vertical condensation/collection tube and connected to a two-way non-rebreathing valve and a mouthpiece. Participants breathed into the tube for 10 minutes while holding the tube in an upright position. Blood samples were collected simultaneous to EBC. Whole blood was obtained by venipuncture in the antecubital space and collected into a sodium heparin vacutainer (Becton Dickinson, Franklin Lakes, NJ). Whole blood samples were centrifuged at 6000g 4°C for 10 minutes to yield plasma. Plasma and EBC samples were then aliquoted and stored at -30° and -80° C, respectively until analysis.

*EBC measures.* A micro pH electrode (Lazar Research Laboratories Inc., model PHR-146B, Los Angeles, CA) coupled to a digital pH meter (Jenco, Model 60, San Diego, CA) was used to measure pH. Prior to these measurements, ultrapure argon gas was

bubbled (de-aeration) through each EBC sample for 90 seconds. All measurements took place within a 2-hour window while samples were stored on ice. Commercially made enzyme-linked immunosorbent assays (ELISAs) were used to measure 8isoprostane (Cayman Chemical Company, Ann Arbor, MI) and myeloperoxidase (R&D Systems, CAT# DMYE00, Minneapolis, MN).

*Plasma and EBC assays.* H<sub>2</sub>O<sub>2</sub> levels were assessed in colorimetric assays (Life Tech, Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit, Cat# A22188, Carlsbad, CA) and optical density (O.D.) values were divided up into tertiles. PTX3 was also measured in both EBC and plasma using commercially made ELISA kits (R&D Systems, CAT# DPTX30, Minneapolis, MN). For SP-D (R&D Systems, CAT# DSFPD0, Minneapolis, MN) experimental reactions, ELISA kit manufacturer instructions were optimized.

*Statistical approach*. We report mean (standard deviation: sd) pre-exposure, immediate post-exposure, and one-hour post-exposure biomarker concentrations for each exposure condition. To take advantage of the crossover design of this study in which biomarker concentrations were obtained from each participant on multiple occasions, the relative change in each biomarker concentration from pre- to post-exposure (or pre- to one-hour post-exposure) was our outcome of primary interest. Relative change was calculated in the following manner: ((post-exposure concentration minus pre-exposure concentration) divided by pre-exposure concentration) multiplied by 100. Due to our small sample size, the presence of outliers, and the skewed distribution of most biomarkers, our analyses investigating the relationship between exposure condition and relative change in biomarker concentration utilized the Skillings-Mack test, a nonparametric analog to repeated measures analysis of variance. Dunnett's post-hoc comparisons were also

used to evaluate if observed immediate post- or 1-hour post-exposure values were significantly different from filtered-air immediate post- and 1-hour post-exposure values. Results were classified as statistically significant when  $P \le 0.05$ . All analyses were performed using Prism (GraphPad, v.5.0a) or Stata (StataCorp, v.9) software.

# RESULTS

Across all trials, the average CO concentrations from filtered-air, 250 µg/m<sup>3</sup>, and 500 µg/m<sup>3</sup> exposures were 0.003 (± 0.007) ppm, 0.87 (± 0.28) ppm, and 1.87 (± 0.65) ppm, respectively. PM<sub>2.5</sub> concentrations from filtered-air, 250 µg/m<sup>3</sup>, and 500 µg/m<sup>3</sup> exposures averaged 5.2 (± 4.9) µg/m<sup>3</sup>, 253.9 (± 5.8) µg/m<sup>3</sup>, and 506.2 (± 4.8) µg/m<sup>3</sup>, respectively. Preliminary data showed that >99% of PM<sub>2.5</sub> mass measured in the dilution chamber resulted from the PM<sub>1</sub> size fraction (data not shown). Means and normalized means (relative change) for all EBC and plasma measures (8-isoprostane, PTX3, SP-D, MPO, pH, and H<sub>2</sub>O<sub>2</sub>) are outlined in **Table 4.3**.

**Table 4.3.** Average pre-exposure, post-exposure, and 1-hour post-exposure values, as well as relative change (%) for all measures, and their standard deviations.

						Relative Change <sup>a</sup>			
			pre	post	one-hour post	post-pre		1-hr post-pre	
Biomarker	Exp	n	mean (sd)	mean (sd)	mean (sd)	mean (sd), %	p-value <sup>b</sup>	mean (sd), %	p-value <sup>b</sup>
8-isoprostane (EBC) pg/mL	А	9	0.917 (0.421)	1.512 (1.227)	0.701 (0.353)	88.9 (151.8)		-6.5 (67.2)	
	В	10	0.651 (0.357)	0.588 (0.248)	0.894 (1.009)	-3.3 (36.1)	0.11	72.2 (221.7)	0.16
	С	10	0.654 (0.233)	0.689 (0.279)	0.838 (0.411)	4.6 (16.4)		31.5 (63.6)	
PTX3 (EBC) ng/mL	А	9	0.051 (0.073)	0.069 (0.112)	0.032 (0.035)	279.9 (619.0)		261.9 (705.9)	
	В	10	0.047 (0.050)	0.059 (0.065)	0.072 (0.100)	167.5 (556.7)	0.66	425.6 (949.6)	0.88
	С	10	0.033 (0.038)	0.048 (0.064)	0.079 (0.137)	135.6 (324.6)		1372.3 (4065.4)	
SP-D (EBC) ng/mL	А	9	0.016 (0.012)	0.019 (0.017)	0.027 (0.030)	11.4 (31.8)		45.1 (69.4)	
	В	10	0.030 (0.049)	0.044 (0.067)	0.019 (0.024)	56.8 (141.0)	0.58	5.6 (39.0)	0.34
	С	10	0.029 (0.042)	0.021 (0.019)	0.039 (0.044)	15.3 (81.4)		109.3 (183.4)	
	А	8	0.016 (0.015)	0.021 (0.026)	0.018 (0.019)	18.5 (66.8)		-5.3 (69.9)	
	в	10	0.052 (0.111)	0.023 (0.024)	0.021 (0.029)	106.3 (297.8)	0.39	110.7 (238.5)	0.34
ng/me	С	10	0.058 (0.112)	0.027 (0.023)	0.030 (0.030)	303.2 (599.3)		430.3 (655.7)	
	А	9	0.729 (0.771)	0.542 (0.663)	0.537 (0.534)	607.0 (1398.6)		654.8 (1995.7)	
H <sub>2</sub> O <sub>2</sub> (EBC)	В	10	0.214 (0.206)	0.814 (1.397)	0.469 (0.465)	761.3 (1628.9)	0.61	350.4 (928.5)	0.80
μΜ	C	10	0.548 (0.561)	0.470 (0.543)	0.738 (0.983)	199.1 (466.6)	0.01	1603.5 (4054.2)	0.00
			. ,	. ,					
	А	9	7.849 (0.342)	7.842 (0.372)	7.803 (0.359)	-0.1 (1.1)		-0.6 (1.3)	
pH (EBC)	В	10	7.968 (0.435)	7.878 (0.381)	7.828 (0.347)	-1.1 (1.8)	0.47	-0.9 (1.8)	0.59
	С	10	7.969 (0.329)	7.873 (0.287)	7.864 (0.304)	-1.2 (1.6)		-1.3 (1.4)	
PTX3 (plasma) ng/mL	Δ	9	0 756 (0 472)	0 695 (0 392)	0 794 (0 388)	9.0 (74.8)		25 7 (76 7)	
	B	10	0.590 (0.315)	0 704 (0 305)	0.807 (0.355)	33 2 (43 9)	0.048	49.5 (71.3)	0.012
	c	10	0.528 (0.328)	0.661 (0.331)	1.205 (1.643)	57.5 (92.4)	0.040	207.1 (498.5)	0.012
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	А	9	4.777 (1.522)	4.487 (1.252)	4.610 (1.418)	-4.6 (8.8)		-3.1 (7.8)	
SP-D (plasma)	В	10	4.343 (1.127)	4.875 (1.488)	5.068 (1.817)	12.2 (15.7)	0.070	14.5 (14.3)	0.022
ng/mL	С	10	4.668 (1.468)	4.410 (1.280)	4.616 (1.299)	-3.2 (15.5)		1.1 (16.6)	
	А	9	19 65 (8 24)	29.65 (31.50)	22 20 (14 22)	67.3 (161.7)		23.8 (75.6)	
H <sub>2</sub> O <sub>2</sub> (plasma) μΜ	в	10	21 68 (14 78)	18 44 (11 35)	17 71 (7 80)	5 1 (88 5)	0.97	-11 4 (32 3)	0.73
	С	10	18.83 (4.67)	18.06 (18.06)	34,75 (60,36)	1.6 (52.7)	0.57	77.5 (299.9)	0.75
Abbraulational Fun auro	- (A	Ellered	ain D. 250 up/m <sup>3</sup> , C. 500 up/	3), and comple obtained i			incurs distals, of		hannat

Abbreviations: Exp, exposure (A, filtered-air; B, 250 µg/m<sup>3</sup>, C, 500 µg/m<sup>3</sup>); pre, sample obtained immediately prior to trial; post, sample obtained immediately after completion of trial; 1-hr post, sample obtained one hour after completion of trial; EBC, exhaled breath condensate; SD, ± standard deviation.

