DNA methylation changes associated with exposure to wildfire smoke in dogs enrolled in the Dog Aging Project

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Public Health

University of Washington
2023

Committee:
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Program Authorized to Offer Degree:
Institute of Public Health Genetics

University of Washington

Abstract

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This study investigates the association between wildfire smoke and DNA methylation changes and how those changes may cause disease. We utilize data from the Precision Cohort in the Dog Aging Project which includes methylome data from peripheral blood mononuclear cells for 527 dogs. Our analysis employs reduced-representation bisulfite sequencing to measure DNA methylation levels. Wildfire smoke exposure was defined using fire report data available from the National Interagency Fire Center. Our results indicate that exposure to wildfire smoke leads to a significant change in DNA methylation, with 667 sites hypermethylated and 198 sites hypomethylated. Functional gene annotation indicates that there are several associations between the upregulation (hyper-) and downregulation (hypo-) of the significant genes. These biological implications include factors contributing to cardiovascular disease, lung disease, and cancer which are diseases known to result from smoke exposure. While the evidence from this study does not prove the causal link between DNA methylation and disease, it does show that there are DNA methylation changes associated with exposure to wildfire smoke.

I. Introduction

Human health is increasingly impacted by wildfires

The human contribution to climate change has led the total area burned by forest fires in the western US to double between 1984 and 2015.[1] Climate models have projected a 50-120% increase in current burned area across the western US by 2100.[2] The main emissions from wildfires include fine particulate matter (PM), carbon monoxide (CO), methane, nitrous oxide (N_2O), nitrogen oxides (NO_x), and volatile organic compounds (VOC).[3] Wildfires contribute 15-30% of atmospheric $PM_{2.5}$ in the United States.[2] The associated costs of vulnerable populations impacted by wildfire smoke increases with exposure.[3] Analysis suggests that wildfire smoke in the Western US contributes $11-20 billion in annual health costs.[3]

The Dog Aging Project

The Dog Aging Project is a nationwide project studying the impact of genes, lifestyle, and environment on aging. For each dog nominated, a Health and Life Experience Survey (HELS) is completed to enroll the dog in the Dog Aging Project Pack (DAP Pack) which currently contains about 45,000 dogs. This initial survey gathers information about general demographics, physical activity, home environment, behavior, diet, medication, and health status. There are also annual follow-up surveys that gather updated information for all of these characteristics. From there, subsets of dogs are invited to join more in-depth studies. One of these is called the Precision cohort which contains about 1,000 dogs that have submitted blood samples for sequencing information. This analysis uses data from this cohort and includes 527 dogs for which methylome, health, and environmental data are available.

Wildfire smoke exposure and disease

Particulate matter (PM) is typically used as a proxy to estimate air pollution resulting from wildfire smoke. Nearly 90% of particulate matter from wildfire smoke is considered fine particulate matter which are $PM_{2.5}$ particles 2.5μm or smaller.[4] The National Ambient Air Quality Standard for a 24-hour average concentration of $PM_{2.5}$ is 35μg/m^3 or below, while ambient concentrations in the vicinity of a wildfire can be as high as 6106μg/m^3 hourly and 394μg/m^3 daily.[5] However, these data can be difficult to track since PM concentrations can vary spatially depending on wind direction and by temperature and humidity.[5]

Exposure to particulate matter (PM) in wildfire smoke is known to cause irritation to the eye and respiratory tract and is a more serious risk for pulmonary function and exacerbation of pulmonary disease.[3–5] To understand this biological relationship, studies have linked wildfire smoke exposure to the release of inflammatory proteins and increased oxidative stress in the body.[3,5,6] Studies have also found significant associations between wildfire smoke exposure and overall systemic inflammation, changes in bone marrow content, and changes in physical strength.[7] There is limited evidence about the health impact of multi-day continuous exposure. However, there is concern that wildfire smoke exposure may affect lifetime disease risk.[4] One study attempting to illuminate this examined adult males exposed to wildfire smoke during the 1997 Indonesian wildfires. This study demonstrated that these men had decreased lung function after 10 years, controlling for the effect of aging.[8]