<sup>a</sup> ((Post-trial minus pre-trial)/pre-trial)\*100

<sup>b</sup> Skillings-Mack tests to determine if relative change from pre-trial measurement varied significantly by exposure condition.

The relative change in pre- to post-exposure (or pre- to one-hour post-exposure) EBC 8isoprostane concentrations did not vary significantly by exposure condition (**Table 4.3.**). Therefore **Figure 4.3.** presents the percent changes in EBC 8-isoprostane for filtered air compared to the combined wood smoke exposures (250 and 500  $\mu$ g/m<sup>3</sup> exposures), for both the immediate post and 1-hour post time points. The relative change from pre- to immediate post-exposure 8-isoprostane levels in EBC following wood smoke exposure were significantly lower than the relative change following filter air exposure. Wood smoke resulted in significantly higher 8-isoprostane than filtered-air at 1-hour postexposure (**Figure 4.3.**).

No significant differences in relative change in EBC MPO (**Figure 4.4.**) or EBC and plasma  $H_2O_2$  (**Figure 4.5.**) concentration by exposure condition were found, although there appeared to be a dose-response curve for the MPO. When investigating the pH measures in the EBC samples, the decrease in the 500 µg/m<sup>3</sup> wood smoke post-exposures was significantly lower (Dunnett's test; p<0.05) than filtered-air post-exposures (**Figure 4.6.**).



**Figure 4.3.** Relative change of 8-isoprostane in EBC as a function of exposure concentration. Dunnett's comparisons were performed comparing the combined wood smoke exposures to their respective filtered-air timepoints (\*, p<0.05).



**Figure 4.4.** Relative change of MPO in EBC as a function of exposure concentration. No significant comparisons (Dunnett's; p<0.05) were observed.



**Figure 4.5.** Relative change of  $H_2O_2$  in EBC (top) and plasma (bottom) as a function of exposure concentration. No significant comparisons (Dunnett's; p<0.05) were observed.

As seen in Table 1 and **Figure 4.7.**, relative changes in PTX3 in EBC were greater than those observed in plasma. However, no significant effect of exposure concentration on relative change in EBC levels of PTX3 was observed (though there does appear to be a dose-response for the 1-hour post measures). In contrast, plasma levels of PTX3 were significantly different between all three levels of exposures at both immediate (p = 0.048) and 1-hour (p = 0.012) post-exposure (**see Table 4.3.**). Also, multiple comparison analyses (Dunnett's Test) showed that 1-hour post-exposure plasma levels of PTX3 were significantly higher (p<0.05) for both 250  $\mu$ g/m<sup>3</sup> and 500  $\mu$ g/m<sup>3</sup> wood smoke exposures, when compared to the same filtered-air timepoints.

As seen in **Figure 4.8.**, relative changes in SP-D concentrations measured in EBC did not differ by exposure condition, even when 250 µg/m<sup>3</sup> and 500 µg/m<sup>3</sup> wood smoke exposures were combined (a similar strategy was used in presenting the results of the 8isoprostane EBC findings in **Figure 4.3.**). Relative changes in plasma levels at 1-hour post did result in significant differences between the three exposure conditions (**Table 4.3.**), though overall trends were not consistent with a normal dose-response relationship. Consistent with other markers, EBC changes were greater than those in plasma.



**Figure 4.6.** Relative change of pH in EBC as a function of exposure concentration. Dunnett's comparisons were performed comparing wood smoke exposures to their respective filtered-air timepoints (\*, p<0.05).



**Figure 4.7.** Relative change of PTX3 in EBC (top) and plasma (bottom) as a function of exposure concentration. Dunnett's comparisons were performed comparing wood smoke exposures to their respective filtered-air timepoints (\*, p<0.05).



**Figure 4.8.** Wood smoke effects on SP-D levels in EBC (top) and plasma (bottom) as a function of exposure concentration. No significant comparisons (Dunnett's; p<0.05) were observed.

## DISCUSSION

The air-lung interface is the boundary between the biological environment and the ambient environment. Foreign substances deposited at this boundary can elicit natural responses that can lead to pulmonary (Alexis et al., 2006; Möller et al., 2010) and systemic (Bagate et al., 2006; Huttunen et al., 2012) measures of inflammation and oxidative stress. Specific markers can be linked to acute and chronic conditions, such as those experienced by wildland firefighters, and offer useful insights into clinical risks. In this study, we report the findings from EBC and plasma measures following human exposures to filtered-air, 250  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>2.5</sub>, and 500  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>2.5</sub> concentrations while exercising on a treadmill for 90-minutes.

Measured CO and PM<sub>2.5</sub> concentrations in these exposure trials were consistent with (and representative of) field research studies where CO and PM levels were recorded during wildland firefighting activities (Adetona et al., 2013). **Table 4.3.** shows that significance in relative percent change was achieved at a variety of time points/exposure concentrations for several markers. However, the main findings from this investigation are that markers of inflammation and oxidative stress, including PTX3, pH, and 8-isoprostane levels in EBC and/or plasma, are sensitive to wood smoke inhalation.

8-isoprostane concentrations (see Figure 4.3.) in EBC did not immediately increase following wood smoke exposures, but were found to be significantly increased 1-hour post exposure, suggesting a latent effect on its release into the lung. In a similar controlled wood smoke exposure study, Riddervold et al. (2012) reported similar EBC results as our filtered-air samples. In contrast, 8-isoprostane levels decreased in all their wood smoke exposures with the exception of their high (400  $\mu$ g/m<sup>3</sup>) wood smoke

exposure. Importantly, the times points used in the Riddervold et al. study (2012) study were 3.5 hours and 6 hours post-exposure, which are hours later than the time points used in the present study.

In other studies, significant increases in urinary 8-isoprostane have been observed following specific exposures, including occupational exposures to welding material (Nuernberg et al., 2008), chronic wood smoke exposures from cookstoves (Commodore et al., 2013), and wood smoke exposures in wildland firefighters (Gaughan et al., 2014). Collectively, these studies suggest that particle exposures, including wood smoke, can result in increased 8-isoprostane. The results in our study also suggest that 8-isoprostane levels in EBC are affected by wood smoke inhalation, including when exercising.

The levels of MPO observed in EBC following wood smoke exposures resulted in a dose-response relationship (see Figure 4.4.). However, no statistical significance was observed. To our knowledge this is the first investigation of the MPO response to wood smoke during exercise. Much like SP-D, the peroxidase enzyme MPO plays an important role in impairing microbial activity. Neutrophils are specially tailored to phagocytose microorganisms followed by a burst of highly reactive oxygen species mediated by MPO (Dubnov et al., 2007). This enzyme is itself an antimicrobial system in which intercellular activity directs MPO and associated reactive oxygen species to the phagosome (Klebanoff, 2005). Extracellular release of MPO can also occur during phagocytosis or when the cell is attempting to phagocytose a large particle (Klebanoff, 2005). Our results suggest that immune cells in the lung were likely uptaking wood smoke particles, resulting in the release of MPO into the lining of the lung. Due to the

apparent, but non-significant, dose-response relationship of MPO following wood smoke exposures, it is possible that these cells are actively releasing MPO into the lung resulting in increased levels in the EBC.

No discernible trends were observed when investigating changes in  $H_2O_2$  in both the EBC and plasma samples (see Figure 4.5.). Studies measuring exhaled levels of  $H_2O_2$ have reported increased concentrations in smokers (Horváth et al., 1998), increased levels in patients with COPD (De Benedetto et al., 2000; Kasielski and Nowak 2001; Kostikas et al., 2003), and even higher  $H_2O_2$  in exacerbated COPD when compared to stable COPD (Dekhuijzen et al., 1996).  $H_2O_2$  levels in EBC did not increase in a cohort of school children exposed to ambient levels (>20  $\mu$ g/m<sup>3</sup>) of wood smoke (Epton et al., 2008), though these levels were much lower than those used in the present study. Plasma levels of  $H_2O_2$  have been shown to correlate with hypertension cases, including those who are predisposed to hypertension (Lacy et al., 1998). The above referenced studies showing increases of  $H_2O_2$  report on chronic underlying conditions. The exposures used in this study may not have resulted in robust changes in  $H_2O_2$  because of the acute nature of the study design and because the study participants were of relative good health. Our results show the most pronounced increase in H<sub>2</sub>O<sub>2</sub> in the 500  $\mu$ g/m<sup>3</sup> exposure at 1-hour post despite it not reaching significance. It is possible that antioxidant activity in the lung was overwhelmed leading to release of  $H_2O_2$ . Future studies could be enhanced by including antioxidant activity measures (e.g., superoxide dismutase, glutathione).

Results showed that there were significant decreases in pH following wood smoke exposures (see Figure 4.6.). Previous studies have evaluated pH in EBC as a marker

of airway inflammation. In studies evaluating impacts of acute lung injury or disease on acidity, decreases in pH were shown to be caused by, or associated with inflammation (Gessner et al., 2003; Kostikas et al., 2002; Vaughan et al., 2003). In a study exposing humans to filtered-air, 200 µg/m<sup>3</sup>, and 400 µg/m<sup>3</sup> wood smoke concentrations for 3.5 hours, "estimated change from baseline" pH levels in EBC increased in all measures except the elevated exposure 6-hours post-exposure (Riddervold et al., 2012). Importantly, the Riddervold et al. (2012) participants were at rest throughout the entire duration of exposure. The similarity of exposure levels between Riddervold et al. (2012) and the present study suggests that other factors beside exposure concentrations may be influencing these different outcomes. The most obvious differences between these two studies is duration of exposure (3.5 hours in the Riddervold et al. (2012) study and 1.5 hours in our study) and the exercise component of our study. Future studies should consider controlling for exertion to further determine how breathing rates and cardiovascular activity influence these outcomes.

Examples of commonly measured pentraxins include C-reactive protein (CRP) and serum amyloid P (SAP). These short chain pentraxins are known to recognize specific pathogen-associated molecular patterns (fungi, yeasts, bacteria, etc.) and play an important role in the innate immune system. They have also been measured to assess risk for cardiovascular disease (Buckley et al., 2009; Ridker et al., 2000). The long chain pentraxin, PTX3, is emerging as an important acute phase protein as concentrations can increase rapidly in response to infections and inflammation (Moalli et al., 2011). This protein has also been shown to be a marker of vascular damage (Presta et al., 2007), sepsis (Mauri et al., 2010), and inflammation (Bevelacqua et al., 2006; Suzuki et al., 2008). Little is known how PTX3 responds to acute wood smoke exposures.

Results from this study showed that PTX3 increased in a concise dose-response manner (in both the EBC and plasma), with significant effects observed in plasma (see **Figure 4.7.)**. The similar trends between PTX3 and MPO (in EBC) might partly be explained by neutrophil release of PTX3 following inflammatory events (Garlanda et al., 2005; Jaillon et al., 2007). Studies suggest that neutrophils release PTX3 into neutrophil extracellular traps further targeting pathogens (Brinkmann et al., 2004). The resulting significant differences between filtered-air and both wood smoke 1-hour post-exposures supports previous studies suggesting PTX3 as an acute phase protein (Moalli et al., 2011). Taken together, these results suggest that PTX3 is an acute phase protein with sensitivity to the concentration of inhaled wood smoke.

Filtered-air and the wood smoke exposures resulted in a rise of SP-D in the EBC, though these results are not significant (**see Figure 4.8.**). The synthesis of SP-D generally occurs in all mucosal tissues as part of the innate immune system and plays a significant role in host defense (Crouch et al., 1992; Madsen et al., 2000). This protein has also been shown to co-locate with IgA further suggesting its role in pathogen clearance and impairing microbial colonization (Madsen et al., 2000; Ni et al., 2005). SP-D also binds to endotoxin (Bufler et al., 2003), reducing potential adverse effects from particle exposure as endotoxin has been shown to contribute to pro-inflammatory outcomes (Kocbach et al., 2008). It is possible that wildfire smoke exposures may induce even greater amounts of SP-D release, as wildfire smoke contains elevated concentrations of endotoxin and induce a greater pro-inflammatory response when compared to residential wood stove emissions (Ferguson et al., 2015b). Similar to our findings, Stockfelt et al. (2012) measured SP-D levels in plasma following human exposures to

wood smoke (i.e., 3-hour sedentary exposures to filtered-air, 146  $\mu$ g/m<sup>3</sup>, and 295  $\mu$ g/m<sup>3</sup>) with no significant increases observed.

# Limitations of study

This present study was designed to specifically mimic wildland firefighting activities, including moderate exercise within a controlled setting. Although the CO and PM<sub>2.5</sub> wood smoke exposures were consistent with field research studies where CO and PM concentrations were recorded during wildland firefighting activities (Adetona et al., 2013), there were still several limitations. Notably, the smoke generated in the Inhalation Facility resulted from a wood stove, and was not "real-world" forest fire smoke (which would contain other fuels such as detritus and soil products, etc.). In addition, the exposure times were relatively short (1.5 hours) compared to wildland fire fighters who are exposed to much longer exposures. Also, the smoke dose may have been lower compared to an accumulation of a full day of work fighting wildfires or the acute exposures they may experience (upwards of 6000 µg/m<sup>3</sup>; (Reinhardt and Ottmar, 2004)). These data should be fully considered in follow up investigations.

## CONCLUSIONS

As wildfires are predicted to increase across the western US in the foreseeable future, so will the potential for occupational wood smoke exposures to wildland firefighters. Importantly, carrying out wood smoke exposure studies in the field poses numerous logistical, financial, and safety challenges. Therefore, conducting controlled smoke exposure studies is critically important, as it provides the opportunity to investigate specific health endpoints with the goal of preventing acute and chronic health effects experienced during occupational firefighting activities.

The main findings of our study were that: (1) MPO levels in EBC showed a nonsignificant increase in a dose-response manner following wood smoke inhalation; (2) significant reductions in pH occurred following wood smoke exposures; (3) PTX3 increased in a dose-response manner in both EBC and plasma (note that significant effects were observed in plasma, suggesting its potential as a sensitive marker of inflammation following wood smoke exposure); and (4) SP-D levels in EBC and plasma tended to be higher following wood smoke exposures when compared to filtered-air samples, though no significance was observed.

Compared to previous wood smoke human exposure studies, this study is novel in that it incorporated exercise into the exposure trials to mimic occupational exertion by wildland firefighting. Previous controlled human wood smoke exposure studies had participants at rest or with light intermitted exercise (Bønløkke et al., 2014; Ghio et al., 2012; Stockfelt et al., 2013; Unosson et al., 2013). Exposures with an exercise component resulted in higher breathing and heart rates that increased particle deposition and blood flow through the lung. To our knowledge, this is also the first controlled human wood smoke study that measured  $H_2O_2$ , PTX3, and SP-D concentrations from both EBC and

plasma samples. In general, these biomarkers resulted in greater magnitude of changes in EBC, but similar pattern of responses were found in plasma. Future studies should investigate these EBC and plasma biomarkers measured during actual wildfire events to validate the use of controlled exposure studies to predict adverse health outcomes from "real world" occupational exposures.
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Chapter 5: Summary, conclusions, and next-steps

**Chapter 2:** Comparison of how ambient  $PM_c$  and  $PM_{2.5}$  influence the inflammatory potential

### Summary

This study investigated current gaps in knowledge for  $PM_c$  including exposure toxicity and PM ratios ( $PM_c$ : $PM_{2.5}$ ) in  $PM_{10}$ . We utilized our studies we utilized a bone marrowderived mouse macrophage *in vitro* system to compare the inflammatory potential of  $PM_c$ ,  $PM_{2.5}$ , and mixtures of the two. The result was a linear increase in interleukin(IL) - $1\beta$  with increasing levels of exposure to winter and summer  $PM_c$ , as compared to  $PM_{2.5}$ , which exhibited logarithmic growth. Also, exposure to  $PM_{10}$  as a function of  $PM_{2.5}$  and  $PM_c$  mass ratios showed that IL-1 $\beta$  and TNF- $\alpha$  levels increased synergistically with a greater burden of  $PM_c$ . Endotoxin content in the PM did not correlate with these results, suggesting that other activators in  $PM_c$  are likely responsible for activating the NF- $\kappa$ B pathway and the inflammasome.