In general, most adults and children can bounce back quickly from acute health effects, while some at-risk groups experience greater and longer-lasting health effects.[4] The primary indicators for an at-risk group are age, health status, and socioeconomic status.[4] The most impacted groups are those with previous diseases (asthma, COPD, cardiovascular disease),

people of low socioeconomic status, children, pregnant women, older adults, and outdoor workers.[3,4] Acute exposure to wildfire smoke can also result in low birth weight and other detrimental birth outcomes.[3,9]

Epigenetics and DNA methylation

It is possible that the diseases that result from air pollution and smoke exposure are epigenetically regulated. The field of epigenetics has developed into a field of novel discovery along with genome- and exome-wide association studies when examining the effects of the environment on human health. Epigenetics can be defined as the processes involved in gene regulation that are independent of the DNA sequence itself.[10] Epigenetic regulation is what allows cells to differentiate beyond pluripotent stem cells to allow for altered activity states.[10] While there are several mechanisms involved in epigenetic regulation, genome-wide patterns of methylation can be informative in determining the basis of disease.

DNA methylation is the addition of a methyl group on the C5 position of cytosine on a CpG site in the DNA sequence. Methylation at these sites is important for transcriptional regulation, silencing of transposable elements, and overall integrity of the genome.[11] Methylation of the CpG sites can modify gene splicing and produce differing mRNAs which can result in phenotypic changes.[12] The patterns of DNA methylation are established during development and determine tissue-specific functions.[10] These patterns are erased in primary germ cells but are recreated via de novo methylation post-implantation and can extend into early childhood.[11,13] However, alterations to DNA methylation patterns after the de novo period can result in disease, including cancer.[10] There are two types of DNA methylation: de novo DNA methylation in which a methyl group is added to an unmethylated DNA sequence, and maintenance of DNA methylation where unmethylated cytosine residues on partially methylated DNA sequences are

methylated after cycles of DNA replication.[11] The methylation maintenance is carried out primarily by DNA methyltransferases.

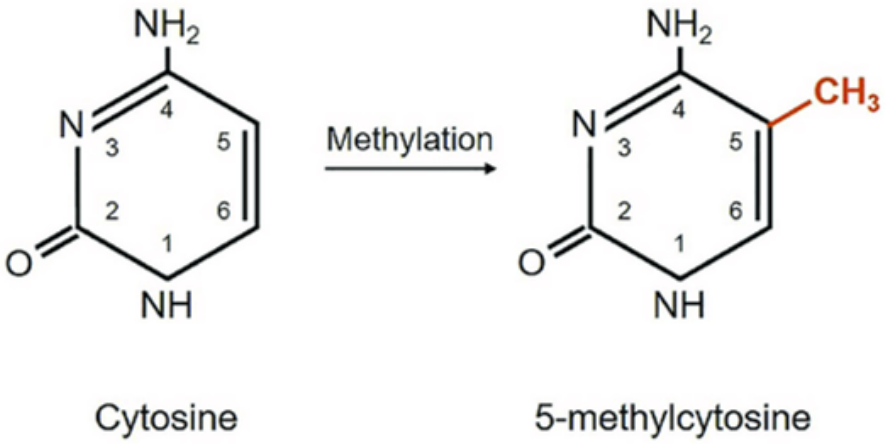

Figure 1 Cytosine is methylated on the C5 location, as numbered above, and is then considered 5-methylcytosine.

Wildfire Smoke, DNA Methylation, and Disease

DNA methylation is a molecular mechanism of genome regulation that can be impacted by the physical environment.[11] While there are many mechanisms of gene regulation, DNA methylation is a practical measure often used in research to understand environmental impacts on the genome. There are different mechanisms in which smoke exposure could alter existing DNA methylation patterns including DNA damage recruiting DNA methyltransferases, upregulation of DNA methyltransferase genes, increased expression of genes allowing for methyl group availability, and DNA binding proteins protecting against methylation.[14] Research has been done examining DNA methylation and its association with wildfire smoke exposure. Analysis of particulate element components using whole blood samples in adults found 343 probes that were differentially methylated due to particulate matter from wood burning, reinforcing how impactful $PM_{2.5}$ is as a primary component of woodsmoke.[15] In inhalation studies where adults were

exposed to simulated smoke, methylation of *IFN-γ* is significantly associated with exposure to $PM_{2.5,}$ indicating that $PM_{2.5}$ exposure might modulate immune function.[16,17]