## Major conclusions:

- This study supports our hypothesis that PM<sub>c</sub> adversely affects macrophage activity with increasing ratios of PM<sub>c</sub>:PM<sub>2.5</sub> in PM<sub>10</sub>.
- There are currently no National Ambient Air Quality Standards (NAAQS) for the coarse fraction, though our results (and in other studies) suggest that a new PM<sub>c</sub> ambient standard should perhaps be considered in addition to the existing daily and annual PM<sub>2.5</sub> NAAQS.

## Next steps:

- Including additional chemical and biogenic characterizations will facilitate future source apportionment studies for different PM fractions, as well as provide more information towards investigating resultant health effects.
- Focus on investigating the role of the biogenic components (including endotoxin) in future studies.
- Evaluation of reactive oxygen species activity in both PM<sub>c</sub> and PM<sub>2.5</sub> in vitro studies should be considered.
- Further seasonal and dose-response relationship studies will aid in determining real-world mixture effects, as well as provide an understanding of the causal health effects from exposure to PM<sub>c</sub>.

**Chapter 3:** Impact of wood smoke source on pro-inflammatory responses in an *in vitro* human macrophage cell model

### Summary

Many uncertainties remain regarding how different sources of wood smoke impact health. Particles were collected from a high emission residential wood stove, an EPAcertified low emission residential wood stove, and during a summer wildfire event (ambient wildfire  $PM_{2.5}$ ). The influences of wood smoke source on inflammation (IL-1 $\beta$ ) and cell viability (MTS assay) were assessed *in vitro*. In a dose-response manner, harvested wildfire smoke  $PM_{2.5}$  stimulated significantly more IL-1 $\beta$  release than both residential wood stove sources and urban particulate matter.  $PM_{2.5}$  from both wood stoves similarly induced the highest levels of cell toxicity compared to wildfire smoke  $PM_{2.5}$ , with overall findings following similar trends in the measured endotoxin content of the particle types. These findings suggest that exposure to different sources of wood smoke  $PM_{2.5}$  result in varying adverse health effects, and additional studies are needed to further understand these relationships and mechanisms.

### Major conclusions:

- Wood smoke source plays a significant role in pro-inflammatory outcomes and cell viability in macrophage-like differentiated THP-1 cells.
- Wildfire smoke PM was a significant inducer of the pro-inflammatory response whereas residential wood stove emissions were not.
- Exposure to residential wood stove emissions also resulted in a decline in cell viability with increasing exposure concentrations.

## Next steps:

- Mechanisms leading to the observed decreased cell viability should be further investigated.
- DAMPS (e.g., chitin, ergosterol, etc.) should be analyzed to determine how potential PM characteristics may be inducing inflammation outside of endotoxin.
- Other chemical constituents of the particles (e.g., elemental and organic carbon, metals, PAHs, etc.) should be fully characterized to increase understanding of how biomass source influences PM properties.
- Additional sources of smoke PM (e.g. smoke emitted during a prescribed burn, agricultural waste burns, etc.) should also be investigated for potential roles in exposure outcome.

**Chapter 4:** Human exposures to wood smoke – rationale, methods, and results.

### Summary

A controlled wildland firefighter exposure study was carried out in a wood smoke inhalation facility. Utilizing a randomized crossover trial design, we exposed 10 subjects once to clean filtered-air, 250  $\mu$ g/m<sup>3</sup>, and 500  $\mu$ g/m<sup>3</sup> wood smoke PM<sub>25</sub>. Subjects exercised on a treadmill at an absolute intensity designed to mimic wildland firefighting for 1.5 hours. In addition to measured  $PM_{2.5}$  smoke concentrations, mean levels of  $CO_2$ , CO, and % relative humidity for each exposure type were recorded, and were representative of occupational 'real-world' wildland firefighting exposures. Pulmonary function was measured, and exhaled breath condensate (EBC) and blood plasma samples were obtained pre-exposure, immediately post-exposure, and 1-hour postexposure. Measures of 8-isoprostane, pH, and myeloperoxidase were measured in EBC while  $H_2O_2$ , surfactant protein D, and pentraxin-3 (PTX3) were measured in both EBC and plasma. Although there were some reductions in FVC,  $FEV_1$ , and FVC:  $FEV_1$ measures following exposures, results of the spirometry testing did not show significant changes in lung function. Significant changes in pH, 8-isoprostane, and PTX3 were observed. PTX3 outcomes suggest a promising role as an acute phase protein that may be particularly sensitive to wood smoke inhalation. The development of this wood smoke inhalational facility will further research into acute and chronic health impacts of wood smoke exposure, and in the future provide a platform to address unique research questions related to wood smoke exposures and associated adverse health effects.

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## Major conclusions:

- The CEHS inhalational facility can successfully be used for human wood smoke exposure studies investigating occupational exposures experienced by wildland firefighters.
- Some reductions in FVC, FEV<sub>1</sub>, and FVC:FEV<sub>1</sub> measures were observed following exposures, though these changes were not statistically significant.
- Main findings from the EBC and plasma assays include:
  - 1. Wood smoke exposures cause significant reductions in EBC pH.
  - 2. In a latent manner, 8-isoprostane levels can significantly increase in EBC.
  - 3. Dose-response outcomes were observed in MPO measures in EBC.
  - 4. Trends of increasing SP-D levels in EBC and plasma following wood smoke inhalation were observed, though not significant.
  - PTX3 displayed a clear dose-response pattern in both plasma and EBC. However, the only significant outcomes were observed in plasma, even though 1-hour post-exposure levels in EBC were tightly associated with 1hour post-exposure plasma levels.

## Next steps:

- Further studies using the human wood inhalation facility should evaluate additional real-world effects, such as the influence of chronic/repeated exposures (further mimicking wildland firefighting) and different levels of exposure, as well as determining the impact of exertion on measured outcomes.
- Modifications in subsequent investigations will likely affect the exposure design, including the need for a greater number of participants to improve statistical comparisons. Also, female participants should be included.

- Future studies should investigate these EBC and plasma biomarkers measured during actual wildfire events (field studies) to validate the use of controlled exposure studies to predict adverse health outcomes from "real world" occupational exposures.
- The PTX3 findings suggest that it may be a particularly sensitive acute phase protein to be investigated in future controlled and field studies related to smoke exposures.

Appendix

## Protocol for collecting mouse bone marrow-derived macrophage cells.

## -By Gini Porter. (6/9/09)

After one week of culturing bone marrow cells harvest on Wednesday.