DNA methylation changes resulting from smoke exposure can also have a detrimental effect on fetuses exposed while still in the womb. Studies were done that analyzed cord blood for DNA methylation changes and demonstrated that hypermethylation of *IFN-γ*, *NR3C1, AARCD3*, *BAI1*, and hypomethylation of *CTNNA2* are all associated with exposure to $PM_{2.5}$ from smoke.[18–20] Changes at these genes could indicate changes in immune response and inflammation in the case of *IFN- γ* and *NR3C1*, or changes in tumor suppression in the case of *AARCD3*, *BAI1*, *CTNNA2*.[12,17,21–24] Additionally, studies have reported differentially methylated regions of *FOXP3* and *IL10*, both genes involved in immune regulation and response, in children with asthma.[25–27] This relationship is possibly related to the hypomethylation of pro-coagulant genes (*SERPINE1*), pro-vasoconstriction genes (*ACE*, *EDN1*), and proinflammatory genes (*CD40LG*).[6]

The average methylation at satellite repeats *LINE-1* and *ALU*, when measured in blood cells, is correlated with environmental exposures including air pollution.[13] This relationship is reflected in studies where hypomethylation of *LINE-1* and *ALU* was found to be generally associated with smoke exposure in adult populations.[6,12] *LINE-1* hypomethylation is also associated with inflammation and increased morbidity and mortality in cardiovascular disease (CVD).[6,28] Because of its established causal relationship with cardiovascular disease, DNA methylation could be a potential tool to understand how methylation of these regions can influence disease and how smoke exposure can impact that relationship.[6,29]

Primary Questions

This analysis will determine if DNA methylation changes are associated with exposure to wildfire smoke and therefore associated with the known health effects resulting from this

exposure, according to our hypothesis as shown in Figure 1. We will assess peripheral blood mononuclear cells sampled from dogs in the Dog Aging Project for these DNA methylation changes, using reduced-representation bisulfite sequencing (RRBS). We will compare these values with $PM_{2.5}$ concentration values retrieved from the Environmental Protection Agency for the geographical area where the dog lives to estimate wildfire smoke exposure. To expand this analysis, we will compare the same geographic $PM_{2.5}$ concentration data with the owner-reported health of the dog to estimate the relationship between DNA methylation, exposure to wildfire smoke, and resulting health.

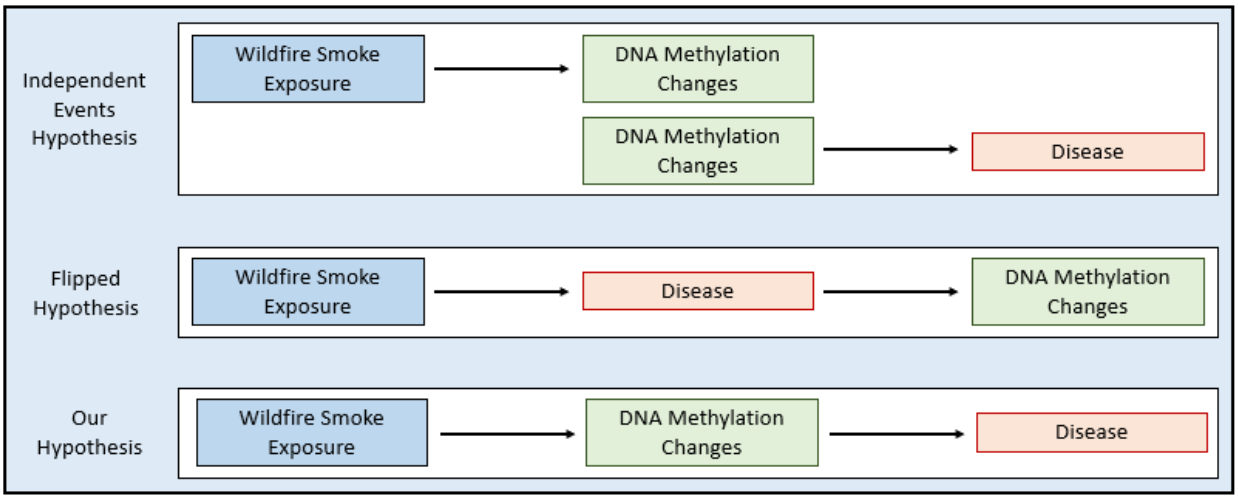

Figure 2 When looking at wildfire smoke exposure and two different outcomes (DNA methylation changes and disease), there are three different causality hypotheses that can be tested. The first is that wildfire smoke exposure causes DNA methylation changes and, independently, DNA methylation changes cause disease. The second is that wildfire smoke exposure causes disease which causes DNA methylation changes. The final, our hypothesis, is that wildfire smoke exposure causes DNA methylation changes which cause disease.

II. Methods

Dog Aging Project Precision Cohort

DNA methylation data are available for 530 dogs in the Precision Cohort of the Dog Aging Project. Specifically, our team collected methylome data for Peripheral Blood Mononuclear Cells obtained from blood samples. The time and location of the collection are recorded for all of the dogs sampled. Additionally, health data are also available for each dog via electronic medical records and the owner-reported Health and Life Experience Survey.

Reduced-Representation Bisulfite Sequencing

We will use bisulfite sequencing in this analysis to measure DNA methylation. This method works by utilizing the fact that the amination reaction between cytosine and 5-methylcytosine proceeds differently when DNA is treated with sodium bisulfite. As a result, after treatment, unmethylated cytosines are converted into uracil residues while 5-methylcytosines remain unaltered. When amplified via PCR and sequenced, the uracil residues are recognized as thymine and are distinguished from 5-methylcytosine.[30] Reduced-representation bisulfite sequencing is a variant of the method that uses restriction enzymes to produce sequence-specific DNA fragmentation.[31] This allows for targeted measurement of methylation at specific regions of interest.

Methylome Data Cleaning and Quality Control

Data processing for bisulfite sequencing depends heavily on read alignment to the reference genome. This process can be carried out with short-read aligners followed by high-throughput sequencing. Aligning the sequence can be done with either wild-card aligners or three-letter aligners. Trimming adapters, aligning sequences, and mapping to the reference

genome were steps performed before this analysis. Quality control measures that are part of this analysis include accounting for sample ID and pool ID.

Wildfires and Air Pollution

Air pollution resulting from wildfire smoke can be assessed in a variety of ways. There were two aspects considered when defining wildfire smoke exposure. There are daily measurements of air quality concentration available from the Environmental Protection Agency for each active air quality monitoring site in the United States.[32] We determined the correct monitoring site for each dog in our population by assessing which monitoring site was the closest to the reported zip code from the HLES. The maximum Air Quality Index (AQI) score was determined for each dog within 30 days before sampling using. We then supplemented these data with reported wildfires from the National Interagency Fire Center.[33] Exposure to a reported fire was defined as a binary by determining if a fire was reported in the country each dog resides in within 30 days before sampling. Due to missing location information, this analysis was only able to utilize data from 527 dogs for which methylome data was available.

Identifying Differently Methylated Genes

Data for this analysis consisted of methylated and total promoter counts for 4,143 genes for all samples. Using these data, we used a generalized linear mixed model fit to a binomial mixed model. The function inputs the methylated promoter counts, the total promoter counts, binary exposure data, and confounding variables. Using these inputs, the model weighs the predictor variable (exposure) with genetic relatedness and independent environmental relatedness to determine if there are promotor sites that are differently methylated between

exposed and non-exposed samples. The directionality of methylation (hyper- or hypo-) was determined using the effect estimates of the binomial model.

Functional Gene Annotation

To determine the functions of the genes of the differently methylated sites, we used two web-based software; the Database for Annotation, Visualization, and Integrated Discovery (DAVID)[34,35] in combination with g:Profiler.[36] The lists of hypermethylated and hypomethylated genes were input separately into DAVID to cluster the genes by functional area. Each list of genes within each function cluster was then input into g:Profiler to cluster further by specific function to gather a clearer picture of the biological implications of the known gene functions.