- 1. Plan experiment and sign up for sterile hood time. (Tuesday)
- 2. Thaw 5ml Trypsin for each flask that you will be collecting cells from.
  - Example: if you have 3 flasks you need 15ml trypsin.
- 3. Warm Sterile PBS and RPMI Media in 37°C water bath for at least 20-30 minutes.
- 4. Remove flasks to be harvested from incubator, spray down and place in sterile hood.
  - If you are harvesting more then one strain of mouse, harvest strains individually being careful not to mix.
- 5. Gather warm PBS and Trypsin from water bath.
  - Wipe off excess water the paper towel.
  - Spray down bottles and conicals with 70% Etoh and place in hood.
- 6. Aspirate media out of flask(s).
  - Tip flask forward and to one corner so media is away from adhered cells and carefully place tip in corner.
  - Be sure not to touch tip to cells or anything in hood.
- 7. Wash cells by adding ~15 ml PBS to flask, tip flask so PBS covers entire bottom of flask and cells.
- 8. Aspirate PBS from flask. Use same procedure as step 6.
- 9. Repeat steps 7 and 8 one more time, for a total of two washes.
- 10. Add ~5ml trypsin to flask.
  - Rotate flask so trypsin coats entire bottom of flask over the cells.
- 11. Place flask in incubator for ~10 minutes.
- 12. Check cells under microscope. Look to see if cells look like little balls and no longer elongated and flat to bottom of flask.
  - If cells are not ready yet, place back in incubator for a few more minutes.
  - Do not leave trypsin on longer then 15 minutes.
- 13. Spray down flasks and place flask in hood.
- 14. Quickly add ~7 ml RPMI media to flask.
  - This stops the reaction between the trypsin and the cells, by diluting the trypsin.
- 15. "Tap flask"
  - Hold flask in one hand and tap side of flask with the palm of other hand.
  - Be careful not to create a lot of bubbles or to get media/cells in the neck or filter of flask.
- 16. Using a blue cell scrapper, gently scrap bottom of flask.
  - Use the media to collect the cells as they are being removed from the bottom of the flask.
- 17. Transfer cells to 50ml conical.
  - Rinse down back of flask with cells/media before transferring to conical.
  - Combine multiple flasks into one conical as long as they are from the same strain of mouse.
  - If more then one conical is needed distribute the amount evenly among conicals. This is for balancing in the centrifuge.

- 18. Spin at 1500rpm for 5 minutes.
  - Be sure that the conicals are evenly balanced. You may need to use a premade balance if you have an odd number of conicals.
- 19. Aspirate off supernatant in hood with aspirator. Leaving cell pellet.
- 20. Resuspend cell pellet in 1ml/flask RPMI media.
  - If you combined more then one flask in one conical. Example 2 flasks into one conical would be resuspended in 2 ml media.
- 21. Count on Hemocytometer or coulter counter. See additional protocol.
- 22. Calculate number of cells.

## General Cell Culture Experiment

- 23. Aliquot cells into eppies at desired concentration.
  - Generally 1 million cells per ml (1.0 x 10^6)
- 24. Add treatments.
- 25. Spin on rotator in incubator.
  - Generally about 1 hour.
- 26. Plate cells in 96-well plate.
- 27. Add additional treatments if any.
- 28. Place 96-well plate in incubator over night. (24 hr)
- 29. Collect supernatants and transfer to new 96-well plate.
- 30. Run assays and elisas on supernatants.
  - If same day or next day store plate at 4°C.
  - If later then 24 hrs freeze plate and store at -20°C.
- 31. Same day: Trypan count on original plate of cells. See **Protocol for collecting mouse bone marrow-derived macrophage cells**.

# Culturing collected bone-marrow cells for macrophage derivation.

# -By Gini Porter and Heather Brunell (3/12/08)

# <u>Monday</u>

- 1) Harvest hind legs from mice-See Additional protocol
- 2) Remove bone from tissue
  - Remove femur and tibia, leave the knee
  - Using forceps and scissors strip tissue away from bone, do not crush the bone.
  - After removing tissue, cut bone straight across and place in separate tissue plate with media.
- 3) Harvest bone marrow from each bone
  - Use a 27.5 gauge needle on a 10 ml syringe
  - Fill the syringe with 10 ml of media (10% RPMI)
  - Rise off the top of the bone to create a spot for the needle to go in
  - Place tip of the needle inside the bone and gently wash out the marrow. Wash into the same tissue plate
  - Flush out the red cells then keep flushing until foam comes out, this is the marrow
  - Flush out one side, then flush out the other
  - Do not re-use media, get new 10 ml
  - Try not to use more than 40-50 ml of media per 3 mice
  - The bone should look clear after flushing
    - Discard bone with the other tissue and put in biohazard
- 4) Using 25 ml pipette suck up all media from plate. Rise down the sides and collect as much media and foam as possible.
- 5) Transfer to a new 50ml conical vial
  - Pipette up and down to expel all liquid and foam from pipette
- 6) Spin down at 1500 rpm for 5 min
- 7) Using vacuum suck off supernatant, leaving cell pellet.
- 8) Resuspend cells in 10 ml media (10%RPMI)
- 9) Do a cell count using 40ul sample in 20ml isotone, 6 drops
- 10)Seed flask at 30 million cells/flask in 20 ml media
  - Example: cell count was 93 million, split into 3 flasks = ~30 million/flask. (# of flasks you seed depends on the cell concentration)
  - Add 17 ml of media to each flask, then add 3.3 ml of suspended cells.
  - Drip cell suspension down the bottom of the flask to prevent splashing
- 11)Label flasks with initials and date and put into incubator

- 12)Incubate for 1 day
- 13)Wipe down hood
- 14)Wash forceps and scissors with soap and hot water, then spray with ethanol and wipe.

Tuesday- Stromal Elimination

- 1) Remove 10% RPMI media from cold room and warm to 37° in water bath for 20-30 min.
- 2) Remove flasks from incubator, spray down and place in hood
- 3) Using 25 ml pipette carefully remove media from each flask
  - Keep cells in media, discard cells stuck to flask
  - Combine media from all flasks into a few 50 ml conical vialsdivided equally
  - Only touch tip of pipette to bottom of flask, not sides or neck of flask
- 4) Spin 50 ml conical down at 1500 rpm for 5 min
  - Remove M-CSF aliquots from freezer to thaw- will need 40ul/flask
  - Get new T-75 flasks
  - Throw away old flasks in biohazard
- 5) Vacuum out supernatant from 50 ml conicals- leaving cell pellet
- 6) Resuspend ALL pellets in the SAME 10 ml of media using 10ml pipette
- 7) Take a cell count: 40ul of sample, 20ml isotone, 6 drops
  - Standard amount: 15 million cells per flask
    - Example: 70 million cells into 5 flasks = 14 million cells which is 2 ml of resuspention into each flask.
- 8) Using 25 ml pipette add appropriate amount of 10% RPMI media (total volume should be 20 ml per flask)
  - Example: 18 ml of 10% RPMI media into each of the new flask. (2ml of cell resuspension makes 20ml per flask)
  - Pipette above the flask neck not in the flask
- 9) Add 40ul of M-CSF per flask to the cell resuspension (not to flasks themselves)
  - Example: 5 flasks at 40ul each is 200ul total
  - Mix by pipetting up and down a few times
- 10)Add appropriate amount of cell suspension to each flask
- 11)Label flasks with cell type, initials, and date
- 12)Let incubate until Friday, check daily

Friday- Spiking Bone Marrow

Spiking with MCSF stimulates bone marrow cells to form macrophages

- 1) Warm media (can warm only the amount you will need)
- 2) Mix MCSF into media
  - Mix 20 ul of MCSF into 2ml per flask

- Example: if you have 5 flasks, mix 100ul MCSF into 10 ml media
- 3) Add 2 ml of media/MCSF to each flask
- 4) Let incubate until Monday.

Monday - 1 Week After Culturing – 50% Change of Media

- 1) Suck up ½ of media from flask using a 25 ml pipette (if total is 20 ml, only take up 10ml)
- 2) Transfer media to a new 50ml conical vial
  - May need to split media between multiple conicals- example: remove 10 ml from 5 flasks for a total of 50 ml, 50 ml total split into 25 ml of media into each 50ml conical. (Balancing for spinning)
- 3) Spin down at 1500 rpm, 5 min.
- 4) Suck off supernatant with vacuum, leaving cell pellet
- 5) Resuspend in 50% of total media in original flask (Example: 5 flask, 10ml removed from each, Then resuspend in 50ml media (10% RPMI)
- 6) Add 20ul of MCFS for each flask to resuspended cells, pipette to mix. (Example: for 5 flasks add 100ul to 50 ml of supernatant).
- 7) Of the resuspension: add an amount, equal to the original amount of media taken, back into the flask. Example: removed 10ml originally, add 10ml of resuspension back into flask.
- 8) Label with date, what done and put back into incubator

<u>Wednesday</u>

\*Harvest cells for experiment-See Additional Protocol

## Protocol for using THP-1 cells in particle exposure model -By Ray Hamilton

**General Description**: Human monocytes from 1 year old male with acute monocytic leukemia exhibiting lymphoblast morphology and expressing Fc; complement (C3b). Antigen expression: HLA A2, A9, B5, DRw1, DRw2. THP-1 is a suspension cell culture that doubles in approximately 26 hours. The cells are phagocytic and lack surface and cytoplasmic immunoglobulin. Differentiation will be induced by 1a, 25-Dihydroxy-Vitamin D<sub>3</sub> at 150 nM. Inflammasome activation can be detected by monitoring IL-1 beta release, but it requires a low-level endotoxin co-exposure to induce pro-IL-1 beta, which is then cleaved by activated caspase 1 as a result of inflammasome NLRP3 assembly.