III. Results

Exposure to Wildfire Smoke

Using data from the National Interagency Fire Center, we determined that of the 527 dogs in this analysis, 134 are considered to be exposed and 393 are not considered to be exposed. The AQI score is calculated and reported using the most prominent criteria pollutant (ex. CO, NO_2, Ozone, PM_{10}, $PM_{2.5}$) present in the air that day. Using this information we plotted the maximum AQI value for each dog between the exposed and non-exposed groups, identifying the defining pollutant of that AQI score as well (figure 3). The difference between exposure groups for $PM_{2.5}$ and PM_{10} was not significant; however, the difference between exposure groups for Ozone was. The highest AQI scores for the exposed group were also defined by PM_{10} and $PM_{2.5}$. The proportions of defining pollutants between groups were explored further and both PM_{10} and $PM_{2.5}$

are a larger proportion of the defining pollutants in the exposed group than in the non-exposed group (figure 3).

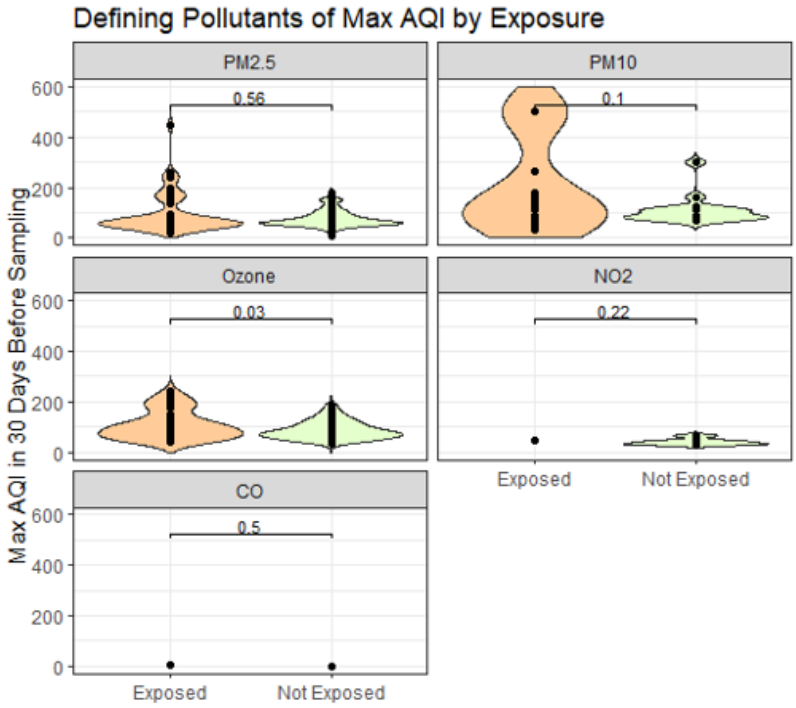

Figure 3 Maximum AQI value from the 30 days before sampling is plotted between exposed and non-exposed groups (p-values obtained using Wilcox ranked-sum test). Defining pollutant for each AQI score is also labelled for each value.

Differently Methylated Sites

After fitting the methylation count data to our model, the methylation at 865 gene sites was identified as significantly different between exposure groups. Using the effect estimates from the binomial model to determine directionality, 667 gene sites were identified as hypermethylated and 198 gene sites were identified as hypomethylated (figure 4).

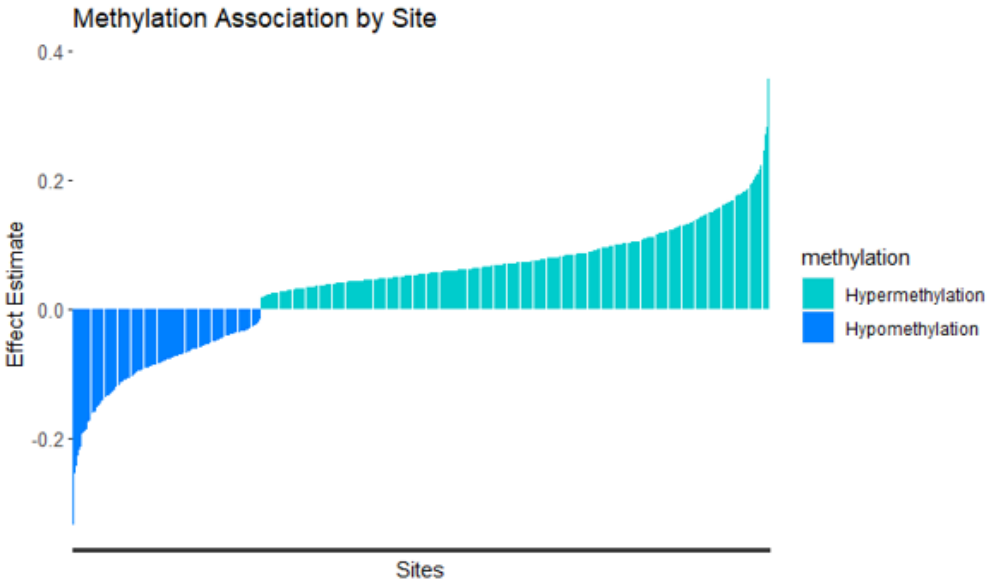

Figure 4 The effect estimates are plotted for each site. A negative effect estimate indicates the site is less likely to be methylated in the exposed group (hypomethylated). A positive effect estimate indicates the site is more likely to be methylated in the exposed group (hypermethylated).

Functional Gene Annotation

After inputting the hypermethylated gene list, the results from DAVID returned six significant annotation clusters, These six clusters represent six different biological associations represented by the list of genes the clusters contain. The clusters returned for the hypermethylated genes include the tumor necrosis factor receptor (TNFR) family, Rap1 signaling pathway, endocrine-related calcium absorption, dilated cardiomyopathy, cAMP signaling pathway, and aldosterone synthesis/secretion. We plotted the relationship between these clusters and the genes within them and determined that there were multiple genes that contributed to several clusters (figure 5).

The TNFR superfamily contains receptor protein domains that bind tumor necrosis factors. These proteins play a main role in cell death pathways and regulating immunity.[37,38] Therefore, the downregulation of these genes could impact adaptive immunity. The Rap1 signaling pathway regulates cell formation and adhesion. As a result, its downregulation has

been shown to promote metastasis and adhesion in melanoma and prostate cancer.[39] Dilated cardiomyopathy is a disease of the heart muscle where the heart muscle is weakened and one or more ventricles are enlarged.[40] Downregulation of calcium absorption results in hypocalcemia. While hypocalcemia is primarily asymptomatic, it can cause muscle weakness, fatigue, seizures, etc.[41] The cAMP signaling pathway is involved in several cell types. The downregulation of cAMP signaling has been shown to result in health complications related to cataracts, diabetes, cancer, and cardiovascular disease.[42,43] Aldosterone regulates serum electrolytes by controlling sodium absorption and potassium excretion in the kidney. Downregulation of aldosterone synthesis can result in hypovolemia, hyponatremia, and hyperkalemia.[44]