Biosafety Level 1.

## Materials necessary for experiment:

## Culture items:

- THP-1 cell line ATCC (cat# TIB-202)
- Complete Growth Media: HEPES-buffered RPMI 1640 supplemented with Lglutamine (MediaTech Cellgro cat# 10-041-CV or similar), 0.05 mM betamercaptoethanol and 10% heat-inactivated fetal bovine serum (PAA Laboratories cat # A15-204, lot # A20407-7003 or similar). Filter media (0.2 μm) before use.
- Freeze Media: Complete growth media with 5% (v/v) DMSO.

## Reagents to be used:

- 1a, 25-Dihydroxy-Vitamin  $D_3$  (50 µg, EMD cat# 679101).
- Phorbol, 12-myristate, 13-acetate (PMA in DMSO) (2 x10 µl at 1 mg/ml (1.62 mM) Sigma cat# P8139).
- Lipopolysaccharide (LPS) (1 mg, Sigma cat# L-4516 from E-coli 0127).
- LDH assay (use Promega CytoTox-96<sup>™</sup> Homogeneous Membrane Integrity Assay cat# G1780).
- MTS assay (use Promega CellTiter-96<sup>™</sup> One Solution cat# G3580).
- Human IL-1beta ELISA (use R & D Systems Human IL-1 beta DuoSet<sup>™</sup> cat# DY 201). In addition to the DuoSet, you will need ELISA plates (Nunc MaxiSorp cat # 439454) and color substrate (ThermoFisher 1-step Ultra TMB ELISA cat # 34028). A 2N sulfuric acid solution is used as a stop solution.

## **Experimental Protocol**

## Cell Culture Methods:

- Quickly thaw the frozen contents.
- Once thawed, the cell suspension should be transferred to a 15 ml tube containing complete media (10 ml) and centrifuged at 300 x g for 5 min.

- The media supernatant should be discarded and the cell pellet should be resuspended in complete media (1 5 ml) and transferred to a T-75 cm<sup>2</sup> vented flask for culture (20 ml total volume) in 37°C humidified environment with 5% CO<sub>2</sub>. The optimal cell concentration is 2 4 x 10<sup>5</sup> cells per ml.
- Avoid having the cell concentration exceed 1 x 10<sup>6</sup> cells per ml. Change the media every 2 to 3 days depending on the cell density and growth rate.
- Keep track of the passages, as these cells can change over time. Do not use a cell lineage for longer than a month before you start a fresh line.

## Differentiation of THP-1 cells:

- THP-1 cells can be differentiated into a macrophage-like cell by Vit D<sub>3</sub>. The stock Vit D<sub>3</sub> should be 100 μM (in 100% EtOH).
- Use 30 µl of the stock Vit D<sub>3</sub> solution per 20 ml (150 nM) in the T-75 flask overnight to create a semi-adherent cell that exhibits macrophage morphology. The cells will become completely adherent during the particle exposure phase of the experiment. Note: The experiment requires approximately 10 x 10<sup>6</sup> cells so prepare enough flasks to have more than this amount to be safe.

## Culturing the THP-1 with the nanoparticles:

- After the cells are differentiated, they will appear to be semi-adherent. Use a rubber cell scraper (**Corning Cat# 3010**) to dislodge the sticking cells and place the resulting cell suspension in a 50 ml polypropylene centrifuge tube.
- The cell suspension is then centrifuged at 300 x g for 5 min. The media supernatant is discarded and the cell pellet is resuspended in 5 ml of complete growth media.
- The cell number is then determined (such as a Z<sub>2</sub> Coulter Counter, but a hemacytometer works also).
- The cells are suspended at 10<sup>6</sup> cells per ml in complete growth media.
- Co-culture with a small amount of endotoxin (LPS) is necessary for a proxy detection of NLRP3 inflammasome activation by IL-1 beta release. The stock solution of LPS should be 1000x at 10 µg/ml in sterile PBS. The final working concentration should be 10 ng/ml (1 µl/ml). This can be added to the complete culture media just prior to the cell/particle exposure. The LPS has no direct effect or interaction effect with the particles on cell viability. A small amount of PMA (10 nM) is added at this time also activate the macrophage-like cells. Use a 10 µM stock solution in 100% DMSO. This will be a 1:1000 dilution.

- Expose cells to nanoparticles in 1.5 ml microfuge tubes. Example: Depending on the number of cells, conditions, etc... we typically place a 350 μl cell suspension into a 1.5 ml tube and then pipette the particles in from a 5 mg/ml stock solution. So 100 μg/ml would be 7 μl into the 350 μl, and 50 μg/ml would be 3.5 μl into the 350 μl, and 25 μg/ml would be 1.75 μl and 10 μg/ml would be 0.7 μl. Larger volumes can be used.
- This cell/particle suspension is then mixed by pipetting up and down as 100 µl is transferred in triplicate into 96-well tissue culture plates and placed in an incubator for 24 hours @ 37° C.

## Assays (MTS and LDH cytotoxicity) and IL-1 beta release:

- At the end of the 24-hour culture period add 10 µl lysis buffer (**cat # G182A**) to the 3-"100% Kill" wells and return to incubator for 40 minutes.
- Thaw out the MTS reagent (CellTiter 96 One Solution) and prepare enough media/MTS reagent solution to do all of the wells plus 3 blank wells and some extra for pipetting error.
- Formula: 0.12 x (number of wells + 4) = ml of solution necessary for experiment. It is always wise to make a little extra volume. If you need 12 ml for example (1 plate), this would be a combination of 10 ml complete media and 2 ml of MTS reagent.
- After 40 minutes in the incubator, spin the plate at 1000 x g for 3 minutes @ RT.
- 50 μl of media per well should be transferred to a fresh plate for the LDH assay. Take care not to disrupt the cells while pipetting or to introduce bubbles into the new plate.
- In the fresh plate, add 50 μl of LDH assay substrate (part# G179A) (made fresh with 11 ml of substrate buffer (part# G180A)) to each well of LDH assay plate. Let plate develop for approximately 10 minutes @ RT (exact times may vary darkest wells should not be > 2.0 OD value).
- While the LDH assay is developing, transfer the remaining media from the original cell culture plate into a fresh plate. Take care to not disrupt the cells while pipetting. Place this plate in cold (-20°C) storage for later IL-1b assay. **Note**: at this point all loose material is gone from the plates.
- The media/MTS solution (120 µl/well) should be added to the original culture plate and this can be placed in the 37° C incubator for an additional 40 60 min depending on development speed. Do this quickly as the cells can be harmed by a protracted period of dryness (exact timings may vary darkest wells should not be > 2.0 OD value).
- Add 50 µl of stop solution (part# G183A) to each well of LDH assay plate and read at 490 nm. The "media only" wells can be subtracted out as background. Our plate-reader lets us designate these as "blanks", and they are automatically

subtracted out from all observations. The sample pseudo-replicates should be averaged and expressed as a percent relative to the "100% total kill" average.

- At the end of the 40 to 60 minute period transfer 100 µl of the media/MTS solution into a fresh plate. The color development in the wells will not be uniform, so it is necessary to pipette up and down once (gently) to mix the color in the media before transfer. Avoid creating bubbles, because they will distort the OD values. If bubbles are present in any wells, you can always spin the plate at 1000 x g for 3 min to get rid of any bubbles before the read.
- Read the plate at 490 nm. The media/MTS solution blank (no cells) average should be subtracted from all samples (see above). Pseudo-replicates should be averaged and expressed as a percent relative to the no-particle control condition (100 %).
- The frozen 24-hr culture media can then be used for the IL-1 beta assessment. From our experience, sample dilutions can range from 1:50 to 1:300 depending on the culture conditions (± LPS, ± particle for example). We suggest 1:100 as a starting place. The standard curve for this assay is relatively small window, so this sample dilution is critical for accurate results. Each lab has to determine the optimal dilution for their samples. For best results, use the R & D instruction sheet provided.