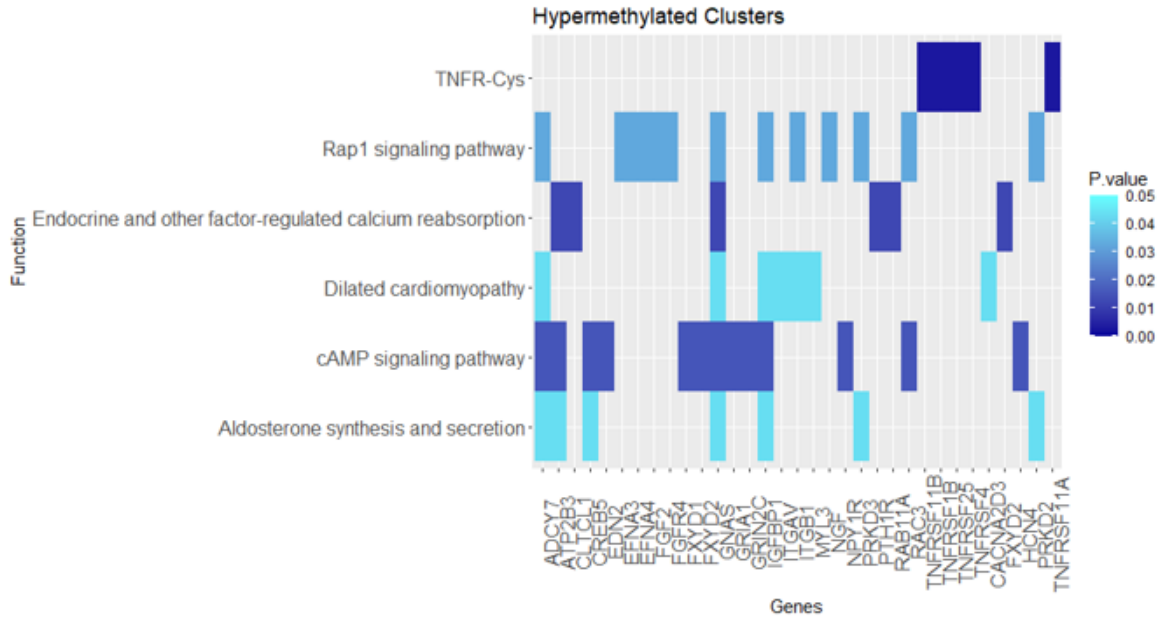

Figure 5 Heatmap for the hypermethylated genes showing the relationship between the genes and the biological attributes they contribute to, displaying the p-value to represent the strength of the contribution.

After inputting the hypomethylated gene list, the results from DAVID returned two significant functional annotation clusters. More specific information was derived within each cluster from the g:Profiler results. We plotted the relationship between the genes within the two clusters and their biological significance (figure 6). Using these relationships, the genes which

contributed the most within each cluster were explored further and we determined three general functional areas: Endocrine, Neurologic, and Cancer.

The genes contributing to endocrine function, *AVPR2,* and *PRLHR*, have been shown to contribute to diabetes insipidus and obesity when upregulated.[45,46] The upregulation of *CHRNA9, HTR1D,* and *HTR2A* can contribute to nicotine dependence, migraines, anxiety disorders, and depression.[47–49] Several of the hypomethylated genes (*PTGER1, GRIN2D, SSTR2, VEGFC, VEGFD*) have also been shown to contribute to various cancers including ovarian seromucinous carcinoma, colorectal cancer, neuroendocrine tumor, lung carcinoma, and breast carcinoma.[50–54]

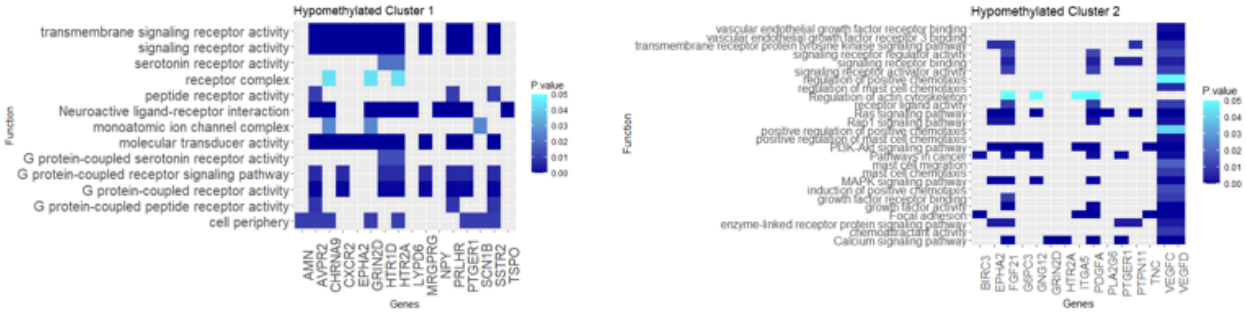

Figure 6 Heatmap for the hypomethylated genes showing the relationship between the genes and the biological attributes they contribute to, displaying the p-value to represent the strength of the contribution.

Translating Gene Function to Known Health Data

There were 83 dogs in this analysis that reported a health condition that was diagnosed after they were sampled, and thus, after their exposure. There were more reported health conditions in the non-exposed group than in the exposed group. The top three reported conditions in the exposed group were skin, eye, and gastrointestinal conditions while the top three reported conditions in the non-exposed group were skin, mouth/dental/oral, and gastrointestinal conditions (figure 7A). There were 10 dogs in this analysis that reported a cancer diagnosis that occurred after sampling. There were dogs that reported multiple cancers, but overall there were more cancers reported in the non-exposed group than in the exposed group.

While the top recorded cancer was of an unknown type, the next top three reported cancers were lymphoma, liver, and skin cancer (figure 7B). The age distributions were determined to be similar between the two groups to account for possible age bias.

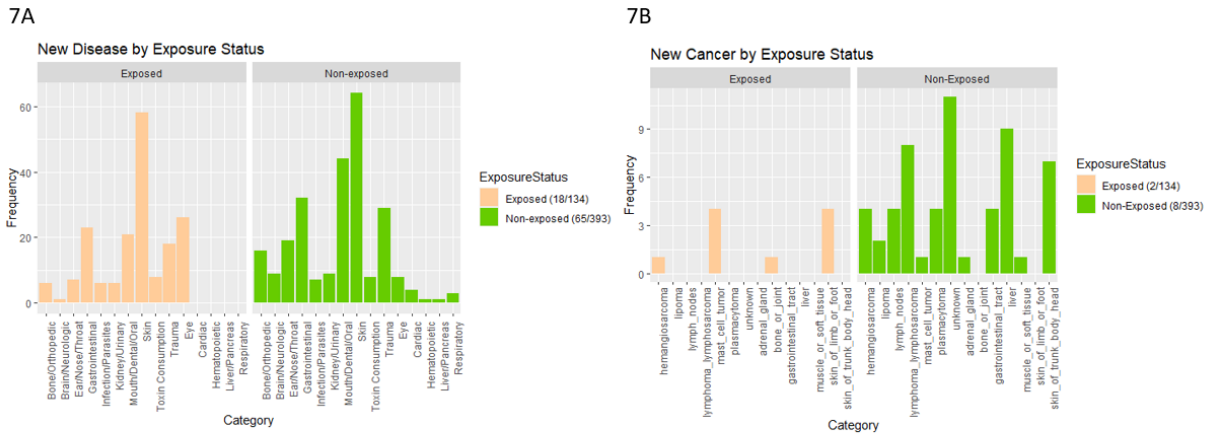

Figure 7A Reported diseases post-sampling between exposure groups (n=83). **Figure 7B** Reported cancer diagnoses post-sampling (n=10). There were dogs that reported multiple health conditions or cancer diagnoses and were counted as such.

IV. Discussion

Conclusions

There is clear evidence that there are methylation changes that occur with exposure to wildfire smoke. There is documentation supporting the role that the genes analyzed play in disease when they are upregulated (hypomethylation) or downregulated (hypermethylation). The diseases that can be caused by changes in these gene regulations are also seen from exposure to wildfire smoke, such as cardiovascular disease, lung disease, and cancer. Despite these results, it is difficult to draw a direct causal link between the changes in methylation and the disease reported in the dogs.

This relationship cannot be reinforced due to the limitations of this project. One limitation is that there is only one sample per dog which limits our ability to see the progression of methylation changes and disease on a longitudinal scale. The association between methylation changes and exposure could be strengthened if samples were taken before and after a known

exposure. Additionally, if there were documented changes in methylation associated with disease over time, there would be a stronger indication of a causal relationship. The exposure was also defined retrospectively based on geographical data, which made it difficult to determine exposure with accuracy. While we were able to determine AQI and fire reports nearest to the dog's location, there are other factors that may augment exposure like true proximity to the fire and time outside during a fire. Sampling was also not done according to the exposure. If the sampling was done on dogs with known exposures or non-exposures, a more accurate picture of methylation change could be obtained.

Future longitudinal studies will soon be able to be conducted as the Dog Aging Project progresses. Annual samples will be collected from the Precision Cohort which will facilitate a longitudinal study. There is also potential for long-term exposure to be studied. This analysis focused on short-term exposure accounting only for exposure within the 30 days before sampling. However, a different approach could account for any exposure that occurred before sampling.

Implications

This analysis contributes to a body of work that examines DNA methylation in association with environmental exposures. There is evidence that DNA methylation is altered by exposure to wildfire smoke. While the link between methylation and disease needs further work to explore, it is still important to consider these changes. There has been a marked increase in wildfires across the western United States in the last 40 years which is projected to increase further.[1,2] More people will be exposed to wildfire smoke and experience resulting health problems. This impact on health needs to be considered in the many discussions to mitigate climate change.

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