	1	2	3	4	5	6	7	8	9	10	11	12
А	Control	Control	Control									
В	100%	100%	100%									
	Kill	Kill	Kill									
С	Media	Media	Media									
	Only	Only	Only									
D	MTS	MTS	MTS									
	Reagent	Reagent	Reagent									
Е												
F												
G												
Η												

# Sample 96-Well Culture Template

- "Control" is the no-particle cell culture
- "100% kill" is the cell culture receiving the 10 µl lysis buffer
- "Media Only" is 100 µl of media without cells (background LDH OD value)
- "MTS Reagent" is the addition of the media/MTS solution to be subtracted out of the final results (background MTS OD value)
- All other wells used for particle-exposed cell cultures in triplicate

# Institutional Review Board (IRB) application:

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**Consent Form:** 



# SUBJECT INFORMATION AND INFORMED CONSENT

### STUDY TITLE: EVALUATION OF EXECUTIVE FUNCTION AND MARKERS OF PULMONARY AND CARDIOVASCULAR STRESS FOLLOWING EXPOSURE TO WOOD SMOKE

**SPONSOR:** Center for Environmental Health Sciences (CEHS) Pilot Grant Program (*pending approval*)

#### INVESTIGATORS: Tony Ward, PhD (406) 243-4092 Charles Dumke, PhD Erin Semmens, PhD Joe Domitrovich, PhD Charles Palmer, PhD

Please read the following information carefully and feel free to ask questions. This consent form may contain words that are new to you. Only sign the final page when you are satisfied procedures and risks have been sufficiently explained.

### REQUIREMENTS

This research study requires that you meet the following criteria:

You must be male, between the ages of 18 and 40, not a regular smoker of any substance, with no signs of cardiovascular risk factors, or pre-existing chronic lung disease, or be exposed to smoke frequently at home or work, and have a maximal oxygen consumption of > 40 ml/kg/min.

### PURPOSE OF THE STUDY

This research study is designed to understand the risk of breathing wood smoke on pulmonary, cardiovascular and executive function risk factors.

### **STUDY PROCEDURES:**

You will be asked to participate in three trials, each one occurring one week apart (i.e., Day 2, Day 3, and Day 4). You will be exposed once to either clean air, 250 µg/m<sup>3</sup> or 500 µg/m<sup>3</sup> wood smoke particles in random order. Day 1 of the study will be used to determine eligibility for Days 2-4. Prior to Day 1, you will be called by phone to verify that you have not developed a respiratory infection or other event that would change your health status from the time of enrollment. You will also be reminded to fast for 3 hours before arriving on Day 1. The phone call to check for respiratory infections or other events will be repeated before each day of the study. If you report a change of health status, the visits will be rescheduled.

Day 1: (McGill Hall, ~1.25 hrs) You will be administered oral and written informed consent. You will then be asked to complete a personal information questionnaire and the Physical Activity Readiness Questionnaire (PARQ). You will then undergo a test to determine your maximum oxygen uptake ( $VO_2$  max). This will be used to determine if you are eligible for the study. This test consists of walking and running on a treadmill, which will gradually increase in intensity until you reach a maximal effort. You will be encouraged to continue to walk/run until volitional fatigue. During the entire testing session on

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the treadmill, you will wear a nose clip and headgear that will support a mouthpiece to allow us to measure the amount of oxygen used during the exercise. Heart rate will be measured using an elastic chest strap that is worn under a shirt. You will be asked to fast (not eat) for at least 3 hours prior to this test. This test will take approximately 45 minutes of which 10-20 minutes will be on the treadmill. Also on Day 1, your percentage of body fat will be determined via an underwater weighing test. Your height and weight will first be recorded in a bathing suit. The underwater weight requires that you submerse in our weighting tank (similar to a hot tub) and maximally exhale as much air as possible while underwater and hold your breath for ~4 seconds while we record the weight. This procedure will be repeated until ~3 consistent measurements have been obtained. A nose clip will be provided upon request. This test will take approximately 30 minutes. Following Day 1, if you meet all the inclusion criteria, scheduling will be completed for Days 2, 3, and 4.

Day 2: (Skaggs, ~3 hrs) You will be asked to provide pre-trial samples and undergo baseline tests, consisting of a 1) urine sample, 2) exhaled breath condensate sample, 3) exhaled nitric oxide (eNO), 4) a blood sample, 5) a baseline executive function test, and 6) baseline lung function measurements (see below for details on each type of sample).

Following these pretest measurements you will be exposed to clean air or wood smoke at a concentration of 250 or 500  $\mu$ g/m<sup>3</sup> for 1.5 hours in a controlled setting. Experimental trials and sample collection will be done in the IPPC facility of the Skaggs Building, rooms 061B and 061C. During exposure, wood smoke will be delivered directly via a modified mask respirator to make it more comfortable. You will be asked to walk on a treadmill at a set rate and incline (3.5 mph and 5.7% grade, <57% of maximum) for 1.5 hours. You will be allowed to ingest water throughout the trials.

Measurements will be done within the exposure chamber to monitor the concentration of wood smoke particles delivered to the modified mask during exposure. Concentrations of smoke will be monitored continuously using a DustTrak II (Model 8530). Temperature, humidity, carbon monoxide, and carbon dioxide in the exposure chamber and the exercise room will be monitored with a Q-Trak. Immediately after the 1.5 hour experimental trials, you will be asked to provide another urine, blood, exhaled breath, and eNO sample, undergo lung function testing, and repeat the executive function test. One hour after completing the trial a third set of biological samples will be collected. After the trial and both sample collection times, the study activities will be complete for the visit.

Days 3 and 4: (Skaggs, ~3 hrs each) You will be asked to arrive at the Skaggs building no less than the seventh day after Day 2 when you will be administered the same protocol as Day 1 at a different randomized exposure, and again on the seventh day (Day 4) after Day 3.

The total time commitment will be approximately 1.5 hours on Day 1 of the study, and approximately 3 hours each on Days 2, 3 and 4. These visits will be carried out over a 4-6 week period. No time will be required outside of what occurs at the study site, except for a brief phone call to verify health status.

Tests 1-5 below will be completed immediately before, after, and 1 hour after the exposure to clean air and wood smoke on Days 2-4.

#### 1) Urine Samples

Urine samples will be collected and stored in laboratories within the Skaggs Building (Center for Environmental Health Sciences, or CEHS) prior to the analysis at the University of Montana for indicators of oxidative stress and creatinine. For this collection you will urinate into a sterile plastic cup.

### 2) Exhaled Breathe Condensate (EBC)

For collection of EBC, you will be asked to breathe normally into a cold tube for 10–15 minutes. When you breathe into this tube the exhaled breath will condense and we will be able to collect the

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condensate. EBC samples will be analyzed at the University of Montana (CEHS) for indicators of respiratory inflammation, including cytokines.

3) Exhaled Nitric Oxide (eNO)

Exhaled NO will be measured using a NIOX Mino instrument. During eNO measurements, you will be asked to inhale and then exhale for 10 seconds into the instrument.

### 4) Blood Samples

Approximately 15 milliliters (or 1.5 tablespoons) of blood will be taken from a vein in your arm under the direction of Dr. Charles Dumke into 2 separate tubes at each time point for a total of 45 ml per visit. Blood samples will be stored in CEHS laboratories prior to the following analyses: a) markers of systemic inflammation and cardiovascular risk factors, including blood counts, CRP, clotting factors and cytokines.

b) cell free plasma and quantitating the changes in expression of a panel of oxidative stress related microRNA (miRNA).

c) circulating white blood cells.

d) methylation levels of genomic DNA of genes selected.

### 5) Lung Function

To obtain this data, you will be asked to forcibly exhale into a device called a spirometer to collect measurements of lung function.

### 6) Executive Function Tests

You will be asked to complete a computerized executive function test before and after each of the three trials (n=6). The test will be administered via touch screen computer using proprietary software from Cambridge Cognition. The Cambridge Cognition tests consist of four subtests: Attention Switching Task, Reaction Time, Rapid Visual Information Processing, and Spatial Working Memory. Time to complete these tests is approximately 20-25 minutes.

### Payment for Participation:

If you complete all aspects of the study, you will be given \$300. If you choose not to complete the entire study for any reason, you will be paid \$100 for each experimental trial initiated (Days 2, 3, 4).

### **Risks/Discomforts:**

For this study, we will use clean air, low (250  $\mu$ g/m<sup>3</sup>), and high (500  $\mu$ g/m<sup>3</sup>) wood smoke levels. The lower level of wood smoke particulate matter exposure in this study (250  $\mu$ g/m<sup>3</sup>) is comparable to levels recorded when wood is burned for cooking or heating purposes in homes without sufficient ventilation (200-500  $\mu$ g/m<sup>3</sup>). This lower level of 250  $\mu$ g/m<sup>3</sup> is also consistent with several other human exposure wood smoke exposure studies that have been reported in the literature. The higher level of exposure (500  $\mu$ g/m<sup>3</sup>) is comparable to human exposure studies where subjects were exposed to an average concentration of 485  $\mu$ g/m<sup>3</sup> over a two hour period. It should be noted that even higher levels of wood smoke exposure have been used in other wood smoke exposure studies (1500  $\mu$ g/m<sup>3</sup> for 2 hours). The Forest Service has collected wood smoke exposure data the last four years, with over 300 subjects in 17 States during actual wild land firefighting activities. Their data is showing average PM exposure to be 300-900  $\mu$ g/m<sup>3</sup>, depending on the operational task on the fire (unpublished data).

A wood smoke exposure study reported that the discomfort from smoke was generally weak with

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exposure at 250 µg/m<sup>3</sup>, with the most prevalent symptom being a mild increase in eye irritation in 10 of 13 subjects. A slight increase in nose irritation was reported by 5 of 13 subjects, and 6 subjects reported the smell of wood smoke to be somewhat unpleasant. These subjects were exposed to similar concentrations of particulate matter for 4 hours, more than double the time proposed in this study, so the discomfort experienced should be comparable. The modified mask used in the proposed study covers just the nose and mouth, and would therefore not result in eye irritation. A research assistant will be constantly monitoring both the particulate matter content and your signs of discomfort during all trials.

To our knowledge no previous literature has assessed changes in executive function following wood smoke exposure in humans. Research suggests that exposure to <300 ug/m<sup>3</sup> does not alter lung function, however little is known about exposures at concentrations that resemble those of wild land firefighters. Firefighters following a shift where they were exposed to 6 hours at wood smoke levels of >1000 µg/m<sup>3</sup> reported 11% difficulty breathing, 13% sore throats and headaches, 20% nasal congestion, 24% cough, 29% sputum production, and 36% were asymptomatic. Dr. Paul Smith (Community Hospital) will be our medical consultant on this project. Dr. Smith is one of only two pediatric pulmonologists in the state and the only pediatric intensive care physician residing in Montana. At his recommendation, if you experience an adverse reaction to wood smoke exposure, the mask will be immediately removed and you will be allowed to rest while breathing clean air. If symptoms persist, you will be escorted to the Curry Health Center for examination and treatment. If necessary, you will be referred to Community Medical Center Emergency room where you will be seen by Dr. Paul Smith.

You may experience discomfort when providing an exhaled breath sample due to the breathing requirements of this technique. When blood samples are collected for this study, you may feel a slight sting or "pinch" in your arm, you may suffer a small bruise, and there is a very slight possibility of infection. Should you notice unusual redness, bruising, or swelling at the blood sampling site you should seek medical attention and contact the study director, Tony Ward.

### **Benefits:**

You will receive no direct benefit from participation in this study. You will receive information on your physical fitness (VO<sub>2max</sub>), and body composition. However, this research will provide useful information about how exposure to smoke can affect acute changes in decision making and memory and other general health indices. This will be particularly useful for wild land firefighters, who are asked to perform strenuous physical activities while being exposed to wood smoke. Results from this study will also provide additional information on the health effects to the general public following wood smoke exposure.

### **Confidentiality:**

You will be randomly assigned an identification number at the start of the study. All samples, questionnaire responses, physiological measurements, and other data collection forms will be labeled with this number. Personal information will also be collected and linked to this ID number. Data will be recorded on the Data Collection Forms and entered in an electronic database that will contain your ID number and no other personal identifiers. Signed informed consent forms and the key linking the identification number to your name and contact number will be under the control of the principal investigator (Ward) and kept separate from data collection materials. After completion of the study protocol, the key linking identifying personal information to their respective study ID number will be destroyed. A separate sheet containing only the contact information with no identification numbers will be kept until data analysis is complete.

### **Compensation for Injury:**

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Although we believe that the risk of taking part in this study is minimal, the following liability statement is required in all University of Montana consent forms: In the event that you are injured as a result of this research you should individually seek appropriate medical treatment. If the injury is caused by the negligence of the University of Montana or any of its employees, you may be entitled to reimbursement or compensation pursuant to the Comprehensive State Insurance Plan established by the Department of Administration under the authority of M.C.A., Title 2, Chapter 9. In the event of a claim for such injury, further information may be obtained from the University's Risk Manager (406-243-2700; kathy.krebsbach@umontana.edu) or the Office of Legal Counsel (406-243-4742; legalcounsel@umontana.edu). (Reviewed by University Legal Counsel, May 9, 2013)

### Voluntary Participation/Withdrawal:

It is important that you realize that you are free to withdraw from the study at any time. If you chose to withdraw please notify Tony Ward (contact info below) as soon as possible. As mentioned above, even if you decide to drop out of the study, you will receive compensation for all the test sessions you complete. A copy of this consent form will be provided for you at your request. In addition, the data collected during this study will be done at no cost to you.

### Questions:

You may wish to discuss this with others before you agree to take part in this study. If you have any questions about the research now or during the study contact Dr. Tony Ward, PhD at (406) 243-4092 (tony.ward@umontana.edu). If you have any questions regarding your rights as a subject, you may contact the chair of the IRB through the University of Montana Research Office at (406) 243-6672.

### Statement of Your Consent:

I have read the above description of this research study. I have been informed of the risks and benefits involved, and all my questions have been answered to my satisfaction. Furthermore, I have been assured that any future questions I may have will also be answered by a member of the research team. I voluntarily agree to take part in this study.

Printed Name of Subject

Subject's Signature

Date

# STATEMENT OF CONSENT TO BE PHOTOGRAPHED DURING DATA COLLECTION

During the study, I understand that pictures may be taken. I provide my consent to having my picture taken during the course of the research study. I provide my consent that my picture may be used in some presentations related to this study. If pictures are used at any time for presentation, names and physiological data will not be associated with them.

Signature

Date \_\_\_\_

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## Recruitment flyers for wood smoke study:



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Locations: Human Performance Lab and Skaggs Building Inhalation Facility

Times: Participants will be enrolled starting in July 2014. The total time commitment is ~4 weeks which includes 4 individual visits (1 day per week).

- Day 1: eligibility will be determined and initial body measures (body fat content and VO<sub>2</sub> max tests) will be recorded (~1.25 hours)
- Days 2-4: subjects will walk on a treadmill while being exposed to low levels of wood smoke or clean air (1.5 hours on treadmill, ~3 hours total).
  - A cognitive function test will be administered and biological samples (blood, urine, and breath) will be collected before and after each 1.5 hours on the treadmill.
- Upon completion of the study, subjects will receive \$300.

<u>Participation Criteria</u>: Each of the subjects will be male, 18-40 years of age, non-smoker, and with no pre-existing chronic diseases such as asthma and/or related lung disease. Participants should not be exposed to smoke frequently at home or work (via cigarettes, wood stoves or other devices). Individuals must be able to complete moderate exercise (walking on a treadmill) on days 2-4.

Results from this study will provide important information about how wood smoke impacts wild land firefighters. This study will also be important in understanding the impact of residential wood smoke on health.



Preliminary mockup of human inhalation system:





Final schematic employed for controlled human wood smoke exposures: