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DNA Methylation in Humans and Dogs: Evaluating the Impact of Nutritional and Particulate Exposures

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Evaluating the Impact of Nutritional and Particulate Exposures

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ABSTRACT

Asthma is among the most common chronic diseases in children, among the leading causes of hospitalization for children, and one of the leading causes of school absenteeism. Discovery of preventable factors and the underlying mechanisms that exacerbate asthma among prevalent cases is important for mitigating the health burden of this childhood disease. Environmental factors such as exposure to particulate matter (PM) or nutritional deficiencies can lead to poor respiratory health among susceptible children. Recent animal studies suggest that PM exposures and dietary nutrients may influence asthma pathways through epigenetic mechanisms. Epigenetic modifications are heritable changes that can regulate gene activity without altering the primary DNA sequence. With respect to allergic or asthma phenotypes, several cytokine and transcriptional factors relevant to the allergy/asthma response pathway and its counter regulation have been shown to be modulated by epigenetic processes. The results of the following research aims may help to elucidate the relationship between PM and dietary exposures, epigenetic responses, and respiratory health outcomes among asthmatic children.

Aims 1 and 2 occur within the context of an ongoing randomized trial of indoor air quality interventions and quality of life among asthmatic children living in homes that heat with wood stoves. Our laboratory has demonstrated that children in wood stove homes are exposed to elevated concentrations of indoor PM smaller than 2.5 microns ($PM_{2.5}$) due to biomass combustion. Aims 1 and 2 take advantage of the extensive research infrastructure currently in place for exposure assessment, health outcomes characterization and biomarker analysis.

Human epidemiological studies are limited by ethical considerations and subject to a number of variables, which make interpretation of results problematic in some cases. Aim 3 is an exploratory aim to establish the utility of using dogs as sentinels for human health effects with respect to $PM_{2.5}$ exposure. Domestic animals, specifically dogs, have been used in this way both in lieu of human studies and in parallel. This multifaceted approach will increase opportunities for success in determining the effects of environmental exposures on human health outcomes in this study and may inform future intervention strategies and study design.

Overall hypothesis: $PM_{2.5}$ and dietary factors will cause hypo- and hypermethylation changes modulating cytokine expression associated with asthma symptoms.

ACKNOWLEDGEMENTS

It is through the support of family and friends along with a good deal of luck that I ended up in Missoula, MT. I feel very fortunate to have completed my graduate education in this beautiful and wild state. My parents really deserve most of the credit, although I am not sure they really know how I ended up in Montana either. Their love of the west surely affected the trajectory of my adulthood. When I decided to follow my passion for science out of the state Ohio, my parents supported the decision without hesitation. I had to be a big brother over the phone and an uncle vicariously through Facebook, but this never seemed to have an effect on the quality of our relationships. Thank you for always believing in me.

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CHAPTER 1

INTRODUCTION

This dissertation describes how exposures to particulate matter (PM) or dietary nutrients influence respiratory health in a cohort of asthmatic children and investigates the potential for these exposures to work through an epigenetic mechanism (i.e. DNA methylation). As a compliment to the research aimed at determining the effect of PM exposure in the cohort of asthmatic children, a sentinel sled dog model will be explored. The introduction will 1) provide an overview of epigenetics and review the current research on DNA methylation and chronic diseases including asthma, 2) describe the potential impact of PM and dietary exposures in the context of asthma, 3) describe the parent study and give context to the subcohort (Aims 1 and 2) and lastly 4) discuss the rationale for animal sentinel models of environmental exposures and health effects (Aim 3).

Chronic Inflammatory Disease: Asthma

According to the World Health Organization (WHO), over the next 20 years there will be a major disease paradigm shift as many countries enter the 'developed' world. The WHO suggests that infectious diseases such as HIV or malaria are becoming less of a health burden worldwide. In contrast, chronic diseases, also called non-communicable diseases, such as heart disease, lung disease, and diabetes are becoming the major causes of death worldwide. At the present time, non-communicable diseases are responsible for 63% of deaths worldwide, which is about double the percentage of deaths attributed to infectious diseases.

The central feature to most of these chronic diseases is inflammation. Inflammation is one of the human body's immediate responses to insult by toxins, toxicants, or physical injury. This mechanism evolved for the short-term healing of damaged tissues (Weiss 2008). While acute inflammation can be helpful and even necessary, chronic inflammation can be destructive and debilitating. Chronic inflammation is linked to several human diseases and in such cases the inflammation is characterized as dysregulated, maladaptive, and persistent. The pathways and molecular processes that lead to the development of asthma, diabetes, obesity, heart disease and other chronic inflammatory diseases (CIDs) have not been fully elucidated, though it is known that chronic inflammation plays a major role. Studies in animals and humans suggest that environmental toxicants are capable of influencing the immunological pathways responsible for the inflammation involved in diseases such as asthma (Crinnion 2012).

Asthma Description and Pathology

Among children, asthma is the most common non-communicable disease. Asthma is characterized by airway obstruction, bronchial hyperresponsiveness, and chronic inflammation (National Heart 2007). Asthma as a disease diagnosis encompasses varying degrees of the above mentioned symptoms and this variability can change within a patient throughout their life (National Heart 2007). Asthma pathology is characterized by spasms by the smooth muscle surrounding the airways, a decrease in size of the inner airway space, infiltration by immune cells, and mucus production (see Figure 1.1.). The manifestation of asthma pathophysiology is driven largely by the underlying inflammation and its interaction with airflow and response to insult (Cohn et al. 2004). Asthma symptoms may include recurrent wheezing, breathlessness, chest tightness, and coughing (National Heart 1997). Rescue medications for these symptoms typically treat the bronchial spasms, but there are medications designed to treat the underlying

inflammation (National Heart 2007). It can take up to weeks for the latter medications to completely resolve the inflammation and patient response is variable (Bateman et al. 2004).

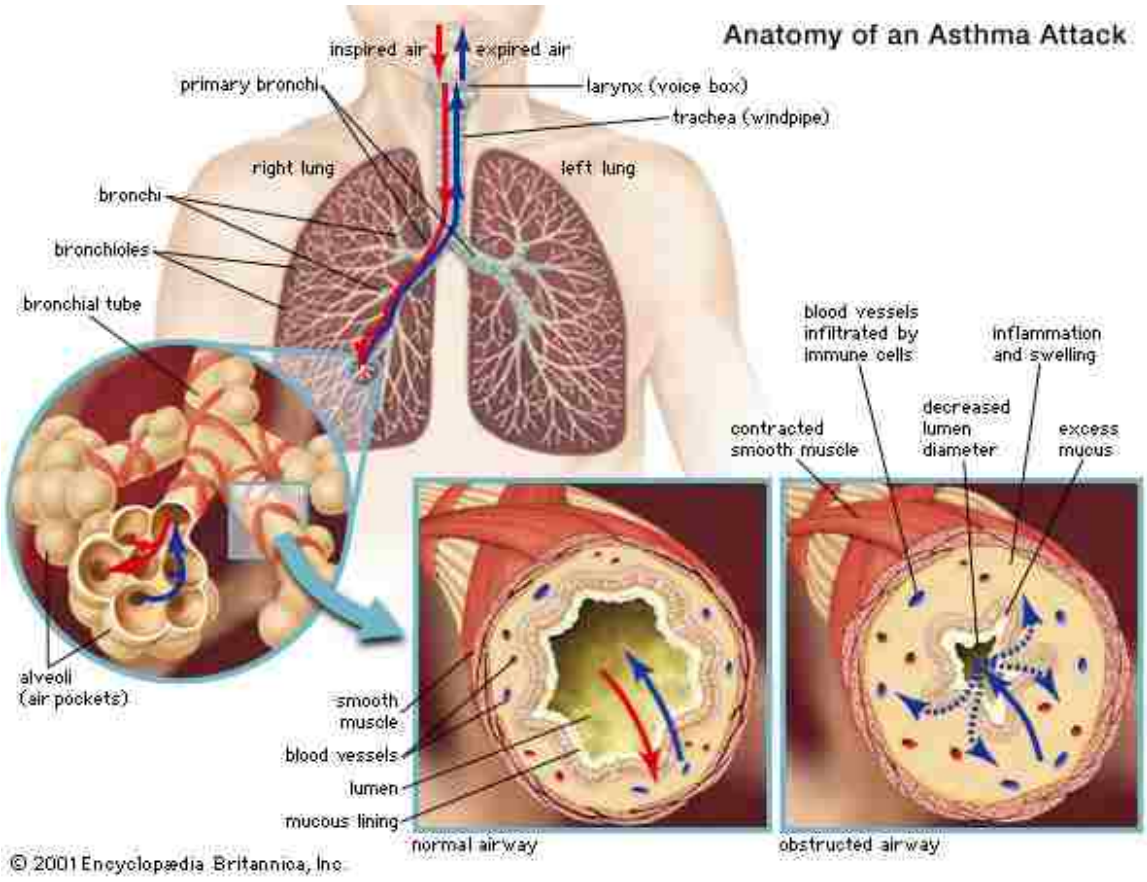

Figure 1.1. Anatomy of an asthma attack.

This figure shows the potential physiological changes that can occur during an asthma attack.

Allergic Asthma and Triggers

Allergic asthma, also known as extrinsic asthma, is the most common form of asthma affecting the majority of the 25 million Americans with the disease (Asthma). Allergic asthma accounts for nearly all cases of childhood asthma in contrast to intrinsic or adult-onset asthma (Pearce et al. 1999). For an individual with allergic asthma, the first time an allergen is inhaled, it is taken up by an antigen-presenting cell in the lining of the airway and presented to a T cell (Allergy). Through series of cell-to-cell interactions, B-cells produce allergen specific antibodies, which are released and prime mast cells. This prepares the immune system for a robust response the next time this allergen is inhaled. This process is called sensitization. According to the CDC, triggers for allergic asthma may include pollen, pet dander, dust mites, cockroach or pest such as mice, mold, ambient PM, chemical irritants and others.

Asthma Mechanism: Th1/Th2 balance

As mentioned above, a hallmark of asthma is infiltration of the airway blood vessels by immune cells. Several cells and cellular factors (see Figure 1.2.) are known to play a role in asthma pathology including eosinophils, T lymphocytes, mast cells, macrophages, neutrophils, and epithelial cells (National Heart 2007). Mast cells and eosinophils are considered the main effector cells and upon stimulation, these cells can secrete inflammatory mediators (Mazzarella et al. 2000). The immune response is orchestrated by T lymphocytes, which are involved in cell-to-cell communication. CD4+ T cells, also known as T helper (Th) cells, have been well studied. This group of cells has been divided into two main categories based on the cytokines and chemokines they produce (Mosmann and Coffman 1989). Th1 cells produce interleukin-2 (IL-2), IL-12, interferon gamma (IFNγ), and other factors. Th2 cells produce IL-4, IL-5, IL-13, and other factors. Importantly, Th2 cells produce little or no IFNγ. Research suggests that

there is interplay between Th1 and Th2 cells through the production of the cytokines and in this way can regulate each other. For example, secretion of IFNγ by Th1 cells activates macrophages and increases their bactericidal activity (Fiorentino et al. 1989), while concomitantly down regulating Th2 cells and their ability to recruit mast cells and eosinophils (Wenner et al. 1996). Th2 cells are suggested to play a major role in the pathogenesis of asthma and many studies have demonstrated a shift in the balance of Th1 toward a Th2 cellular phenotype in the asthmatic airway (Mazzarella et al. 2000).

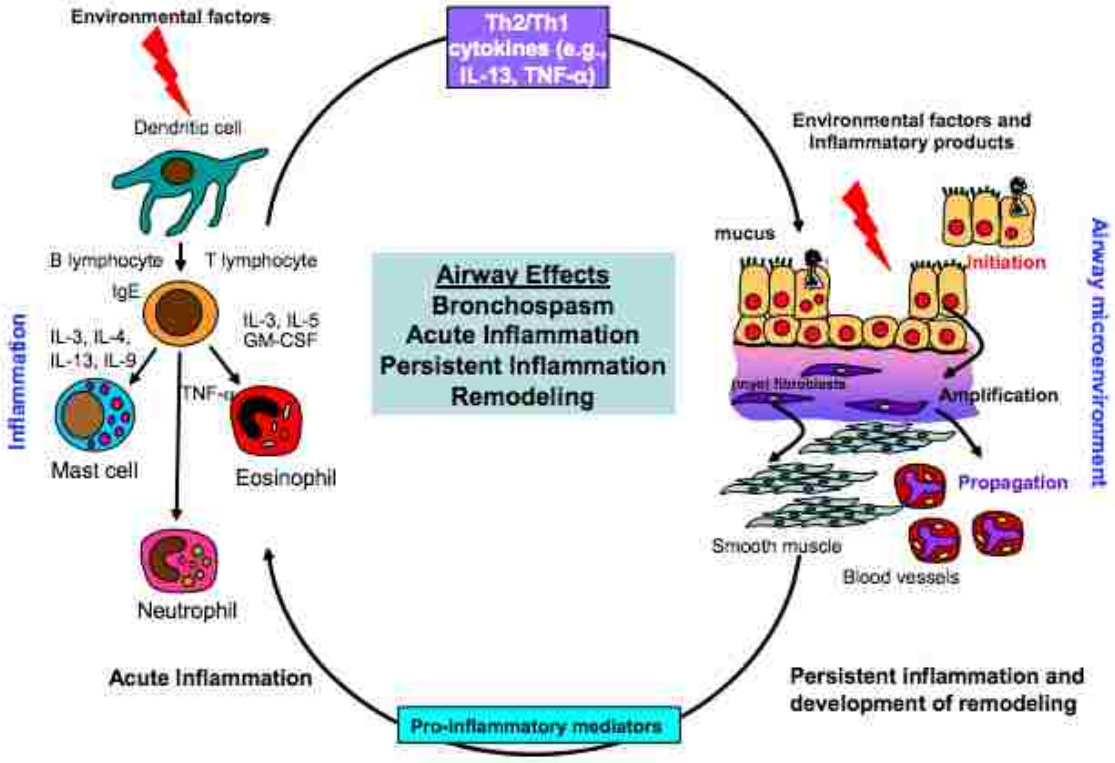

Figure 1.2. Airway microenvironment and inflammatory mediators.

A model depicting the factors involved in persistent inflammation in the lung environment after an insult.

Asthma Epidemiology and Economic Burden

The incidence of asthma is increasing worldwide and here in the US. According to the WHO, approximately 235 million people suffer from asthma globally with about 25 million of those people living in the US. Asthma is a common disease among children. About 1 in 10 children have asthma in the US (http://www.aaaai.org/). Asthma disproportionately affects some ethnic and racial minority groups, with African Americans currently having a higher incidence of asthma in the US compared to Caucasians (Association 2010). Epidemiologists have proposed that this disparity may be due to genetic predisposition to asthma, while others have suggested environmental factors such as stress, socioeconomic status, and access to healthcare as the causes. In addition to the pain and suffering felt by the affected individual and their family, there is a great economic burden placed on society directly through medical costs and indirectly through work and school absence due to illness or death (www.state.gov). The cost of asthma in the US as of 2007 was $56 billion annually (http://www.aaaai.org/).

Epigenetics Background

In 2003 the Human Genome Project officially concluded and hopes of 'cracking' the genetic code allowing scientist to predict and prevent human disease did not come to fruition. The human genome research produced information on about 20,000 genes and it became apparent that genetic variability alone was not sufficient to explain many of the complex and phenotypically diverse chronic diseases. When considering disease states and their association with single nucleotide polymorphisms in genome wide association studies, it was discovered that genetic components explain less than 20% of phenotypic variation (Wallace 2010)

Gene-environment Interactions

Studies in monozygotic twins looking at disease discordance have brought the importance of environmental exposures to the forefront. In this context 'environment' is anything non-genetic (i.e. diet, physical activity, and other lifestyle factors in addition to environmental toxins). Disease discordance is the idea that two people with the exact same genome are born disease-free, but later in life one, but not both, develops a disease. Genes alone are not enough to explain the cause of the disease in this case, so researches have hypothesized that it could be an interaction between genes and the environment. Studies of this nature have been done looking at a number of diseases including asthma (Runyon et al. 2012). At the interface of genes and environment is the human epigenome.

The Epigenome

There are many metaphors used to describe the way that the epigenome relates to the genome. For example, some have referred to the genome as the hardware and the epigenome as the software. The hardware cannot be operated correctly without the software. The epigenome tells the genome when, where, and how to operate. It is the epigenome that allows two cells in the human body, each with the exact same set of genetic information, to be phenotypically different cells with very different jobs; a liver cell and a red blood cell for example.

Epigenetic Marks are Heritable and Reversible

Epigenetic marks are modifications that can be added to the genome resulting in changes in gene expression. These marks do not alter the genetic code and are heritable through cell division exclusive of genetic factors (Cortessis et al. 2012). Consistent with the example used above, the regiment and location of epigenetic marks

and resulting gene expression in the liver cell compared to the red blood cell is different. Most of the epigenetic alterations that occur throughout the life of an organism are natural and necessary, however some aberrant epigenetic marks can occur. Unlike the inherited genetic code, which remains static, epigenetic marks are plastic and dynamic (Rozek et al. 2014). This fact is the reason for the recent increase in interest and funding in the field of epigenetics (see Figure 1.3). Epigenetic marks have the potential to serve as biomarkers of disease or exposure and even as targets for therapy.

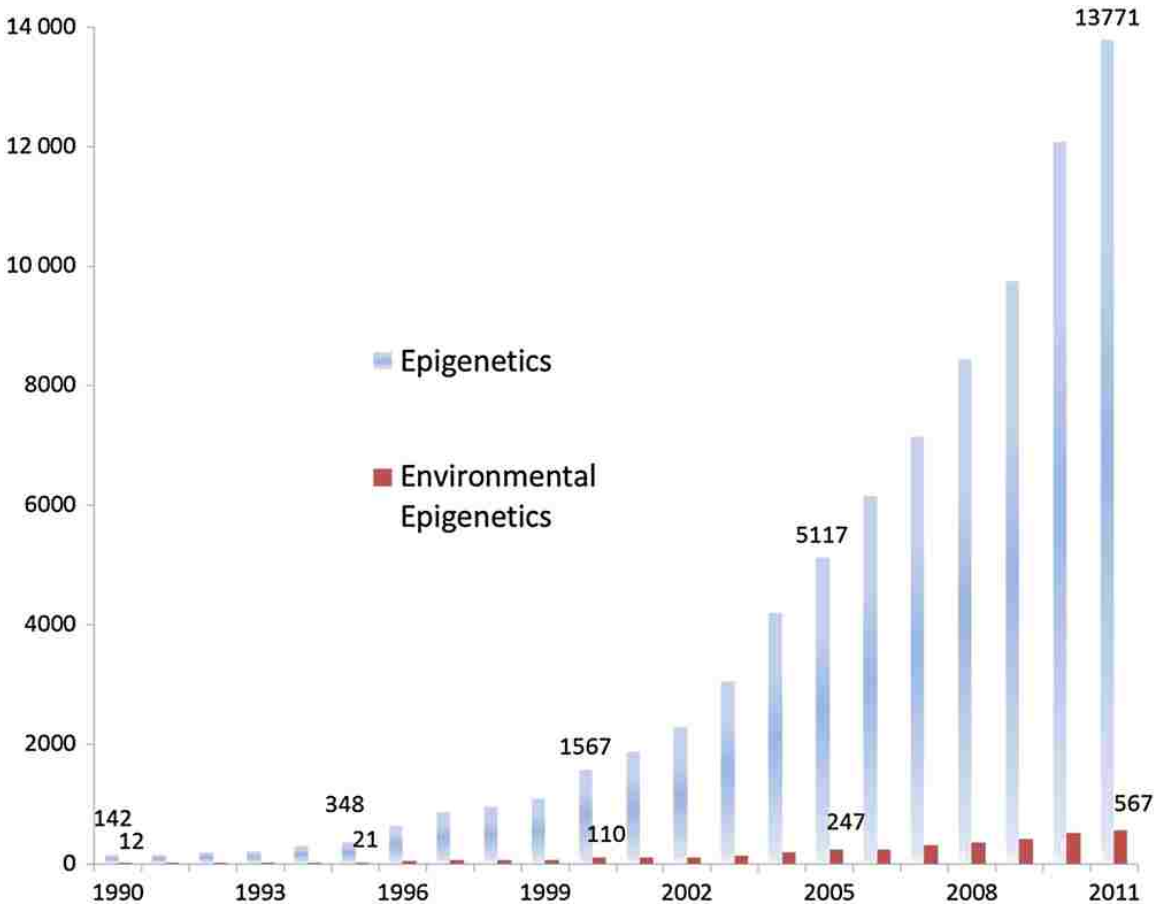

Figure 1.3. Increases in epigenetic research papers over time.

This graph shows the rise in epigenetics research over the last two decades and highlights the need for more environmental epigenetic studies. From Burris et al. 2013.

Types of Epigenetic Modification

Several different types of epigenetic alterations have been identified, but the most commonly studied are micro RNAs, histone modifications, and DNA methylation (see Figure 1.4). Micro-RNAs are a class of non-coding RNAs that control genetic expression by binding to mRNAs and repressing protein production (Wei et al. 2015). Recent findings suggest that micro-RNAs may be important regulators of cytokines involved in T-cell polarization and the allergic response (Tay et al. 2014). Histone modifications and histone variants are epigenetic modifications that serve a wide range of purposes from nucleosome stability to chromatin dynamics and play a critical role in gene expression (Li and Fang 2015). The most commonly studied epigenetic mechanism is DNA methylation, which has received more attention and funding than histone modifications or micro-RNAs (Rozek et al. 2014). This is largely due to the difficulty in working with RNA. DNA is more stable, which makes storage and handling easier. The availability of high throughput methods for DNA methylation analysis that require small amounts of sample at a relatively low cost is also a driving factor for the discrepancy (Burris and Baccarelli 2014).

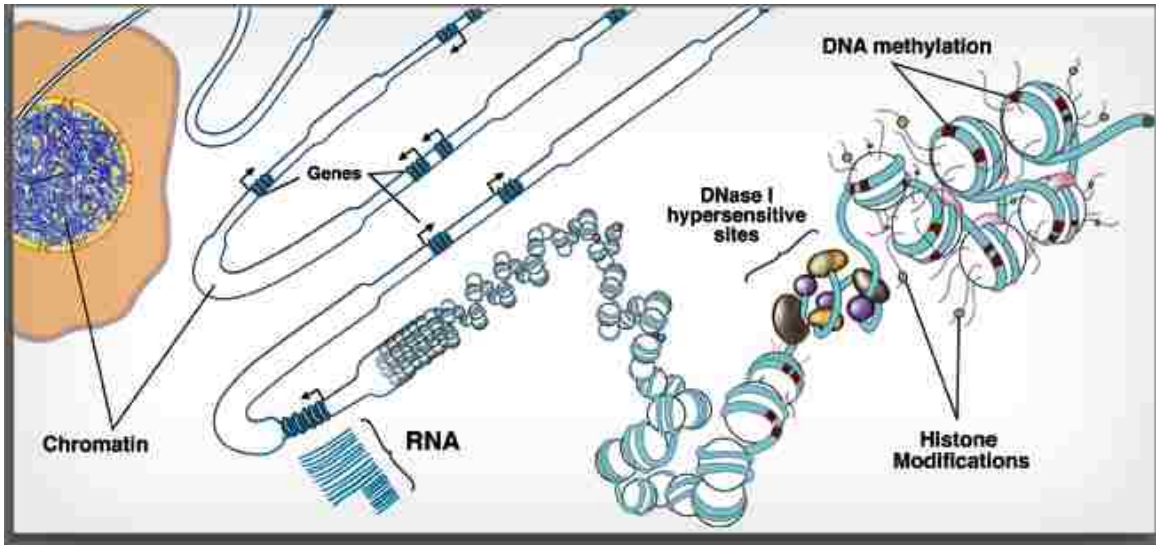

Figure 1.4 Epigenetic Mechanisms.

This figure show how several different types of epigenetic marks contribute to the overall chromatin structure and their potential role in gene transcription.

http://www.nih.gov/news/health/feb2015/niehs-18.htm

DNA Methylation

DNA methylation is a phenomenon where a methyl group is covalently bonded to a cytosine-guanine dinucleotide, also called a CpG. Genomic regions with high CpG densities are called CpG islands. In mammals, methylation typically occurs in these islands, which are disproportionately found in gene promoter regions relative to the whole genome (Jimenez-Chillaron et al. 2012). The "promoter region" is a somewhat ambiguously labeled region approximately 200-3000 base pairs upstream of the start site for a particular gene. Modification of this region can induce or inhibit transcription activity at a given locus. Some researchers suggest that in addition to the promoter region, the CpGs near the start site may also be important (Kwon et al. 2008). Under normal circumstances the majority of these CpG islands are hypermethylated, which typically equates to suppression of the associated gene (see Figure 1.5). Methylation of the CpG requires an enzyme called DNA methyltransferase, which is able to transfer a methyl group from S-adenosylmethionine (SAM) to the fifth carbon of cytosine. SAM is enzymatically made available through a process called one-carbon metabolism in the presence of micronutrients such as folate or choline. Dietary intake of such nutrients has become a focal point of epigenetic research.

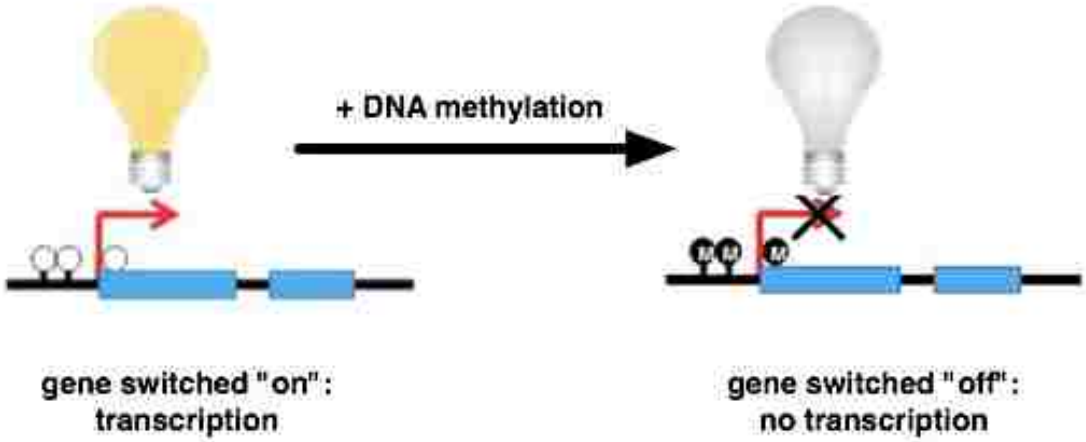

Figure 1.5. DNA Methylation paradigm.

A simple model showing how DNA methylation can influence gene transcription. The lit bulb represents gene expression and the dim bulb represents gene suppression. This paradigm is true most of the time, however, there are instances were DNA methylation can result in gene expression. https://www.elitenetzwerk.bayern.de

Aberrant Global DNA Methylation

Aberrant methylation can lead to altered phenotypes (Wolff et al. 1998). Data from cancer-related research has shown that genomic reductions in methylation, also called global hypomethylation, are a trademark of this altered cellular phenotype (Wu et al. 2011). In animal models, hypomethylation is associated with increased mutation rates and genomic instability (Chen et al. 1998; Eden et al. 2003). Similarly, hypomethylation is also associated with genomic instability in human cancer tissue (Rodriguez et al. 2006). It has been established that genomic instability in the context of cancer is principally the result of demethylation in intergenic and intronic regions where repeated sequences and transposable elements are located (Wilson et al. 2007). After initial observations in the cancer field, it was found that global changes in methylation can impact non-cancer diseases as well. For example, immunodeficiency-centromeric instability-facial abnormality (ICF) syndrome is a rare human disease where DNA methyltransferase 3B (DNMT3B) is mutated resulting in global hypomethylation (Ehrlich 2003). Loss DNMT3B function leads to immune dysfunction, compromised lymphocyte function, and chromosome rearrangement in these patients.

Aberrant Gene-specific DNA Methylation

Aberrant gene-specific methylation can result in up-regulation of genes that are typically suppressed and suppression of genes that are typically up-regulated. Epigenetic modification within the CpG island of the promoter region is thought to interfere with binding of transcription factors and increase affinity for other epigenetic modifiers and co-repressors (Ji and Khurana Hershey 2012; Kuroda et al. 2009). Removal of these marks is thought to make transcriptional binding sites more accessible and prime the system for increased protein production. Leaders in the field have noted the need for

future molecular epidemiology studies to validate the epigenetic changes with RNA or protein expression (Rozek et al. 2014).

Key Windows for Exposure-related Epigenetic Changes

It is suggested that there are key developmental periods, or windows, that are important for epigenetic programming. With the exception of imprinted genes, methylation patterns are reset during early development of the fetus and exposures during this period of epigenetic programming may affect disease susceptibility later in life (Tang and Ho 2007). Considerable effort has been exerted to determine the epigenetic effects of the maternal environment on the offspring. These studies were initially conducted in animal models and have more recently transitioned into human retrospective, cross-sectional, and case control studies. For example the agouti mouse gene has been exploited to show that gestational exposure to differing amounts of dietary nutrients can have phenotypic effects in the offspring (Waterland and Jirtle 2004). The Dutch Famine of 1944 was an unfortunate incident but also a remarkable natural experiment. Children born to mothers during the famine, in their first trimester of pregnancy compared to their third trimester, have differential methylation patterns at an imprinted gene related to insulin production (Heijmans et al. 2008). There are numerous examples of studies, which have investigated human perinatal exposures and found correlations with risk of disease later in life. Attention is only just recently being placed on exposure-epigenetic relationships in later windows of life. Researchers have investigated how environmental exposures such as pesticides (Collotta et al. 2013), lead (Li et al. 2013), mercury (Goodrich et al. 2013), diisocyanate (Ouyang et al. 2013), and nano particles (Stoccoro et al. 2012) are associated with epigenetic modifications in adult human populations. These studies show that cumulative estimates of exposure have the potential to cause

epigenetic modifications at the global and gene specific level suggesting that these later windows of exposure are also important.

Epigenetic Plasticity

Authorities in the field of epigenetics have suggested that there are likely to be at least two categories of epigenetic "marks" (Nadeu 2013). This paradigm is applicable to DNA methylation and other epigenetic alterations. The paradigm suggests there are gene specific marks that happen very early on during natural development, and though these marks can hypothetically be reversed, it is highly unlikely that they ever will (Robertson 2005). Additionally, there are other gene specific marks that can be more flexible and may be reversed more readily due to a number of factors and in response to a number of potential exposures. Other authors have noted this dichotomy and called for more research to identify and characterize the nature of plasticity of the latter. It is currently unclear whether transient DNA methylation is a common feature of gene-environment interactions (Youngson and Morris 2013).

DNA Methylation Analyses

There are many ways to measure DNA methylation and there is no 'best technique'. A researcher must consider the overall study question, the funding available, bioinformatics capabilities, and most importantly what information is available in the current literature. Some methylation analysis techniques are quantitative while others are qualitative. The study model, matrix, or DNA quality/quantity may restrict the use of certain techniques. To cast the broadest net, a genome-wide study could be done using microarray technology. Assays like the Infinium450kb Chip have revolutionized the field of epigenomics, however assays like this can produce terabytes of data, which can be

challenging to store, process, and interpret. Unlike the chip arrays, which are extremely sensitive and produce CpG specific data for nearly all the CpGs, there are other techniques like the Luminometric Methylation Assay (LUMA) or 5-methylcytosine Assay (5-mC) that evaluate global methylation as an average percentage. The latter assays are less helpful for generating hypothesis driven target genes compared to the chip arrays but are relatively inexpensive. Repetitive elements like LINEs and SINEs can be used as targets for analyzing global methylation. ALU for example is a CpG-rich SINE that contains approximately one-third of all human CpG dinucleotides (Yang et al. 2004). LINE-1, a retrotransposon-containing repetitive element, comprises about 20% of the human genome and is normally hypermethylated (Beck et al. 2010). Both ALU and LINE-1 have been used as proxies for human global methylation analysis. Workflow including storage and processing of DNA samples is highly dependent on the downstream assay that will be utilized. Bisulfite-PCR pyrosequencing is a common strategy and necessary for the LINE-1 assay. This method exploits sodium bisulfite's ability to selectively deaminate cytosine but not 5-methylcytosine to uracil (Clark et al. 1994).

The technology in the field of epigenetics is quickly evolving and with these advances comes a call for more mechanistic studies. As these high-throughput methods become more cost effective it will be possible to move from studying mixed cell populations to sorted cell populations and generate cell specific epigenetic profiles. Current techniques require cautious interpretation because changes in methylation patterns may simply represent a shift in cell population (Rozek et al. 2014). Advances in this area of epigenetic research will help move the field from descriptive studies toward more mechanistic studies.

Novel Interdisciplinary Findings in Epigenetics

In this relatively novel field, epigenetic mechanisms have been implicated in diseases from prostate cancer to obesity. It is a field that has helped to bridge gaps between disciplines. There have been some very interesting findings from interdisciplinary studies looking at the link between social and behavioral factors and epigenetic regulation. The molecular mechanisms underlying gene expression following social and behavioral factors are not well understood (Rozek et al. 2014). Novel research is often met with skepticism and that was the case for a couple of scientists at McGill University. In the early 2000s, Michael Meaney, a behavioral scientist, and Moshe Szyf, a molecular biologist, were trying to figure out why pups of attentive mother rats grew up to have less stress compared to pups of neglectful mothers. They determined that these behavioral differences had to do with the levels of glucocorticoid receptor, a stress hormone (Weaver et al. 2004). Szyf determined that contact between the mother and pup could result in demethylation at the gene that coded for the stress receptor and concluded this could lead to more receptor. This novel finding was met with extreme skepticism and the publication was rejected from several journals including Nature and Science. Methylation was thought to be static and the journals were hesitant to stray from this dogma (Buchen 2010). After two and half years the paper was published in the journal Dialogues in Clinical Neurosciences.

The Frontier of Epigenetics

The frontier of epigenetics is the transgenerational inheritance of epigenetic marks. Examples of environmental exposures that are suggested to produce transgeneration epigenetic changes can be found in Table 1.1. This is probably the most fiercely debated subject in the field of epigenetics because it unearths the teachings of Lamarckian science (Hughes 2014). If trangenerational inheritance were possible

through an epigenetic mechanism, then it would mean that an organism could pass on traits that it acquired in its lifetime to its progeny (Szyf 2015).

Examples Epigenetic Transgenerational Inheritance.

Environmentally induced transgenerational inheritance	Reference
Vinclozolin induced epigenetic transgenerational adult onset disease in rats (F1–F4)	Anway et al. (2005)
Transgenerational response in longevity to nutrition (F0-F2)	Kaati et al. (2007)
Tumor susceptibility in *Drosophila* (F1–F3)	Xing et al. (2007
Nutrition induced transgenerational obesity in mice (F1–F3)	Waterland et al. (2008)
BPA-induced transgenerational testicular abnormality (F1–F3)	Salian et al. (2009)
Stem cell culture induced adult onset disease (F0–F4)	Lee et al. (2009)
Dioxin induced transgenerational uterine abnormality (F1–F4)	Bruner-Tran and Osteen (2010)
Stress induced behavior alterations (F0-F2)	Matthews and Phillips (2010)
Transgenerational glucose intolerance (F0–F2)	Pentinat et al. (2010)
Transgenerational effects of morphine or thyroxine on hypocampus, birth weight and behavior (F0–F3)	Vyssotski (2011)
Transgenerational effects of chemotherapy in mice (F0-F6)	Kujjo et al. (2011)
Transgenerational effects of obesity on female body size (F0–F3)	Dunn and Bale (2011)

Table 1.1. Examples of Epigenetic Transgenerational Inheritance.

From Guerrero-Bosagna et al. 2012

The phenomenon was first observed in plants. There is an epigenetic mark at the locus that controls tomato ripening, and this mark is passed on to the next generation (Manning et al. 2006). Following this finding in plants, large epidemiological studies in Europe started to publish data implicating transgenerational inheritance could occur in humans but these were (and still are) highly controversial findings because of the nature of epidemiological studies. The difficulty with epidemiological studies is that it is often hard to control for confounding variables. Because post-conception exposures to the mother could influence epigenetic data, it has become more commonplace to see studies focused on paternal exposures and associations with disease risk in the offspring. Recent data from members of the Dutch Famine cohort have provided convincing evidence that under-nutrition can have effects on future generations. Children and grandchildren of men exposed to famine have higher risk for diseases such as heart disease and obesity relative to offspring from non-exposed men (Kaati et al. 2002; Pembrey et al. 2006).

In addition to human studies, researchers have also tried to show that this phenomenon is occurring by conducting the studies in the more controlled environment of a rodent model. For example, Vinclozolin, a fungicide and known endocrine disruptor, has been used to show transgenerational effects in male rat sperm and these effects can be seen out to the F4 generation (Anway et al. 2005). Using this model transgenerational inheritance, changes have also been detected in the prostate (Anway and Skinner 2008) and the brain (Skinner et al. 2008). These particular studies have not helped to reconcile the debate, however. After two groups failed to reproduce this data, it was found that a member of the Skinner lab had fabricated data in a related paper. Skinner and his laboratory did show that they were able to reproduce the retracted data (Skinner et al. 2013). The debate over this frontier of epigenetic research remains and the

mechanism is still unclear. The data exists to suggest that in addition to imprinted genes there are several types of epigenetic marks that escape reprogramming during development (see Figure 1.6.) (Hughes 2014).

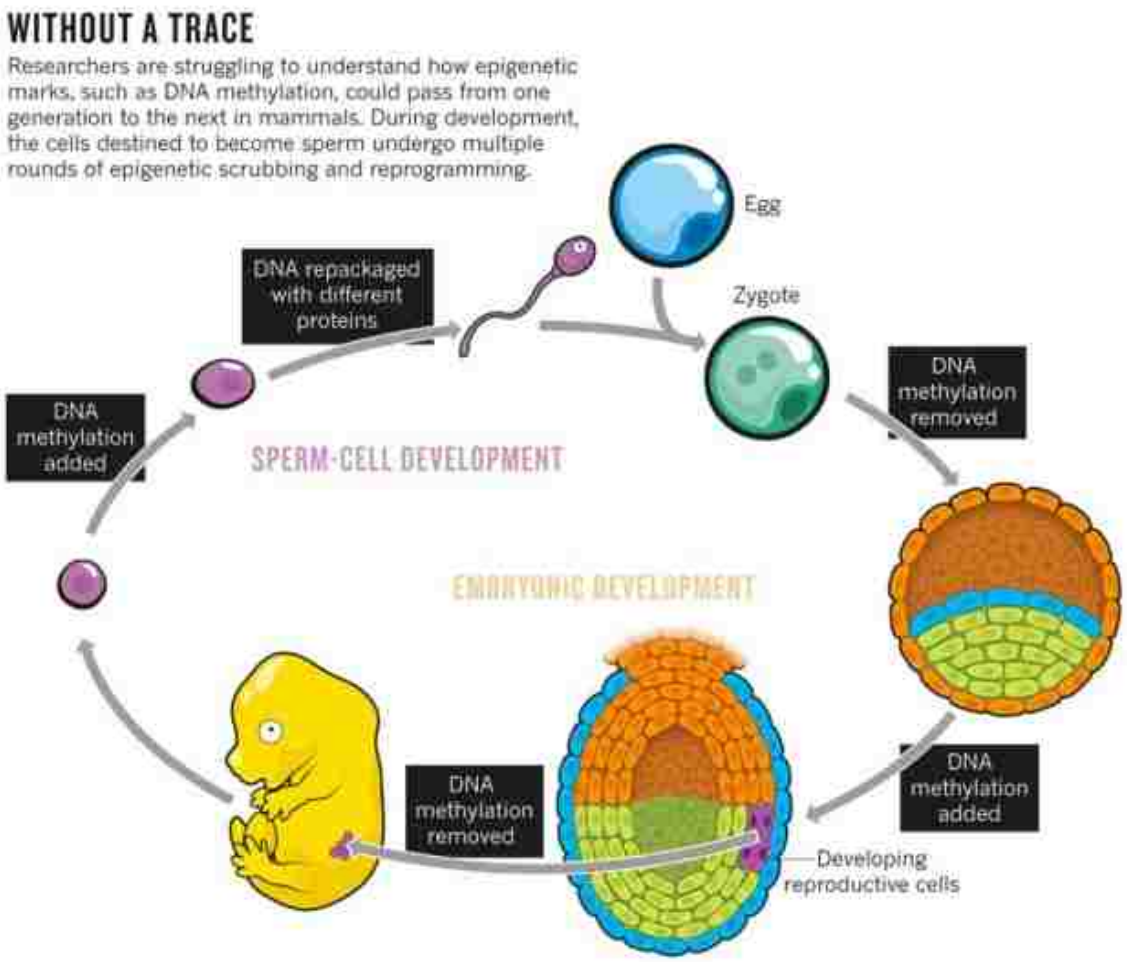

Figure 1.6. Resetting epigenetic marks during development.

This figure depicts the reprogramming points that the epigenetic marks would need to elude to be inherited by the offspring. From Hughes 2014 Nature Sins of the Father.

Epigenetics and Asthma

For Aims 1 and 2 we will investigate the effects of dietary exposure and airborne PM exposure on a representative measure of global and gene specific methylation. LINE-1 is discussed above and the potential for global methylation to affect immune function has also been covered. In the context of asthma LINE-1 methylation may be an indicator of asthma severity and could be related to health outcomes. Interferon gamma (IFNγ) was chosen for its involvement in the asthma pathway as a Th1 cytokine. The differentiation and maintenance of T cells is dependent on the balance between Th1/Th2 (Robinson 2010). Several studies have shown that IFNγ plays a key role in the counter-regulation of Th2 polarization (Crinnion 2012). A study of asthma discordance in twins showed that IFNγ gene expression is suppressed in asthmatics compared to non-asthmatics (Runyon et al. 2012). Modulation of the methylation profile in the promoter region of IFNγ has been shown to affect T cell polarization and/or differentiation in humans (cytosine phosphate guanine (CpG) -53 and -186) (Dong et al. 2013). Tang et al. has shown that IFNγ hypermethylation is associated with reduced expression in an *in vitro* model (Tang et al. 2012). This study also showed that there were differential patterns of IFNγ methylation in cord blood samples and these patterns were associated with maternal PAH-exposure. Tang et al. did not show that these patterns were reflective of gene expression in the cord blood but suggests this is potentially the case based on the *in vitro* model. The promoter region of the IFNγ gene has three proximally located CpG sites (see figure 1.7.), two of which have been shown to be mediated by environmental exposures.

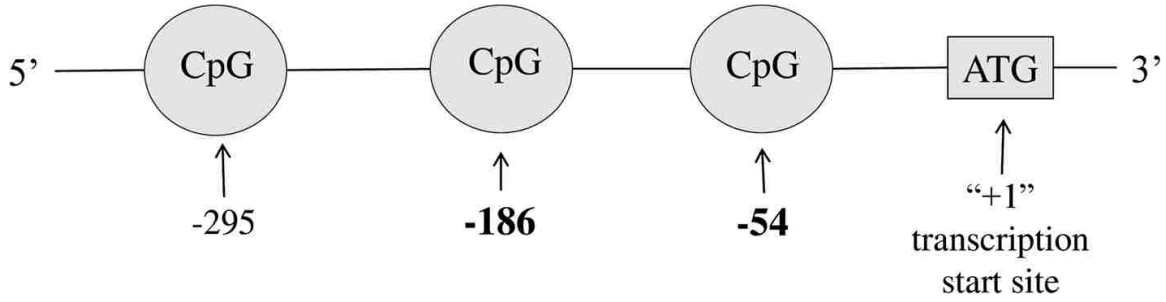

Figure 1.7. IFNγ CpG locations.

Schematic of the CpG positioning for the promoter region of IFNγ. Adapted from Lovinsky-Desir et al.

Rationale for Chosen IFNγ Promoter CpG Sites

IFNγ CpG -54 (-53 is the corresponding murine CpG) and -186 have functional relevance to allergic outcomes in animal models (Liu et al. 2008a; Brand et al. 2012) and humans (Runyon et al. 2012; Kohli et al. 2012). In an asthmatic cohort, Lovinsky-Desir et al. showed that there are differential methylation profiles for these CpGs relative to age, sex and tissue type (Lovinsky-Desir et al. 2014). In the case of buccal cells, children had more methylation than adults at these two CpG sites. In contrast to CD4+ lymphocytes, buccal cells methylation at these two CpG sites were not significantly different between males and females. Methylation for CpG -186 was significantly correlated between CD4+ lymphocytes and buccal cells, but this was not the case for CpG -54.

Particulate Matter Exposure

Although it was nearly 2,500 years ago that Hippocrates wrote *On Air, Water, and Places* and noted that a person's health could be affected by the air they breathe, the idea to protect an individual's right to clean air and preserve the environment only came about recently. There are two catastrophic historical events that receive credit for raising public health concern regarding air quality. Concomitantly, these events also mark the start of air pollution research. In 1948, a pollution event occurred in Donora, Pennsylvania that killed 20 people and hospitalized 7,000 (1948). 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. In 1952, another pollution event called The Great Smog of 1952 occurred in London, England (Metoffice.gov.uk). This event killed between 4,000 and 12,000 people. The full impact of this event was not realized until months later.

Definition of Particulate Matter

Particulate Matter (PM) is a small subdivision of solid matter that is suspended in gas or liquid. Airborne PM is a complex mixture of chemical species that is determined by the mechanism of formation, source, and environmental conditions. Primary PM is unchanged from the time it was generated while secondary PM is formed through atmospheric reactions. PM is categorized into three main types 1) PM_{10} which is all particles 10 micrometers in aerodynamic diameter and smaller, 2) $PM_{2.5}$ which is all particles 2.5 micrometers in aerodynamic diameter and smaller, and lastly 3) Coarse Fraction, which is the particles that are between PM_{10} and $PM_{2.5}$. $PM_{2.5}$ is especially important in the context of lung health because this subset of particles can bypass the body's natural inhalation pathway defenses (i.e. nose hair and mucociliary escalator) and deposit in the alveolar spaces. Depending on the chemical composition of the particles and the body's reaction, this insult can start a chain of events leading to inflammation and even permanent structural changes such as fibrosis (EPA.gov). PM has been shown to affect other organ systems and is therefore a major human health concern (see figure 1.8.). Research suggests that there are susceptible populations (i.e. young, old, and those with pre-existing conditions) with regard to PM exposure and the Environmental Protection Agency (EPA) sets their standards to protect these individuals.

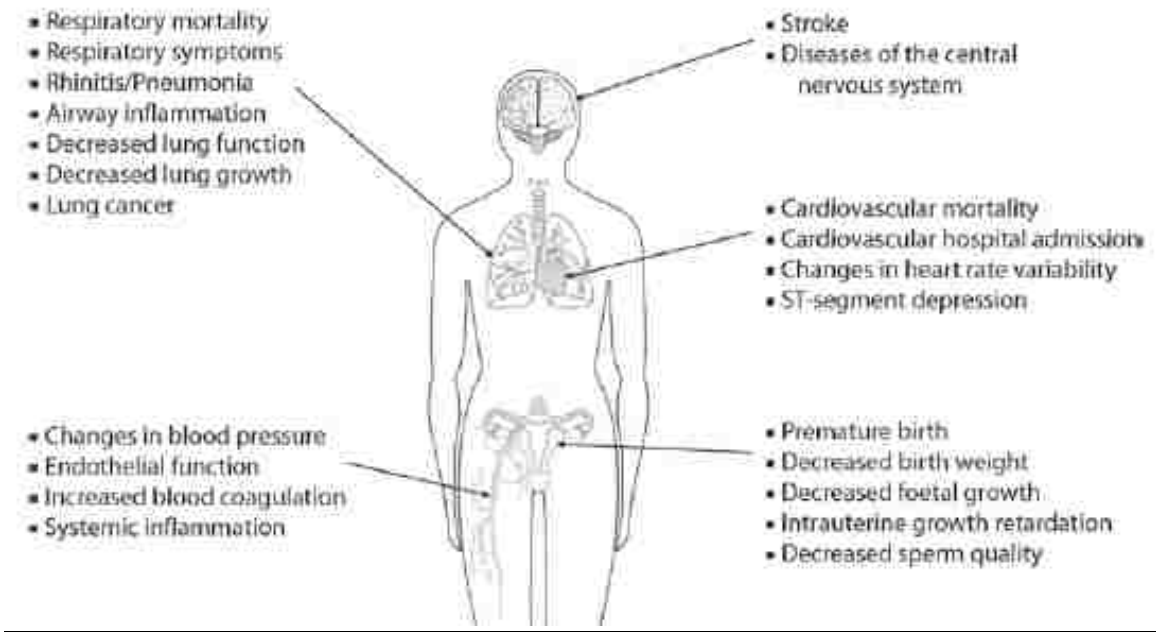

Figure 1.8. Potential health effects of PM exposure

from Peters et al. 2011

Particulate Matter Regulations

The Clean Air Act was enacted by Congress in 1970 and this was mostly prompted by visible pollution in many major cities (WWW.EPA.gov/air). The latest amendments (1990) to the Clean Air Act require the EPA to set National Ambient Air Quality Standards for pollutants that are harmful to human health. There are currently six pollutants that meet this criteria and particle pollution is on this list. Particle pollution is divided into $PM_{2.5}$ and PM_{10}. The annual standard for $PM_{2.5}$ is 12μg/m^3 and the 24-hour standard is 35μg/m^3 (WWW.EPA.gov/air/criteria). Only a 24-hour standard is set for PM_{10}, which is 150μg/m^3.

Particulate Matter and Health Outcomes

PM is a key indicator of air pollution exposure and has the potential to significantly impact human health (Kim et al. 2015). Certain populations are disproportionately affected therefore research and subsequent regulations should consider these populations. Children are especially susceptible to PM exposure because humans lungs are not fully developed until age 6 (Schwartz 2004). In addition to differences in physiology, children also have behavioral differences (i.e. activity level, time spent outdoors, etc.) compared to adults that make them vulnerable to PM exposure-related health effects.

The following studies are examples of studies that have investigated the relationship between PM pollution and respiratory health in children with the majority of these studies occurring in the urban setting. Researchers in the Netherlands looked at panels of children 7 to 11 years of age and found that associations between poor respiratory health and air pollution exposure were stronger among asthmatic children compared to healthy children (van der Zee et al. 1999). A 7-week study with repeated measures

found that increased exposure to ambient air pollution was positively associated with exhaled nitric oxide (Fischer et al. 2002), which is a good marker of airway inflammation in children with asthma (Kharitonov et al. 1995). In a panel of over 200 school aged children, researchers in Brazil found that air pollution, including $PM_{2.5}$ generated from seasonal fires, was negatively associated with peak expiratory flow (PEF), a marker of respiratory health (Jacobson Lda et al. 2014). Gleason et al. investigated the relationship between summertime emergency department visits for asthmatic children and levels of $PM_{2.5}$ and found that the association was the strongest when a pollution event occurred on the same day as the emergency department visit (i.e. no lag time). A recent study in approximately 1,500 school aged non-asthmatic children in Taiwan considered the relationship between lung function and air pollution including $PM_{2.5}$. The authors concluded that sub-chronic exposure to $PM_{2.5}$ was associated with reduced lung capacity in children 6 to 15 years of age. Additionally, the results of this study suggest that children 6 to 10 years of age may have airway obstructive patterns that are associated with acute exposure to $PM_{2.5}$ (Chen et al. 2015).

Impact of Community Based Strategies to Reduce Ambient Particulate Matter

Concern for the impact of ambient PM on human health has grown over the last decade and intensified with formation of the Environmental Protection Agency (EPA) in the mid-20^{th} century. Major reductions in ambient PM are the result of changes in residential fuel sources (i.e. coal to gas or electric), improvements to automobile engine design and fuel, and regulations placed on industrial emissions (Lioy and Georgopoulos 2011). Recently, there have been a small number of studies that have investigated the impact of strategies aimed at reducing biomass generated ambient PM at the community level (Johnston et al. 2013). The majority of these studies have reported improvements in health-related outcomes associated with reductions in PM.

Opportunity for Indoor Particulate Matter Intervention

Ambient pollution has been the focus of the EPA, but the indoor environment has become the target for some respiratory health related interventions because people today spend up to 90% of their time inside (Klepeis et al. 2001). While it is not perfect, the home environment represents a more controlled research setting for determining the effectiveness of indoor intervention strategies. Several strategies for reducing indoor PM have been employed. For example, researchers have evaluated strategies to remove cockroach, mouse, or dust mite allergens, strategies to change subject behavior (i.e. having a parent of an asthmatic child smoke outside), removing or changing out an outdated heating source, and even multifaceted strategies that are tailored specifically to the subject (Matsui 2014). With respect to asthma, several reviews have established the efficacy of air filtration units as a viable intervention strategy for reducing indoor PM exposure and improving respiratory health (McDonald et al. 2002; Fisk 2013).

Particulate Exposure and IFNγ Methylation

The mechanistic link between PM exposure, persistent inflammation, and asthma exacerbations is not fully understood. Some environmental asthma triggers such as environmental tobacco smoke and traffic-related pollutants are known to induce epigenetic changes that may be important for the regulation of inflammatory mediators (Lovinsky-Desir and Miller 2012). The relationship between PM exposure and the Th1/Th2 balance may be important in the context of asthma and epigenetics. A study in a mouse allergen model demonstrated that particulate matter exposure was associated with epigenetic effects at promoter sites of IL-4 and IFNγ. Diesel exhaust particulate exposure coupled with allergen-induced hypermethylation at three promoter sites for IFNγ and hypomethylation at one promoter site for IL-4 (Liu et al. 2008b). These

epigenetic outcomes would theoretically result in suppression of the Th1 pathway and promotion of the Th2 pathway, respectively.

Nutritional Exposure

People are not exposed to single insults in a bubble. On the contrary, people are exposed to a concert of insults daily. Humans are exposed to a myriad of chemicals and dietary factors through the consumption of foodstuffs daily and this may be one of the most important and ubiquitous exposures occurring in the 21st century (Cordain et al. 2005). From the time of the industrial revolution, human lifestyle has become urbanized (Popkin 1999) and this has led to changes in activity level and diet (Chilton et al. 2014). A number of epidemiological studies have investigated the role of nutrition and specific nutrients in the development of chronic inflammatory diseases (CIDs). Evidence from these studies suggest that over-nutrition and gene-diet interactions may be driving a persistent systemic inflammatory state and contribute to diseases such as cardiovascular disease (CVD), diabetes, asthma, allergies, cancer, skin and digestive disorders, and neurological diseases (Ferrante 2007; Hamminga et al. 2006; Forsythe et al. 2008; Nguyen et al. 2009; Calle and Thun 2004; Naderali et al. 2009; Leveille et al. 2005).

Currently, researchers believe that nutrition, obesity, inflammation, and disease are all interconnected in a dynamic fashion. The adaptation of the Western diet (i.e. high fats, sugars, and processed foods) is associated with a rise in obesity and inflammatory disease and exposure to these dietary factors seem to affect certain populations in a disproportionate way. Obesity may be a susceptibility factor for nutritional-related exposures leading to inflammation as well as part of the pathway. Early life exposure to dietary factors could be responsible for 'epigenetic programing' and this may affect

disease risk later in life (Chilton et al. 2014) or even in the following generations (Hughes 2014). It is possible that recent changes to our diet are causing deleterious epigenetic changes leading to poor health outcomes.

Dietary Patterns and Human Health

The current body of literature on diet and human health can be broken down into two levels of study; research investigating the effect of dietary patterns on health and research investigating the effect of specific nutrients on health (James 2009). In some cases the latter informs studies on the former.

The health benefits of the Mediterranean diet (MD) have been known for half a century. The Seven Countries Study, which began in the 1940's, was the first study to systematically examine the effect of diet (including MD) and lifestyle on heart disease. The MD is consistent with the traditional diet of southern Italy, Greece, and Spain, which includes high intake of olive oil and unrefined grains (Spreadbury 2012). MD typically contains very low saturated fats and cholesterol and very high monounsaturated fats, fiber, and carbohydrates (Sexton et al. 2013).

The Western diet pattern is that of the developed world (i.e. Europe, United States, etc.). This diet is characterized by convenience and highly processed foods high in saturated and trans fats. The body of evidence supporting the deleterious effect of the Western diet on health is ever growing, but possibly the most persuasive data comes from studies looking at immigrants. When immigrants from developing countries move to more developed countries they typically adopt some the host country's dietary habits resulting in a natural experiment (Holmboe-Ottesen and Wandel 2012). It has been found that these ethnic minority groups are at risk for developing obesity, type 2 diabetes and CVD

(Abate and Chandalia 2001; Tillin et al. 2005; Fischbacher et al. 2007; Jenum et al. 2005). A meta-analysis of the available data has shown that the risk of these diseases is correlated with the adoption of a diet high in energy, low in fiber, and low in plant-based micronutrients (Holmboe-Ottesen and Wandel 2012).

A recent study analyzed data from the 4th Portuguese Annual Health Survey (Barros et al. 2015). The study population was nearly 33,000, and within this population the prevalence of asthma was 5.3%. The authors used Latent Trait Models to classify dietary intake into patterns. After controlling for confounders, the authors found that two dietary patterns were associated with asthma. The dietary pattern "high fat, sugar, and salt" was positively associated with asthma prevalence as well as severity. The dietary pattern "fish, fruit, and vegetable" was negatively associated with asthma. These results corroborate findings from a recent review by Brigham et al. that concluded that there is evidence to suggest that the Western diet pattern is associated with asthma morbidity (Brigham et al. 2014). Gregory Diette from Johns Hopkins Bloomberg School of Public Health shared interim finding at the 2015 Children's Environmental Health Network Conference from a clinical trial of dietary interventions in school aged asthmatic children in the context of an indoor air pollution monitoring program in inner city Maryland. His research suggests that a prudent diet may be protective for asthma exacerbations from indoor air pollution, however, this data has not been finalized.

Importance of the ω6:ω3 Ratio

One of the most obvious differences between Western and non-Western diets is the increase in the ω6:ω3 ratio (Chilton et al. 2014). The essential fatty acids were first discovered in 1929 by Evans and Burr, who showed that mammals lacked the enzymes necessary to synthesize ω6 or ω3 fatty acids. These ω3 fatty acids can be extended to

make eicosapetaenoic acid (EPA) and docosahezaenoic acid (DHA) by enzymes called desaturase and elongase (Simopoulos 2008). EPA and DHA can be used to make E and D-series resolvins which have anti-inflammatory effects (Bosma-den Boer et al. 2012). For more than a half-century, scientists have suspected that deficiencies in essential fatty acids could cause CIDs (HM 1953). According to Kromhout et al. controversial results have been published since the year 2000 regarding ω3 supplementation (Kromhout et al. 2012). The authors note that issues with omega 3 dose and source, statistical power and study design have made the data difficult to interpret. Cordain et al. estimate that some ancestral populations may have consumed as much as 58% of their energy as fat, however the 'quality' of the fat was better and still fell within the recommendations for ω6:ω3 ratio and saturated fat intake (Cordain et al. 2000). Data showing that populations such as the Greenland (Kromann and Green 1980) and Yup'ik (Makhoul et al. 2011) Eskimos consume high levels of ω3 and have lower levels of systemic inflammatory markers suggests an important role for the ω6:ω3 ratio. The ω6:ω3 ratio is potentially a very important player in the pathway leading to metabolic inflammation, or metaflammation. This type of inflammation is unique to obesity and is defined by chronic low-grade inflammation resulting from nutritional factors. For example, linoleic acid (LA) can be converted in the liver to leukotoxins (LTX) by CYP2C9, which can further be modified by an epoxide hydrolase into leukotoxin diol (LTXD) or iso-LTXD (Bosma-den Boer et al. 2012; Ozawa et al. 1988). *In vitro* models have shown that production of these metabolites can lead to systemic inflammation through the production of reactive oxygen species (ROS) and activation of transcription factors like NF-κB and AP-1 (Moghaddam et al. 1997; Viswanathan et al. 2003). In the context of respiratory health, there does not appear to be a consensus in the literature about the benefits of the ω3:ω6 ratio, however, there is a call for more research because a dietetic approach may be better tolerated and more cost effective than currently

available asthma management tools (D'Auria et al. 2014). The mechanism whereby respiratory health is improved by eating a diet high in ω3 is likely too complex and possibly dependent on the severity of asthma among other factors.

Dietary Fiber and a Potential Role for Inflammation

Dietary fiber has recently received a rapid increase in attention for its potential role in immunoregulation. Dietary habits can influence the microbial diversity of the gut, which can contribute to low-grade inflammation through immune dysregulation. Dietary fiber is at the interface between the gut microbiota and normal immune function (Kuo 2013). It is estimated that there are more than 100 trillion resident microbes in the human gut and as much as 150-fold more genes in the 'microbiome' compared to the host genome(Qin et al. 2010). The diversity and health of the gut microbiota is dependent on the nutritional intake of the host and fiber appears to play a key role in ensuring that nutrients reach the large intestine where the majority of the microbes reside (Kuo 2013). Therefore it is possible that the recent trends toward more processed foods in the Western diet could be compromising normal host immune function by undernourishing the gut microbiota. In this way, fiber has been used as a treatment for inflammatory diseases (Thorburn et al. 2014). Kan et al. investigated fiber intake and respiratory health measures and chronic obstructive pulmonary disease. In a population of nearly 12,000 men and women from the Atherosclerosis Risk in Communities (ARIC) study, the authors found that dietary fiber intake was positively associated with several respiratory health metrics including percent predicted FEV_1 (Kan et al. 2008). The current literature supports the idea that fiber may be beneficial in controlling asthma in children.

Diet and Epigenetics

Another way the diet may be influencing the development and/or exacerbation of CIDs, particularly asthma, is through DNA methylation. Methyl groups can be added to cytosines in a cytosine guanine dinucleotide. If this occurs in a functionally relevant region, this epigenetic mark can affect gene expression (Sharma and Litonjua 2014). Dietary methyl donors play an important role in one-carbon metabolism and the production of S-adenosylmethionine, which along with a methyltransferase is required for DNA methylation (see Figure 1.9.). Intake of methyl donors can influence patterns of methylation resulting in differences in gene transcription. Landmark studies, such as those looking at the methyl donor folate and its protective role for neural tube defects (Pitkin 2007), highlight the importance of these micronutrients. However, recently there has been some research to suggest that the windows of exposure need to be revisited (Rozek et al. 2014). The perinatal window may be the most sensitive time for the effects of these methyl donors and the therapeutic range may be more narrow than originally thought. The findings from postnatal studies of folate and asthma have been mixed and the animal research has not helped untangle the relationship (Sharma and Litonjua 2014). In the context of asthma, even clinical trials of folate provide mixed results; evidence of the complexity of disease and also the mechanism.

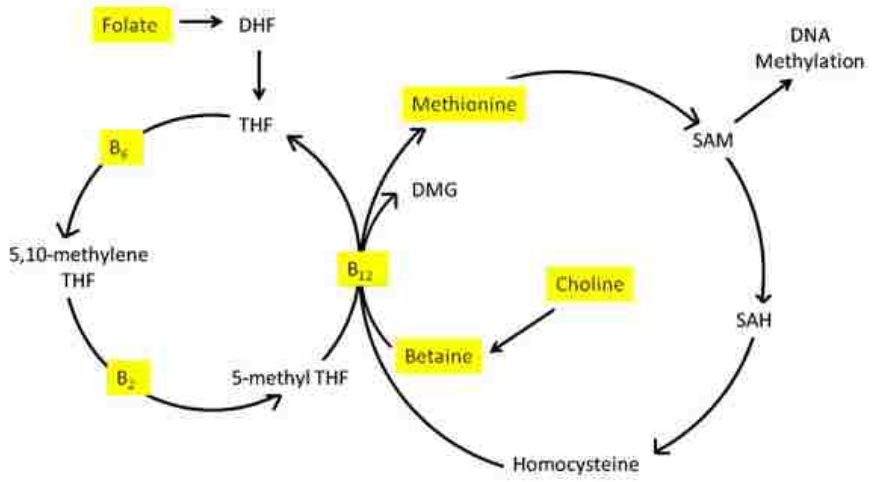

Figure 1.9. Methyl donation.

This is a simplified model depicting one-carbon metabolism. DNA methylation is dependent on the availability of dietary methyl donors, highlighted yellow, to produce S-adenosylmethionine (SAM).

IFNγ Methylation and Dietary Exposure

To our knowledge there have not been any studies evaluating dietary exposures in humans in the context of IFNγ promoter methylation. Recently, Meng et al. showed that IFNγ production is influenced by dietary variables and may predict upper respiratory tract infection incidence (Meng et al. 2015). The authors did not investigate methylation in the IFNγ promoter region, however, this is one mechanism that could explain how exposure influences cytokine production.

ARTIS: Description of Parent Study

Aims 1 and 2 of my dissertation will occur within the context of this randomized trial and more specifically in final two years of this five-year study. The rationale and methods for the Asthma Randomized Trial of Indoor Wood Smoke Study (ARTIS) have been described previously (Noonan and Ward 2012). The objective of this study is to assess the effectiveness of residential intervention strategies for reducing wood stove generated $PM_{2.5}$ and quantify the resulting improvement in health outcome measures among asthmatic children living in the home. Similar studies have investigated PM reduction strategies in the urban environment (Johnston et al. 2013) and in the context of cookstoves (Alexander et al. 2014). This novel study will be the first to investigate wood stove generated PM reduction strategies. The analysis for my study will take advantage of a very robust data collection strategy (see Figure 1.10.).

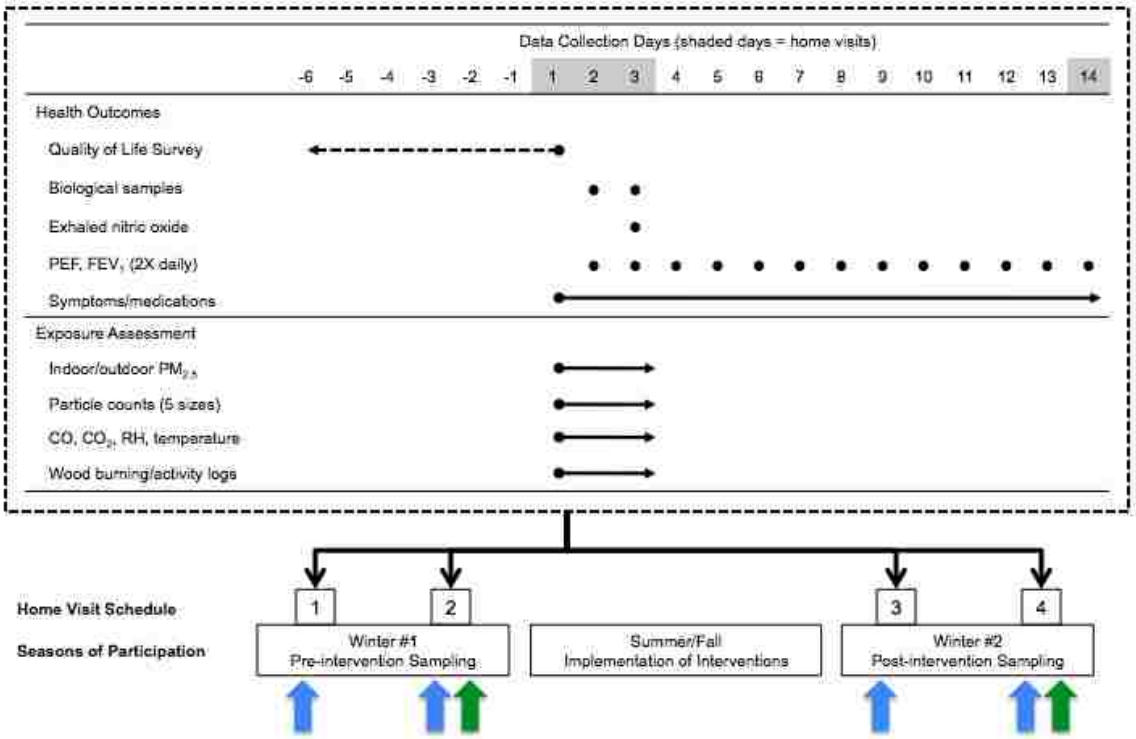

Figure 1.10. ARTIS data collection strategy.

Four sampling episodes occur at each participant home. Two sampling episodes occur during the pre-intervention winter, and two sampling episodes occur during the post-intervention winter. Household interventions (i.e., active air filter or placebo filter) are introduced during the summer or fall between the two winter sampling seasons. Each sampling episode, days 1-3 in this Figure, includes data collection for both health outcomes and exposure assessment. The blue and green vertical arrows represent the addition of buccal cell collection and food frequency questionnaire administration respectively. These two metrics take place during days 1-3.

Opportunity for an Animal Sentinel for Human Health

There are often practical, logistical, and ethical considerations that make human community-based studies challenging. Additionally, humans often have practices that are potentially confounding such as tobacco use, legal and illegal use of drugs, varying transportation and movement. In such cases, there is utility in establishing a sentinel model for human health that could reduce the likelihood of potential confounding from these factors (Reif 2011).

An animal sentinel is an animal that shares the same environment as humans and whose health is informative for human health. In some cases the sentinel species may be more susceptible to the exposure or disease and in other cases may have a greater risk for exposure due to a difference in behavior (e.g., more breaths per minute translates to greater delivered dose for inhalation exposures). A wide range of sentinel animals have proved useful including mammalian and nonmammalian speicies, domestic or companion animals, food animals, fish, amphibians, and wildlife (van der Schalie et al. 1999). The classic example of a human sentinel is the canary in the coalmine shaft (Reif 2011). In the early 1900's miners would take a canary down the mineshaft with them because the bird was more susceptible to carbon monoxide exposure and would show signs of sickness before the miners. This would give the miners time to escape to fresh air.

Sentinel animals have been used to monitor for human health hazards (i.e. the canary), for risk assessment through field research programs, or serendipitous observation has signaled the alarm for human health. Feral cats happened to be an example of the latter in Minamata Bay (Aronson 2005). The dancing cats of Minamata were a sign that the fish supply had become toxic. After an outbreak of anthrax in Russia, cattle and other

livestock were used as sentinels because they were more susceptible (Bezdenezhnykh and Nikiforov 1980). Additionally, crows have been used as a sentinel of West Nile Virus infection (Julian et al. 2002).

It could be argued that dogs, more than any other animal, have gone through a very intertwined evolution with humans over the last 5,000-15,000 years, which is the estimated time that dogs became domesticated (Boyko 2011). In this time of co-evolution, dogs have shared the human environment and likely the same pathogens and these shared exposures likely shaped both species' immunological responses. This makes the domestic dog a very plausible sentinel species. In contrast to laboratory rodent models, which are typically used in acute high dose studies, domestic dogs are chronically exposed to many of the same environmental contaminates as their owners (Bukowski and Wartenberg 1997).

An early observation on the effect of air pollution on dogs was made by a Russian scientist (Leake 1960). He suggested that lung cancer occurred more often in dogs from larger cities compared to rural areas. In the early 70's Reif et al. conducted a retrospective study that looked at radiographic screenings for chronic pulmonary disease (CPD) in urban and rural domestic dogs. In one of the study areas, the authors found that older urban dogs had a higher prevalence of CPD (Reif and Cohen 1970). In a follow up study of 1,892 dogs from three teaching hospitals, the author's results showed that the prevalence of CPD was higher among older dogs from high pollution zones in two of the urban cities. Dogs in Mexico City were studied in the early 2000's and the researchers found that compared to dogs in more rural areas, urban dogs had structural lung changes likely due to an inflammatory response to chronic particulate and ozone exposure (Calderon-Garciduenas et al. 2001). A follow up study was conducted and

found that this pollution could cause neuroinflammatory effects. A study of dogs and cats in Pakistan showed that hair samples from domestic animals could be used as biomarkers for indoor air pollutants (Ali et al. 2013). The authors concluded that dogs and other domestic animals were viable biosentinels for human health.

It is unlikely that any sentinel species will ever be the sole determinant of health risk for humans, but animal sentinels can clearly inform risk assessments, provide early warning signs, and help monitor post-remediation exposure. Further, the use of animal models is critical to expanding our understanding of epigenetic regulation of gene expression and its contribution to complex disease phenotypes such as asthma (Dolinoy and Faulk 2012). Models that incorporate outbred strains and domestic animals may be particularly informative. To succeed in elucidating the epigenetic mechanisms of complex diseases, Dolinoy suggests researchers must integrate animal models, human clinical research, and human epidemiological studies into the overall approach.

Introduction Summary

Our research strategy employs a multifaceted approach to determine the respiratory health impacts of diet and wood smoke particulate exposure on human health. The following research will add to the body of literature by investigating a potential epigenetic mechanism whereby environmental exposures influence respiratory health outcomes.

Project Overview

Title: DNA Methylation in Humans and Dogs: Evaluating the Impact of Nutritional and Particulate Exposures

My overall hypothesis is that $PM_{2.5}$ and dietary factors will cause hypo- and hypermethylation changes modulating cytokine expression associated with asthma symptoms.

Specific Aim 1 (Chapter 2): Among asthmatic children (n=32), assess with repeated measures the impact of diet on (a) health outcome measures (i.e. quality of life and markers of respiratory health) and (b) methylation outcomes (i.e. LINE-1 and IFNγ). Data from winter 1 and winter 2 visits will be used. However, only visits that include all health outcome measures, diet assessment, and buccal collection were included in the final analysis. I hypothesize that intake of nutrients that are involved in methyl donation (i.e. folate, methionine, choline, B vitamins, etc.), reduction of oxidative stress (selenium), and linked to reduction of systemic inflammation (high in dietary fiber and ω3 compared to ω6) will be positively associated with health outcome measures. Further, I hypothesize that these nutrients will be associated with more LINE-1 methylation and less methylation at CpG promoter sites of IFNγ, a Th1 response pathway related gene.

Specific Aim 2 (Chapter 3): Among asthmatic children (n=37), assess with repeated measures the impact of indoor $PM_{2.5}$ exposure on buccal cell DNA methylation and health outcome measures. Data from winter 1 and winter 2 visits will be used, however, only visits that include all health outcome measures and buccal collection will be included in the final analysis. I hypothesize that $PM_{2.5}$ will be negatively

associated with health outcome measures. Further, I hypothesize that $PM_{2.5}$ will be negatively associated with methylation percentages of LINE-1 and positively associated with methylation at CpG promoter sites of IFNγ, a Th1 response pathway related gene.

Specific Aim 3 (Chapter 4): Identify three kennels of Alaskan sled dogs that are predicted to represent "high", "medium", and "low" winter-time ambient $PM_{2.5}$ exposures and determine if whole blood global DNA methylation differs by kennel. I hypothesize that $PM_{2.5}$ will be negatively associated with methylation percentages of LUMA. Dogs in the high exposure kennel will have less global methylation compared to the dogs in the low exposure kennel.

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CHAPTER 2

Assessing the Impact of Dietary Nutrients on Respiratory Health Outcome Measures and DNA Methylation in Asthmatic Children

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Abstract

Asthma is an increasingly common chronic disease among children, and data point toward a complex mechanism involving genetic, environmental and epigenetic factors. Epigenetic modifications such as DNA hypo- or hyper-methylation have been shown to occur in response to environmental exposures including particular dietary nutrients. Current research suggests that children may be especially susceptible to these exposures.

Within the context of the Asthma Randomized Trial of Indoor wood Smoke (ARTIS) study we investigated relationships between diet, DNA methylation and asthma outcomes. Asthma health measures included a quality of life (PAQLQ) instrument and two-week daily monitoring of diurnal peak expiratory flow (dPEF) and forced expiratory volume in first second (FEV1). Dietary intake was assessed using the 2004 NutritionQuest Block Kids Food Frequency Questionnaire (FFQ). Buccal cells were collected using cytology brushes, and methylation of the LINE-1 repeated element and IFNγ (CpG -186 and -54) was measured by Pyromark Q96 (Quigen, Germantown, Maryland).

Data were collected on 32 asthmatic children living in wood stove homes. The mean (sd) age was 12.8 (2.5), ranging from 8-17 years, and 47% were male. Buccal samples (n=125) were collected 1 to 4 times per subject (n=32). Mean (sd) LINE-1 methylation was 65.3% (3.4) with a range of 56.1 – 73.2%. Mean (sd) IFNγ CpG -54 was 79.6% (4.5) with a range of 68.6 - 92.4%. Mean (sd) IFNγ CpG -186 was 70.1% (6.6) with a range of 49.1 - 81.6%.

In this cohort, methyl donors appear to be beneficial for respiratory health (i.e. higher percent predicted FEV_1) and PAQLQ. Several dietary constituents were associated with both global and gene specific methylation. LINE-1 methylation was positively associated with intake of protein and several methyl donors. The two IFNγ CpG sites that were investigated appear to be affected by intake of methyl donors in an opposite fashion. This was supported by our investigation of the relationship between methyl donors and the ratio of IFNγ CpG -54 to -186 (Me% 54/186). Methylation at IFNγ CpG -186 was associated with more dPFV and lower PAQLQ. While methylation at IFNγ CpG -54 was not associated with respiratory health, a higher Me% 54/186 was associated with less dPFV and better quality of life. We explored the influence of diet on respiratory health measures through a DNA methylation pathway by crude mediation analysis. This analysis did not support this hypothesis, however, further investigation is needed.

INTRODUCTION

Asthma is an environmentally triggered disease and dietary nutrients represent a very important environmental exposure. Dietary manipulation has been used successfully to reduce symptoms of diseases, including asthma, however, few human studies have focused on elucidating the mechanistic pathways linking diet to chronic disease. One potential mechanism whereby diet affects respiratory health is through epigenetic modulation of inflammatory cytokines.

Potential Impact of Diet on Asthma and Respiratory Health Outcomes

Significant observational data exists to suggest that dietary status and intake of particular nutrients can affect respiratory health outcomes. Recent studies suggest that some dietary nutrients may be protective for respiratory health (Thuesen et al. 2010; Mehta et al. 2010; Barros et al. 2008; Barros et al. 2011; Castro-Rodriguez et al. 2008; Chatzi et al. 2007, 2008; de Batlle et al. 2008; Garcia-Marcos et al. 2007; Romieu et al. 2009). In a study of nearly 12,000 men and women from the ARIC study, fiber was associated with several positive respiratory health measures including FEV_1 (Kan et al. 2008). Interestingly, the authors were able to show that fiber from fruit and not cereal was responsible for the association. A cross-sectional study by Berthon et al. showed that among asthmatics, a high fat diet was associated with increased airway eosinophilic inflammation, and low fiber was associated with poor lung function (Berthon et al. 2013). A group of researchers from Portugal found that, within a population of adult asthmatics, intake of a diet with a high ω6 to ω3 fatty acid ratio increased the odds of having uncontrolled asthma and intake of ω3 fatty acids predicted low exhaled nitric oxide (Barros et al. 2011). Supplementation of dietary folic acid and folates have been very important in the prevention of neural tube defects in the United States, however, some researchers have began to wonder if there may be a risk for unexpected effects because

folic acid is an important methyl donor (Lovinsky-Desir and Miller 2012). Human studies have produced mixed results regarding maternal folic acid supplementation and asthma development (Lovinsky-Desir and Miller 2012; Cortessis et al. 2012). A study in the murine model found that pups born to dams that ate a diet supplemented with methyl donors including folic acid had higher rates of allergic airway disease (Hollingsworth et al. 2008). In this study, the allergic airway disease was related to differences in T-cell maturation. Hollingsworth et al. also found differential methylation patterns for *Runx3*, a regulator of T-cell differentiation, in the exposure group relative to control.

Potential for an Epigenetic Mechanism

Several dietary nutrients are known to play a role in epigenetic mechanisms (see Table 2.1 and Figure 2.1). It is unclear if epigenetic changes are in the causal pathway whereby diet influences asthma outcomes or if these changes are markers of effect. There is likely a case to be made for each of the aforementioned circumstances depending on which gene and what exposure is measured. For the current study, interferon gamma (IFNγ) was chosen for its involvement in the asthma pathway as a T helper (Th) cell 1 cytokine. IFNγ and its role in the Th1/Th2 balance can be found in the overall introduction. After reviewing the literature, only one study has investigated the effect of diet on IFNγ production in humans (Meng et al. 2015) and this study did not consider DNA methylation. This study showed that cells, which were extracted and purified from non-asthmatic adults, produced differential amounts of IFNγ and these amounts were associated with intake of specific dietary variables. Further, it was found that dietary status and resulting IFNγ levels could predict upper respiratory tract infection incidence.

Nutrient	Food Origin	Epigenetic Role
Methionine	Sesame seeds, brazil nuts, fish, peppers, spinach	SAM synthesis
Folic Acid	Leafy vegetables, sunflower seeds, baker's yeast, liver	Methionine synthesis
Vitamin B12	Meat, liver, shellfish, milk	Methionine synthesis
Vitamin B6	Meats, whole grain products, vegetables, nuts	Methionine synthesis
Choline	Egg yolks, liver, soy, cooked beef, chicken, veal and turkey	Methyl donor to SAM
Betaine	Wheat, spinach, shellfish, and sugar beets	Break down the toxic byproducts of SAM synthesis
Genistein	Soy, soy products	Increased methylation, cancer prevention, unknown mechanism
Sulforaphane	Broccoli	Increased histone acetylation turning on anti-cancer genes
Butyrate	A compound produced in the intestine when dietary fiber is fermented	Increased histone acetylation turning on 'protective' genes, increased lifespan (shown in the lab in flies)
Diallyl sulphide (DADS)	Garlic	Increased histone acetylation turning on anti-cancer genes

Table 2.1. Dietary nutrients and their potential epigenetic role.

Adapted from http://learn.genetics.utah.edu/content/epigenetics/nutrition/

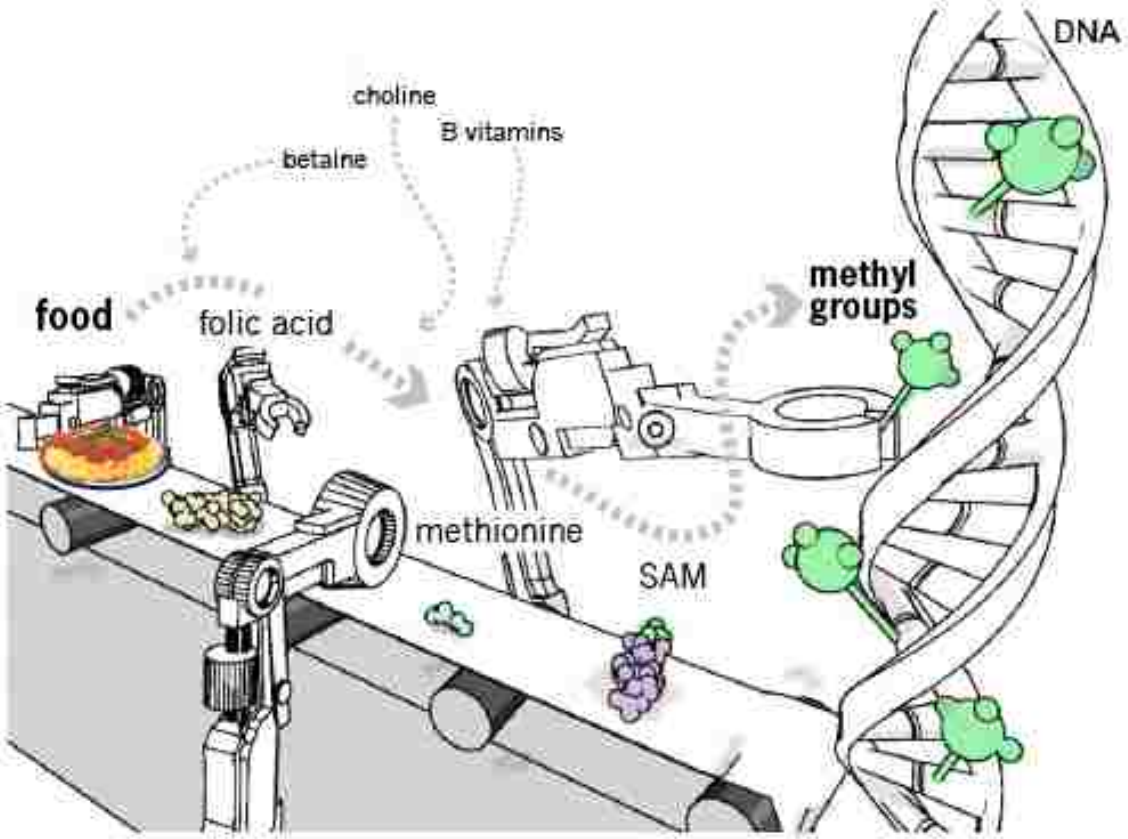

Figure 2.1. Dietary methyl donors.

This cartoon shows how constituents of food can be involved in methyl donation.

http://learn.genetics.utah.edu/content/epigenetics/nutrition

We hypothesized that within this asthmatic cohort of children there would be dietary nutrients associated with better respiratory health outcomes. These dietary nutrients are expected to be components of a healthy diet (i.e. high in fiber, omega 3, and low in saturated fats) compared to the modern Western diet (i.e. low in fiber, omega 3, and high in saturated fats). Additionally, nutrients such as folate, betaine, methionine, and choline would be associated with better respiratory health outcomes because of their methyl donating capabilities. Methylation at the IFNγ CpG sites will serve as an indicator of the Th-1/Th-2 balance. Within this asthmatic cohort, methylation profiles will likely be biased by Th-2 polarization however some nutrients may be inversely associated with methylation of IFNγ even within this context.

METHODS

Subject Recruitment

The ARTIS study included 114 subjects (children ages 6-17) from 97 homes. In homes where two asthmatic children were recruited to the ARTIS study, the child with the worst asthmatic symptoms was designated as the primary asthmatic. The subcohort in this epigenetic study included 32 primary asthmatic subjects. These subjects were given information about the buccal and FFQ collection and analysis and agreed to participate. In addition to the general ARTIS informed consent procedures, children were separately assented to participate in the epigenetics study and parents signed a separate permission form.

Dietary Nutrient Collection

Dietary data was collected using the 2004 Block Kids food frequency questionnaire (FFQ) (NutritionQuest, Berkley, CA) to characterize dietary intake among subjects. This instrument has been validated in children, ages 6 – 17 years old (Block et al. 1994; Block and DiSogra 1995; Block et al. 1992; Block et al. 1990). Correlation coefficients for total energy, fat, saturated and monounsaturated fat, carbohydrate, fiber and calcium range from 0.40 to 0.50 (unpublished Abstract presented at the 4th International Conference on Dietary Assessment Methods). A recent study compared the Block Kids FFQ to two 24-hour recalls in 83 children from age 10 to 17 years. Correlations for nutrients ranged from 0.29 to 0.69 and for food groups from -0.03 to 0.74 (Cullen and Watson 2008). The questionnaire includes 77 food items and takes 25 to 30 minutes to complete. The questionnaire was administered to each individual subject by trained staff using serving size visual aides provided by NutritionQuest. Parents or guardians were asked to be present during the interview to assist their child with portion size recognition and remembering foods they ate during the last week. All completed questionnaires

were sent to NutritionQuest for processing and calculating nutrient intake estimates. These data were returned to the University of Montana for analysis.

Health Outcome Measures Collected in Parent Study

The Pediatric Asthma Quality of Life Questionnaire (PAQLQ)

PAQLQ is a 23-item asthma-specific battery which provides domain scores for symptoms (10 items), activity limitation (5 items), and emotional function (8 items) (Juniper et al. 1996). The total PAQLQ score and each domain score are calculated as mean scores ranging from one to seven with seven as the optimal score. This questionnaire was directly administered to children twice per winter period. The PAQLQ has been validated as an evaluative tool to measure within subject changes over time due to treatment, and changes in this scale of 0.5 or more points can be directly translated as a clinically significant outcome (Juniper et al. 1996).

PiKo-1 Meter

Using the PiKo-1 meter (Ferraris) subjects performed a test two times daily, in the morning and in the evening for a period of two weeks. These two-week periods were initiated at the beginning of each air sampling event. For each sample event the instrument records the best result for both peak expiratory flow (PEF) and FEV_1. Outcomes from these measures include average morning PEF and FEV_1, average evening PEF and FEV_1, and diurnal PEF variability (dPFV).

Global and Gene-specific Methylation Analysis

Buccal Cell Collection and DNA extraction

Buccal cells were collected, stored, and processed according to a Gentra Puregene Buccal Cell DNA Kit (Qiagen, Valencia, CA). Briefly, the cells were collected from the

subject's cheek using a cytology brush (see Figure 2.2) and stored in a buffer solution at room temperature until all samples were collected. In compliance with this protocol, all samples were processed within 24 months from the day of collection. To extract the DNA, the cells were lysed, the proteins were removed, and the DNA was washed. The quantity of the purified DNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and then stored at -20°C. In the event that the primary sample did not yield sufficient DNA, secondary samples meant for RNA analysis were used. Pilot data confirmed that DNA could be successfully extracted using a modified protocol. At the time of collection, samples meant for RNA extraction were collected and put immediately in RNAlater solution (instead of cell lysis buffer) and placed in a -80°C freezer upon returning to the university. The modified protocol for DNA extraction involved centrifuging the sample for 20 minutes at 20,000 x g to create a cell pellet and discarding the RNAlater-containing supernatant. From this point, the Gentra Puregene Kit could be followed as written starting with the addition of the cell lysis solution.

a. b.

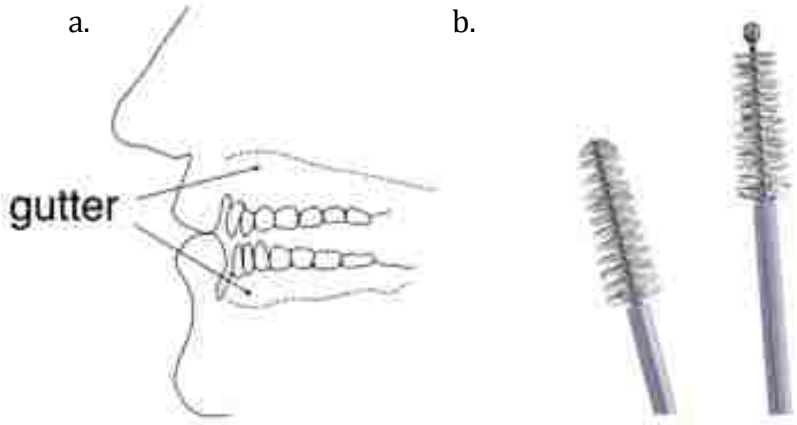

Figure 2.2. Location of buccal cell collection and brush.

The buccal cells were collected from the 4 gutters of the mouth (Figure 2.3.a.). Cells were collected on a cytology brush with a gentle scraping technique (Figure 2.3(.b.).

Bisulfite Treatment and Methylation Analysis

DNA bisulfite treatment was carried out using the EZ DNA Methylation-Direct Kit (Zymo Research, CA, USA) according to the manufacturer's instruction. Briefly, buccal cell DNA denatured at 98°C for 10 min was incubated with modification reagents for 2.5 h at 64°C and cleaned and desulphonated. The bisulfite-modified DNA was stored at -20°C. PCR amplification and pyrosequencing steps have been described previously (Yang et al. 2004). Briefly, after sodium bisulfite conversion, the DNA was PCR amplified using a forward and reversion primer. Gel electrophoresis was used to ensure proper amplification. The PCR product was visualized using a Pyromark Q96 MD (Qiagen, Germantown, MD). Each sample was PCR amplified using previously published primer sets for LINE-1, IFNγ -54, and IFNγ -186 (see Table 2.2). Samples were run in duplicate for each assay until the coefficient of variation multiplied by 100 (%CV) was less than 5. Epitect (Qiagen) bisulfite treated controls, which include a methylated and unmethylated human genome sample, were used on each plate.

Gene/region	Assay	Primers	PCR Conditions
LINE-1	PCR and Pyrosequencing	F: 5'-TTTGAGTTAGGTGTGGGATATA-3'	95°C, 15 min; 44 cycles of 95°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec; 72°C, 10 min; 4°C hold
		R: 5'-biotin-AAAATCAAAAAATTCCCTTTC-3'	
		S: 5'-AGTTAGGTGTGGGATATAGT-3'	
IFNλ CgG -186	PCR and Pyrosequencing	F: 5'-biotin-AGATGGTGATAGATAGGTAGGGATGATA-3'	95°C, 15 min; 45 cycles of 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec; 72°C, 10 min; 4°C hold
		R: 5'-TCCCACCAAAATAACACAAATAAACAT -3'	
		S: 5'-AAATAAACATAATAAATCTATCTCA-3'	
IFNλ CgG -54	PCR and Pyrosequencing	F: 5'ATGTGTTGTATTTTTTTTGGTTGTTGGTAT-3'	
		R: 5'-biotin-TATCATCCCTACCTATCTATCACCATCTC-3'	
		S: 5'-ATTGAAGTTTTTTGAGGATT-3'	

Table 2.2. Primers and amplification conditions for PCR and pyrosequencing experiments

Other Covariates

Anthropometric measures were determined by trained staff using a digital scale and stadiometer. Consistent with other studies of adolescent children, BMI percentile was calculated. Age, ethnicity, and gender were captured by the food frequency questionnaire.

Statistical analysis

Prior to analysis in SAS v9.3 (Cary, NC), applicable dietary data were adjusted to total energy. Dietary nutrients were converted to tertiles of intake for comparison with outcomes. Selected dietary nutrients were considered separately and evaluated for associations using linear regression for continuous health outcome measures and logistic regression for categorical health outcome measures. Similarly, selected dietary nutrients were considered separately and evaluated for associations using linear regression for global methylation and gene-specific methylation.

dPFV was dichotomized using the cut point of 20% (Jamison and McKinley 1993). In a study comparing asthmatics to normal subjects, Jamison et al. showed that the peak flow variability of > 20% could be used as a discriminating index and this measurement was more sensitive than other measurements tested such as forced expiratory volume. For our study peak flow variability < 20% represents well controlled asthma. PAQLQ scores were dichotomized at the median value of 5.8 (on a scale from 1-7) with higher values indicating better quality of life. Morning and evening FEV_1 were dichotomized using the cut point of 80%, which is the cut point for moderate asthma in adolescent children according to the Asthma Severity and Control Monitoring Guide.

To account for repeated measures within subject, a generalized estimating equation (GEE) normal distribution model was used to determine if associations existed between dietary nutrients and measures of methylation. In this model a parameter estimate of zero is the null value and values above zero indicate a positive association between dietary nutrients and the corresponding epigenetic marker. A GEE binomial distribution model was used to determine if associations existed between dietary nutrients and categorical respiratory health measures. This model estimates odds ratios with one being the null value, and odds ratio estimates less than one suggests that the dietary nutrient is protective for the corresponding asthma outcome measures. For clarification, an odds ratio is informative about the relationship between an exposure and an outcome. More specifically, an odds ratio tells you what the odds are for an outcome in the context of an exposure compared to the odds for that outcome without the exposure.

RESULTS

A subcohort of 32 asthmatic children from the ARTIS study participated in this study of diet, respiratory health, and epigenetics. The age of the children ranged from 8 to 17 and 47% were male. The study population was 94% non-Hispanic white. The mean (sd) BMI percentile was 70.6 (20.1) and 34% (n=11) were above the 85th percentile, which is considered overweight according to the Centers for Disease Control (CDC). Buccal samples were collected 1 to 4 times for each of the 32 subjects (n=125 samples). 52 total observations (includes repeated measures), where buccal sample, FFQ, and spirometry measurements were recorded, were used for analysis. Approximately 63% (n=20) of subjects had a year 1 and year 2 sample. Mean (sd) LINE-1 methylation was 65.3% (3.4) with a range of 56.1 – 73.2%. Mean (sd) IFNγ CpG -54 was 79.6% (4.5) with a range of 68.6 - 92.4%. Mean (sd) IFNγ CpG -186 was 70.1% (6.6) with a range of 49.1 - 81.6%. Baseline respiratory health measures and PAQLQ values can be found in Table 2.3. Baseline dietary nutrient values can be found in Table 2.4.

The relationship between potential confounding variables (i.e. age, and sex) and methylation and respiratory outcomes were investigated. Age was associated with higher percent methylation at the IFN CpG sites. For each year increase in age, methylation at IFNγ CpG -186 was 0.5 percentage points higher (95%CI: 0.29, 1.01) and Me% 54/186 was 1.01 units higher (95%CI: 1.00, 1.02). Age and sex were associated with PAQLQ. For each year increase in age, there was 26% lower odds of having below median PAQLQ (95%CI: 44%, 3%). For boys, there were 491% higher odds of having below median PAQLQ relative to girls.

In bivariate analysis of dietary constituents and DNA methylation or asthma outcomes, age and sex did not appreciably influence parameter estimates except where noted

below. Table 2.6, 2.7, and 2.8 presents bivariate analysis results unadjusted for age and sex.

Global and Gene-specific methylation

There was a modest positive association between IFNγ CpG -54 and LINE-1 methylation (p= 0.08). IFNγ CpG -186 was positively associated with LINE-1 methylation (p= 0.02). IFNγ -54 CpG and IFNγ CpG -186 were not associated (p= 0.86). Me% 54/186 tended to be negatively associated with LINE-1 methylation, however this relationship was not significant (p= 0.30).

A percentage point increase in LINE-1 methylation was linked to a 4% (95% CI: 2%, 7%) greater odds of high dPFV (i.e., dPFV > 20%). LINE-1 methylation was not associated with PAQLQ or FEV_1.

A percentage point increase in methylation at IFNγ CpG -186 was linked to a 2% (95% CI: 0%, 3%) greater odds of high dPFV and a 2% (95% CI: 0%, 3%) greater odds of below median PAQLQ (i.e. QOL < 5.85). Respiratory health measures were not significantly associated with methylation at IFNγ CpG -54, but Me% 54/186 was associated with better asthma outcomes. A unit increase in Me% 54/186 was linked to a 41% (95% CI: 63%, 7%) lower odds of high dPFV and 44% (95% CI: 64%, 13%) lower odds of below median PAQLQ.

Dietary nutrients with respect to methylation

Several dietary constituents, comparing highest tertile to the lowest tertile of intake, were associated with higher average LINE-1 methylation. For example, mean LINE-1 methylation among those in the highest tertile of folate intake was 2.1 percentage points

higher (95% CI: -0.03, 4.2) than LINE-1 methylation among those in the lowest tertile of folate intake. Similarly, among children in the highest tertiles of intake compared to those in the lowest tertile for selenium, folate, and two measures of choline, LINE-1 methylation was higher (see Table 2.7).

Several dietary constituents were positively associated with methylation at IFNγ CpG -54. Among those in the highest tertile of omega 3 to 6 ratio the mean methylation at IFNγ CpG -54 was 3.1 percentage points (95%CI: -0.1, 6.3) higher than the mean for those in the lowest tertile. Similarly, those in the highest tertile of folate, phosphotidylcholine, and vitamin B-6 also had higher mean IFNγ CpG -54 methylation compared to those in the lowest tertile (see table Table 2.7). In contrast to the relationship of diet and IFNγ CpG -54, several constituents were negatively associated with methylation at IFNγ CpG -186. Among those in the highest tertile of fiber the mean methylation at IFNγ CpG -186 was 5.4 percentage points (95%CI: 10, -0.8) lower than the mean for those in the lowest tertile. Similarly, those in the highest tertile of methionine, total choline, betaine and vitamin B-6 had lower mean IFNγ CpG -186 methylation compared to those in the lowest tertile (see Table 2.7). Those in the middle, rather than the highest, tertile of folate and vitamin B-2 had lower mean IFNγ CpG -186 methylation compared to those in the lowest tertile.

Intake of monosaturated fat and saturated fat was negatively associated with Me% 54/186. Intake of fiber, folate, methionine, free choline, phosphotidylcholine, total choline, betaine, vitamin B-2, and vitamin B-6 was positively associated with Me% 54/186.

Dietary nutrients with respect to markers of respiratory health

Those in the highest tertile of protein intake had 80% lower odds of having poor percent predicted FEV_1 (i.e., FEV_1 < 80%) (95% CI: 94%, 10%) compared to those in the lowest tertile of protein intake. Similarly, those in the highest tertile of monosaturated fat, saturated fat, methionine, free choline and the middle tertile of betaine had lower odds of having poor percent predicted FEV_1 compared to those in the lowest tertile (see Table 2.6). Those in the middle tertile of selenium intake had 80% lower odds of having below median PAQLQ (95% CI: 97%, 10%) compared to those in the lowest tertile of selenium intake. Similarly, those in the middle tertile of methionine, glycerophosphocholine, total choline, and betaine had lower odds of having below median PAQLQ compared to those in the lowest tertile (see Table 2.6). These results remained robust after adjusting for age and sex (data not shown). The relationship between PAQLQ and the following nutrients: fiber (middle and high), free choline (middle), glycerophosphocholine (high), vitamin B-2 (middle and high), and vitamin B-6, were no longer significant after adjusting for age and sex. Dietary nutrients were not significantly associated with dPFV.

Do nutrients affect respiratory health through an epigenetic mechanism?

The relationship between diet, respiratory health and DNA methylation was explored through crude mediation analysis. Individual methylation variables were added to the binomial GEE model where significant relationship existed between a diet variable and a respiratory health variable. The results of the mediation analysis did not suggest that the diet variables selected were affecting respiratory health through modulation of DNA methylation.

	N	Mean	SD	Minimum	Maximum
Age	32	12.81	2.47	8	17
Gender					
Male	15 (47%)				
Female	17 (53%)				
Ethnicity					
Non-Hispanic white	30 (94%)				
BMI percentile	32	70.64	24.14	5.83	98.97
dPFV	32	20	14	2	66
Morning FEV_1	32	81.72	19.72	26.88	112.92
Evening FEV_1	32	82.32	19.33	19.26	110.10
PAQLQ	32	5.68	1.05	3.10	7.00

Table 2.3. Population characteristics
Definitions: BMI, body mass index; dPFV, evening to morning peak flow variability; FEV_1, forced expiratory volume in 1 second; PAQLQ, pediatric asthma quality of life questionnaire

Nutrient	N	Mean	SD	Minimum	Maximum	Low Tertile	Medium Tertile	High Tertile
%kcal Monosaturated Fat	32	12.51	1.54	9.53	15.21	< 11.86	11.86 - 13.09	> 13.09
%kcal Saturated Fat	32	11.83	1.53	7.78	13.84	< 11.26	11.26 - 12.71	> 12.71
%kcal Protein	32	14.61	2.14	9.44	19.76	< 13.58	13.58 - 15.68	> 15.68
%kcal Omega 3	32	0.60	0.14	0.38	0.96	< 0.53	0.53 - 0.66	> 0.66
%kcal Omega 6	32	6.12	1.56	3.68	10.97	< 5.63	5.63 - 6.82	> 6.82
Omega 3/6	32	0.10	0.02	0.06	0.14	< 0.09	0.09 - 0.1	> 0.1
Selenium (mcg)	32	75.70	32.66	26.53	153.87	<53.34	53.34 - 77.4	> 77.4
Fiber (gms)	32	13.39	5.65	5.34	24.04	< 10.24	10.24 - 13.28	> 13.28
Vitamin D (IU)	32	185.83	106.10	18.19	365.67	< 125.17	125.17 - 192.83	> 192.83
Vitamin B2 (mg)	32	1.81	0.76	0.59	3.40	< 1.35	1.35 - 2.02	> 2,02
Vitamin B6 (mg)	32	1.48	0.58	0.62	2.68	< 1.1	1.1 - 1.5	> 1.5
Vitamin B12 (mcg)	32	4.42	2.11	1.63	10.49	< 3.16	3.16 - 4.91	> 4.91
Methionine (g)	32	1.31	0.55	0.47	2.40	< 1.01	1.01 - 1.33	> 1.33
Betaine (mg)	32	111.97	54.87	34.58	278.05	< 72.9	72.9 - 121.77	> 121.77
Free Choline (mg)	32	60.49	24.74	19.89	119.01	< 44.97	44.97 - 62.69	> 62.69
Glycerophosphocholine (mg)	32	49.65	23.36	9.95	90.46	< 37.79	37.79 - 61.3	> 61.3
Phosphocholine (mg)	32	11.72	5.07	3.62	22.99	< 9.84	9.84 - 12.91	> 12.91
Phosphatidylcholine (mg)	32	92.49	41.77	30.72	191.03	< 58.25	58.25 - 94.63	> 94.63
Total Choline (mg)	32	225.33	89.28	97.86	381.08	< 172.35	172.35 - 226.16	> 226.16
Folate (mcg)	32	445.35	179.05	166.17	862.97	< 330.23	330.23 - 457.03	> 457.03

Table 2.4. Dietary Nutrient Measurements

	N	Mean	SD	Minimum	Maximum
LINE-1 (%)	32	65.30	3.39	56.05	73.23
IFNγ -54 (%)	32	79.57	4.51	68.59	92.38
IFNγ -186 (%)	32	70.11	6.56	49.05	81.64
IFNγ -54/-186	32	1.15	0.15	0.93	1.85

Table 2.5. Global and gene-specific methylation measurements with repeated measures

		dPFV	p value	PAQLQ	p value	Morning FEV1	p value	Evening FEV1	p value
		cut point is 20%		median split at 5.85		cut point is 80%		cut point is 80%	
		1 is >20%		1 is < 5.85		1 is <80%		1 is <80%	
%kcal protein	high	0.6 (0.1, 3.3)		0.4 (0.1, 1.3)		0.2 (0.06, 0.9	0.03	0.3 (0.05, 1.3)	
	medium	0.7 (0.2, 2.4)		1.0 (0.5, 2.0)		0.4 (0.1, 1.5)		0.3 (0.06, 1.4)	
%kcal monosaturated fat	high	1.2 (0.2, 7.0)		1.0 (0.4, 2.9)		0.2 (0.06, 1.1)	0.06	0.5 (0.1, 1.8)	
	medium	0.9 (0.2, 3.6)		1.1 (0.3, 3.4)		0.4 (0.1, 1.5)		0.9 (0.3, 2.4)	
%kcal saturated fat	high	0.9 (0.2, 4.2)		1.3 (0.5, 3.3)		0.2 (0.05, 0.9)	0.03	0.3 (0.09, 1.3)	
	medium	0.5 (0.1, 2.3)		1.1 (0.4, 3.0)		0.2 (0.06, 0.8)	0.02	0.4 (0.1, 1.2)	0.09
omega 3 to 6 ratio	high	1.5 (0.3, 6.8)		1.3 (0.5, 3.2)		1.2 (0.3, 4.8)		0.9 (0.4, 2.0)	
	medium	1.4 (.3, 6.3)		0.6 (0.3, 1.2)		2.3 (0.7, 8.4)		1.2 (0.6, 2.2)	
selenium	high	2.0 (0.4, 9.3)		0.4 (0.1, 1.5)		0.6 (0.2, 2.4)		0.5 (0.2, 1.5)	
	medium	1.6 (0.3, 8.5)		0.2 (0.03, 0.9)	0.04	1.0 (0.3, 4.0)		0.6 (0.2, 1.7)	
fiber	high	1.0 (0.3, 4.2)		0.3 (0.07, 1.2)	0.08	0.6 (0.2, 2.1)		0.4 (0.1, 1.5)	
	medium	1.2 (0.3, 5.7)		0.2 (0.04, 1.09)	0.06	1.3 (0.4, 3.8)		1.1 (0.7, 1.9)	
folate	high	1.0 (0.3, 3.8)		0.8 (0.3, 2.4)		1.2 (0.3, 4.3)		0.7 (0.3, 1.8)	
	medium	1.0 (0.2, 4.7)		0.6 (0.1, 2.2)		1.5 (0.4, 6.4)		0.6 (0.2, 1.6)	
methionine	high	0.5 (0.1, 2.5)		0.3 (0.09, 1.3)		0.4 (0.07, 1.8)		0.3 (0.06, 1.2	0.09
	medium	1.2 (0.3, 4.5)		0.2 (0.04, 0.7)	0.02	0.5 (0.1, 2.1)		0.3 (0.06, 2.0)	
free choline	high	1.9 (0.4, 8.4)		0.6 (0.2, 1.5)		0.3 (0.05, 1.3)		0.1 (0.01, 1.3)	0.08
	medium	1.7 (0.3, 9.3)		0.3 (0.1, 1.0)	0.06	1.6 (0.5, 4.8)		0.9 (0.4, 2.1)	
glycerophosphocholine	high	1.0 (0.2, 3.8)		0.2 (0.02, 1.1)	0.07	0.7 (0.2, 2.2)		0.8 (0.4, 1.6)	
	medium	0.6 (0.1, 2.8)		0.1 (0.02, 0.9)	0.04	1.1 (0.4, 3.5)		1.1 (0.7, 1.8)	
phosphocholine	high	1.4 (0.4, 4.5)		0.3 (0.08, 1.4)		0.8 (0.3, 2.7)		1.1 (0.5, 2.6)	
	medium	0.8 (0.2, 3.4)		0.2 (0.04, 0.9)	0.04	1.5 (0.3, 6.4)		2.0 (0.5, 7.4)	
phosphotidylcholine	high	1.6 (0.3, 7.9)		0.5 (0.2, 1.9)		0.4 (0.08, 1.9)		0.4 (0.1, 1.3)	
	medium	1.9 (0.5, 7.0)		0.7 (0.4, 1.3)		2.4 (0.7, 8.4)		0.6 (0.2, 1.9)	
total choline	high	1.9 (0.4, 8.4)		0.4 (0.1, 1.2)		0.3 (0.07, 1.4)		0.2 (0.03, 1.4)	
	medium	1.7 (0.3, 9.6)		0.1 (0.03, 0.7)	0.02	1.5 (0.5, 4.5)		0.8 (0.4, 1.7)	
betaine	high	0.5 (0.2, 1.6)		0.5 (0.2, 1.2)		1.0 (0.2, 4.0)		0.6 (0.1, 3.8)	
	medium	0.6 (0.2, 1.9)		0.2 (0.06, 1.0)	0.05	3.9 (1.0, 15.8)	0.05	1.5 (0.5, 4.6)	
vitamin b-2	high	0.8 (0.2, 3.6)		0.2 (0.04, 1.2)	0.08	0.7 (0.2, 2.8)		0.6 (0.2, 1.4)	
	medium	0.6 (0.1, 3.1)		0.2 (0.06 ,1.1)	0.07	1.2 (0.3, 3.9)		1.3 (0.7, 2.2)	
vitamin b-6	high	2.0 (0.3, 11.2)		0.2 (0.04, 1.3)	0.09	1.2 (0.3, 5.4)		0.6 (0.3, 1.5)	
	medium	1.7 (0.4, 6.8)		0.5 (0.2, 1.1)		0.8 (0.2, 3.3)		0.6 (0.2, 1.7)	
vitamin b-12	high	0.8 (0.1, 4.6)		0.3 (0.5, 2.0)		0.8 (0.2, 3.5)		0.5 (0.2, 1.7)	
	medium	1.0 (0.2, 4.2)		0.6 (0.1, 3.1)		0.5 (0.2, 1.8)		0.7 (0.2, 2.1)	

Table 2.6. Bivariate analysis of diet and respiratory health variables.
Unadjusted results from GEE binomial distribution model evaluating associations between selected dietary nutrients and respiratory health outcomes. Dietary nutrients were evaluated as highest or medium tertile relative to lowest and results are given as the odds ratio followed by the 95% confidence interval. Definitions: dPFV, evening to morning peak flow variability; PAQLQ, pediatric asthma quality of life questionnaire; FEV_1, forced expiratory volume in the first second.

		IFN -54	p value	IFN -186	p value	IFN CpG Average	p value	IFN -54 / -186	p value	LINE-1	p value
%kcal protein	high	1.9 (-0.9, 4.6)		-2.4 (-6.1, 1.3)		-0.6 (-2.9, 1.8)		0.1 (-0.04, 0.2)		2.1 (-0.03, 4.2)	0.05
	medium	1.0 (-1.9, 3.9)		1.1 (-2.4, 4.6)		1.1 (-1.5, 3.7)		-0.01 (-0.09,0.06)		2.6 (0.4, 4.8)	0.02
%kcal monosaturated fat	high	-1.3 (-4.4, 1.8)		-0.6 (-4.9, 3.7)		-1.4 (-4.3, 1.5)		-0.03 (-0.1, 0.08)		0.8 (-1.2, 2.8)	
	medium	-2.1 (-4.9, 0.7)		3.3 (-0.6, 7.2)		0.3 (-2.4, 3.0)		-0.08 (-0.2, -0.002)	0.05	0.7 (-1.1, 2.5)	
%kcal saturated fat	high	0.2 (-2.1, 2.4)		3.2 (-2.6, 9.0)		0.8 (-2.0, 3.6)		-0.1 (-0.2, 0.003)	0.05	1.1 (-1.0, 3.2)	
	medium	1.7 (-1.3, 4.7)		2.0 (-2.6, 6.6)		1.3 (-1.3, 4.0)		-0.05 (-0.2, 0.04)		1.0 (-1.1, 3.2)	
omega 3 to 6 ratio	high	3.1 (-0.1, 6.3)	0.05	2.6 (-2.0, 7.2)		2.6 (-0.1, 5.3)	0.06	-0.00 (-0.1, 0.1)		1.2 (-1.3, 3.8)	
	medium	2.4 (-0.3, 5.1)	0.08	1.3 (-4.5, 7.2)		1.4 (-1.8, 4.5)		0.00 (-0.1, 0.1)		1.3 (-0.7, 3.2)	
selenium	high	1.4 (-1.7, 4.5)		-1.6 (-5.3, 2.0)		-0.3 (-2.9, 2.4)		0.06 (-0.03, 0.1)		1.9 (-0.3, 4.1)	0.09
	medium	1.8 (-1.2, 4.7)		-1.4 (-4.2, 1.4)		0.2 (-2.5, 3.0)		0.05 (-0.02, 0.1)		0.8 (-1.4, 3.0)	
fiber	high	0.8 (-2.4, 3.9)		-5.4 (10.0, -0.8)	0.02	-2.4 (-5.3, 0.5)		0.1 (0.02, 0.2)	0.02	2.1 (-0.6, 4.7)	
	medium	-0.5 (-3.4, 2.5)		-1.5 (-4.2, 1.1)		-1.0 (-3.4, 1.5)		0.01 (-0.04, 0.06)		0.5 (-1.6, 2.5)	
folate	high	4.2 (1.8, 6.5)	0.0006	1.3 (-2.4, 5.0)		2.0 (-0.2, 4.2)	0.08	0.06 (-0.02, 0.1)		2.1 (0.3, 3.9)	0.02
	medium	2.0 (-0.7, 4.8)		-3.4 (-6.1, -0.6)	0.02	-0.7 (-3.1, 1.6)		0.09 (0.03, 0.2)	0.004	0.3 (-1.4, 2.0)	
methionine	high	1.3 (-2.0, 4.5)		-3.1 (-6.6, 0.4)	0.08	-1.3 (-4.1, 1.4)		0.08 (-0.00, 02)	0.05	1.2 (-1.2, 3.5)	
	medium	0.5 (-2.5, 3.5)		-1.4 (-5.0, 2.2)		-0.9 (-3.6, 1.7)		0.05 (-0.03, 0.1)		1.3 (-1.0, 3.5)	
free choline	high	1.3 (-1.6, 4.1)		-2.2 (-5.8, 1.3)		-0.8 (-3.3, 1.7)		0.06 (-0.02, 0.1)		1.8 (0.08, 3.4)	0.04
	medium	1.8 (-1.1, 4.6)		-2.1 (-5.1, 0.9)		-0.2 (-2.9, 2.4)		0.06 (-0.00, 0.1)	0.06	-0.03 (-1.7, 1.7)	
glycerophosphocholine	high	0.5 (-2.4, 3.3)		-2.9 (-6.5, 0.6)		-1.5 (-3.7, 0.6)		0.07 (0.02, 0.2)		0.4 (-1.7, 2.4)	
	medium	1.5 (-1.0, 4.0)		1.4 (-1.9, 4.8)		1.6 (-1.1, 4.3)		0.00 (-0.05, 0.05)		1.0 (-0.8, 2.7)	
phosphocholine	high	0.8 (-2.1, 3.7)		-1.1 (-4.4, 2.2)		-0.6 (-3.0, 1.9)		0.04 (-0.03, 0.1)		0.8 (-1.0, 2.5)	
	medium	0.7 (-2.0, 3.3)		0.3 (-3.2, 3.7)		0.3 (-2.4, 3.0)		0.01 (-0.07, 0.09)		-0.4 (-2.6, 1.9)	
phosphotidylcholine	high	2.4 (-0.3, 5.1)	0.09	-2.1 (-5.5, 1.4)		-0.01 (-2.6, 2.5)		0.07 (-0.00, 0.2)	0.07	1.1 (-1.0, 3.1)	
	medium	0.7 (-2.4, 3.8)		-0.9 (-6.3, 4.5)		0.00 (-3.1, 3.1)		0.03 (-0.07, 0.1)		-0.8 (-3.0, 1.3)	
total choline	high	1.6 (-1.2, 4.4)		-3.5 (-6.8, -0.1)	0.04	-1.1 (-3.4, 1.2)		0.09 (0.01, 0.2)	0.03	2.4 (0.4, 4.3)	0.02
	medium	1.7 (-1.3, 4.8)		-2.3 (-4.9, 0.4)	0.09	-0.5 (-3.1, 2.1)		0.07 (0.00, 0.1)	0.04	1.5 (-0.5, 3.6)	
betaine	high	1.9 (-0.8, 4.6)		-3.4 (-7.4, 0.5)	0.09	-0.9 (-3.5, 1.6)		0.09 (0.0, 0.2)	0.02	1.3 (-0.7, 3.3)	
	medium	2.2 (-0.4, 4.7)		-2.0 (-4.3, 0.4)		0.05 (-2.2, 2.3)		0.07 (0.02, 0.1)	0.003	1.1 (-0.9, 3.1)	
vitamin b-2	high	1.7 (-0.8, 4.2)		-2.2 (-5.8, 1.4)		-0.7 (-2.9, 1.5)		0.07 (-0.01, 0.1)		1.3 (-0.5, 3.0)	
	medium	0.7 (-1.7, 3.1)		-3.4 (-7.1, 0.2)	0.07	-1.3 (-4.0, 1.3)		0.08 (0.00, 0.2)	0.05	-0.2 (-2.0, 1.7)	
vitamin b-6	high	2.9 (0.7, 5.2)	0.01	-3.7 (-6.9, -0.5)	0.02	-0.3 (-2.4, 1.9)		0.1 (0.05, 0.2)	0.001	1.5 (-0.6, 3.6)	
	medium	0.1 (-3.0, 3.3)		-4.7 (-9.1, -0.4)	0.03	-1.8 (-4.6, 1.0)		0.1 (0.00, 0.2)	0.04	0.7 (-1.6, 3.0)	
vitamin b-12	high	2.1 (-0.7, 4.8)		-1.4 (-4.7, 1.9)		0.1 (-2.3, 2.6)		0.06 (-0.02, 0.1)		0.8 (-1.3, 2.9)	
	medium	1.4 (-1.6, 4.4)		1.0 (-2.1, 4.1)		0.9 (-1.6, 3.3)		0.007 (-0.06, 0.08)		1.2 (-1.1, 3.5)	

Table 2.7. Bivariate analysis of diet and methylation variables.
Unadjusted results from GEE normal distribution model evaluating associations between selected dietary nutrients and methylation outcomes. Dietary nutrients were evaluated as highest or medium tertile relative to lowest and results are given as the odds ratio followed by the 95% confidence interval.

		dPFV	p value	PAQLQ	p value	Morning FEV1	p value	Evening FEV1	p value
		cut point is 20%		median split at 5.85		cut point is 80%		cut point is 80%	
		1 is >20%		1 is < 5.85		1 is <80%		1 is <80%	
IFN -54		1.00 (0.98, 1.03)		0.99 (0.97, 1.02)		1.01 (0.99, 1.04)		1.01 (0.98, 1.03)	
IFN -186		1.02 (1.00, 1.03)	0.01	1.02 (1.00, 1.03)	0.01	1.01 (0.99, 1.03)		1.01 (0.99, 1.02)	
IFN CpG Average		1.02 (1.00, 1.05)	0.06	1.02 (0.99, 1.04)		1.02 (0.99, 1.05)		1.01 (0.98, 1.04)	
IFN -54 / -186		0.59 (0.37, 0.93)	0.02	0.56 (0.36, 0.87)	0.01	1.05 (0.49, 2.23)		0.98 (0.56, 1.71)	
LINE-1		1.04 (1.02, 1.07)	0.001	1.0 (0.96, 1.03)		0.99 (0.95, 1.03)		0.98 (0.95, 1.02)	

Table 2.8. Bivariate analysis of methylation and respiratory health variables.
Unadjusted results from GEE binomial distribution model evaluating associations between methylation variables and respiratory health outcomes. Results are given as the exponentiated point estimate followed by the 95% confidence interval. Definitions: dPFV, evening to morning peak flow variability; PAQLQ, pediatric asthma quality of life questionnaire; FEV_1, forced expiratory volume in the first second.

DISCUSSION

Within this cohort of childhood asthmatics, we sought to identify dietary nutrients that may be beneficial for respiratory health. In addition we measured LINE-1 and IFNγ (CpG -54 and -186) methylation levels in hopes of identifying a potential mechanism whereby diet directly influences factors in the allergic asthma pathway.

Diet and respiratory outcomes

Percent predicted FEV_1 in our cohort was influenced by several dietary nutrients including macro and micro nutrients. Intake of methyl donors appears to be beneficial for this respiratory health marker. Below median PAQLQ was associated with low intake of several nutrients including selenium, fiber, and methyl donors, however, the associations with selenium and fiber were no longer significant when adjusted for age and sex. The relationship between PAQLQ and several methyl donors were robust even after adjusting for age and sex. Methyl donors have been implicated as having a protective role for airway inflammation (Haberg et al. 2009). Interestingly dPFV was not associated with any of the selected dietary nutrients in this cohort.

The data for the respiratory health measures in this population were not normally distributed, therefore we chose to dichotomize on specific cut points. A cut point of 20% was used for dPFV (i.e. high peak flow variability is > 20%) but this may warrant further investigation because depending on the study methods a cut point of 8% to 20% has been recommended (Reddel et al. 2009). A cut point of 80% was chosen for percent predicted FEV_1, which is an accepted guideline for discriminating between mild asthmatics and persistent asthmatics (Miller et al. 2005). For PAQLQ, we conducted a median split. It should be noted that the median split was conducted because the data were skewed toward a high PAQLQ score and therefore the median was also relatively

high.

One limitation of our study is that the FFQ that was used did not originally include several of the methyl donor metrics that we were interested in. NutritionQuest included these seven metrics (i.e. methionine, free choline, glycerophosphocholine, phosphocholine, phosphotidylcholine, total choline, and betaine) in the analysis at our request. While this investigation of the relationship between methyl donors and health outcomes is a novel aspect to our study it is also a limitation because the FFQ was not validated with these metrics. Therefore, the interpretation of these metrics and their relationships with our outcomes of interests should be done with caution.

Dietary nutrients with respect to Global methylation

We found that %kcal of protein, selenium, folate and two measures of choline were positively associated with LINE-1 methylation, a proxy for global methylation. Selenium is a potent antioxidant and may play a role in respiratory health through systemic reduction of oxidative stress (Norton and Hoffmann 2012). Folate and choline have been studied extensively for their role in one carbon metabolism and methyl donation. In a subcohort from the COMIR study, researchers looked at white blood cell DNA from participants and found that folate from fortification was positively correlated with LINE-1 methylation (Spearman $r = 0.21$, $p = 0.007$) and suggested that folate fortification of cereals and other grains may need to be revisited (Zhang et al. 2012). Protein could be an important proxy measurement for the child's dietary pattern but it is difficult to make this designation using this FFQ. In some studies, high protein in the context of a Western diet has been shown to cause global hypomethylation, while a prudent diet has been shown to protect against hypomethylation (Zhang et al. 2011). Protein can come from a variety of sources and one possibility is that these children are eating protein from more

wild/organic sources. The FFQ that was used does not differentiate between certain sources of the meat, like a deer steak versus a store-bought beef steak for example.

Dietary nutrients with respect to IFNγ CpG methylation

The omega 3 to 6 ratio and intake of several methyl donors were positively associated with methylation at IFNγ CpG -54. The fatty acid ratio may be an important determining factor in the body's ability to resolve inflammation (Bosma-den Boer et al. 2012) and research suggests that increasing the omega 3 to 6 ratio can improve measurements of systemic inflammatory markers (Urpi-Sarda et al. 2012). Children who had more fiber and methyl donors in their diet had less IFNγ CpG -186 methylation. These negative associations should be cautiously interpreted because it is unlikely that intake of dietary nutrients are resulting in direct demethylation at any CpG site. The literature suggests that hypomethylation in the IFNγ promoter region will result in increased transcription but the mechanism for active demethylation is not clear. One mechanism whereby diet could cause passive hypomethylation at an IFNγ CpG site would be through down regulation of a methyltransferase involved in maintenance DNA methylation (Jin et al. 2014). Further investigation into the methylation patterns of methyltransferases and other genes involved in the Th1/Th2 balance within the context of asthma and diet are needed.

Global methylation with respect to respiratory health

We found that methylation was positively associated with a marker of poor respiratory health, dPFV. Global methylation is difficult to interpret in the context of respiratory health and may be even more complex in this cohort of asthmatic children. Few studies have looked at buccal DNA LINE-1 global methylation in healthy children. A study of 57 healthy girls aged from 6-15 investigated LINE-1 global methylation in saliva samples and the average (SD) was 75.2 (3.4) (Wu et al. 2014). In our cohort of asthmatics, the

mean (sd) LINE-1 methylation from buccal cells was 65.3% (3.4), which is considerably lower. Our data did not suggest that there was a significant difference in LINE-1 methylation between boys and girls. The cells collected from buccal and saliva should be similar and would likely not account for such a discrepancy. In a study of 35 asthmatic children aged 8 to 11 years, the mean (sd) LINE-1 methylation from nasal cells was 72.3 (4.1) (Baccarelli et al. 2012). It is difficult to compare buccal cells and nasal cells, however. These results suggest that our cohort has low LINE-1 methylation measurements on average even compared to other asthmatic children. Regarding global methylation and diet, researchers from the MESA study found no association between methyl donors and LINE-1 methylation in an adult atherosclerotic population, but the authors did report a correlation between BMI and LINE-1 methylation (Perng et al. 2014). We did not see a correlation between BMI and LINE-1 methylation in our cohort of asthmatic children.

By convention, an increase in global methylation is thought to protective, while a shift toward genome-wide hypomethylation is often associated with a poor health outcome or disease (Rozek et al. 2014). However, DNA methylation is dynamic and global methylation is a reflection of the epigenetic changes occurring at many gene locations. It is unclear if the changes that occur in response to acute exposures would be measurable or if these changes would be muted by the more pernicious epigenetic changes that are acquired over longer periods. The latter would suggest global methylation is more vulnerable to chronic exposures. Our data suggests that global methylation is positively related to a marker of poor respiratory health, however, the average global methylation of the cohort was very low relative to other similar cohorts.

IFNγ CpG methylation with respect to respiratory health outcomes

Children with more IFNγ CpG -186 methylation had more dPFV and below median PAQLQ scores. Methylation at IFNγ CpG -54 was not associated with the selected measures of respiratory health. Children with a higher Me% 54/186 had less dPFV and above median PAQLQ scores.

IFNγ CpG -54 methylation was not correlated with IFNγ CpG -186 by general model of linear regression where clustering by subject repeated measure was accounted for. This is in contrast to Lovinsky-Desir et al. who found these CpG sites were weakly correlated ($r = 0.24$, $p = 0.04$) in a cohort of adult and child asthmatics. There was a strong positive association between IFNγ CpG -186 and LINE-1 methylation, while there was only a weak association between IFNγ CpG -54 and LINE-1 methylation.

One limitation of our study is that we did not measure RNA expression of IFNγ. However, if IFNγ is considered a proxy for Th1/Th2 balance and important for respiratory health, then methylation at IFNγ CpG -186 may be an important biomarker. IFNγ CpG -186 methylation in this population could be influencing respiratory health by suppressing IFNγ production. Our data suggest that IFNγ CpG -54 is having either no effect or potentially the inverse effect, meaning that methylation at this site could cause increased expression. The IFNγ CpG -54 to -186 ratio was included in our analysis after finding that dietary nutrients were affecting methylation at the sites inversely. The data to support this proposed relationship is modest but the idea that dietary nutrients may be affecting two neighboring CpGs in the promoter of a gene in opposite ways is very interesting. The findings suggesting there is differential methylation patterns at these two CpG sites is inconsistent with prior studies (Runyon et al. 2012; Kohli et al. 2012). In contrast to our study, both Runyon et al. and Kohli et al. were evaluating DNA

methylation in T cells purified from whole blood samples. Therefore, it is possible that DNA methylation is different between these two matrices. It is possible for methylation of a functional CpG to cause more transcription (Ngo and Sheppard 2015) and it can happen as a result of having more methyl donors in the diet (Li et al. 2014). Without RNA expression data, it is difficult to determine what impact methylation at these CpG sites is having. It is intriguing that differential patterns were found at two proximally located CpGs and that the ratio, which appears to be influenced by diet, was associated with respiratory health and quality of life.

CONCLUSIONS

In this cohort, methyl donors appear to be beneficial for respiratory health (i.e. higher percent predicted FEV_1) and PAQLQ. Several dietary constituents were associated with both global and gene specific methylation. LINE-1 methylation was positively associated with intake of protein and several methyl donors. The two IFNγ CpG sites that were investigated appear to be affected by intake of methyl donors in an opposite fashion. This was supported by our investigation of the relationship between methyl donors and the ratio of IFNγ CpG -54 to -186 (Me% 54/186). Methylation at IFNγ CpG -186 was associated with more dPFV and below median PAQLQ. While methylation at IFNγ CpG -54 was not associated with respiratory health, a higher Me% 54/186 was associated with less dPFV and above median PAQLQ. We explored the influence of diet on respiratory health measures through a DNA methylation pathway by crude mediation analysis. This analysis did not support this hypothesis, however, further investigation is needed.

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CHAPTER 3

Assessing the Impact of Indoor $PM_{2.5}$ Exposure on Respiratory Health Outcome Measures and DNA Methylation in Asthmatic Children

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ABSTRACT

Biomass fuels are used worldwide and wood is the most common of these fuel sources. Wood is of particular importance in the Rocky Mountain region not only because it is abundant and relatively easy to harvest but also because it is the primary fuel source for residential heating in rural areas where the infrastructure for gas does not exist. Historically, studies that target particulate exposure focus on urban sources such as diesel exhaust, so the ARTIS study will provide useful data to fill the knowledge gap on the efficacy of indoor PM intervention strategies in the Rocky Mountain region where wood smoke PM may be more important than other sources of PM such as diesel exhaust. PM is a known trigger for asthma exacerbation, however the mechanism has not been fully elucidated. Epigenetic modifications such as DNA hypo- or hyper-methylation have been shown to occur in response to environmental exposures including PM and it is possible that DNA methylation is involved in this mechanism.

Within a subcohort of asthmatic children from the Asthma Randomized Trial of Indoor wood Smoke (ARTIS) study, the relationship between PM, DNA methylation, and respiratory health was evaluated. Buccal cell DNA was analyzed for percent methylation of LINE-1 as a proxy for global methylation and for two IFNγ promoter CpG sites. $PM_{2.5}$, fine particle counts, and coarse particle counts were assessed. Asthma health measures included a quality of life (PAQLQ) instrument and two-week daily monitoring of diurnal peak expiratory flow (PEF) and forced expiratory volume in first second (FEV1).

Data were collected for 37 asthmatic children living in wood stove homes. Mean (sd) LINE-1 methylation was 65.0% (3.5) with a range of 55.5 – 74.6%. Mean (sd) IFNγ CpG -54 was 79.0% (4.9) with a range of 64.7 – 97.1%. Mean (sd) IFNγ CpG -186 was 71.0% (6.5) with a range of 49.1 – 95.9%. In this cohort, age and sex were associated

with both IFNγ methylation and respiratory health. The filter intervention in the ARTIS study successfully reduced wintertime measurements of indoor PM in the homes of childhood asthmatics and these reductions were associated with better respiratory health outcomes. Methylation at CpG sites within the promoter of IFNγ was associated with indoor PM measurements and respiratory health measures, however we did not find that PM was affecting respiratory health through this epigenetic pathway. Further investigation into RNA expression of IFNγ and other genes related to Th1/Th2 balance will help to clearly define the interplay between indoor PM exposure and immunoregulation among asthmatic children.

INTRODUCTION

Wood Smoke Particulate Matter

About half the world's population uses a biomass fuel source to heat or cook with, and among these sources wood is the most common. Biomass fuels are relatively inefficient and create more air pollution compared to other fuels, but these sources of fuel are cheap. In contrast, sources like gas and electricity are more efficient and create less air pollution, but are also more expensive and often less accessible for rural communities (Sood 2012). The smoke generated from burning wood and other biomass combustion sources can be a complex mixture of pollutants including particulate matter, carbon monoxide, oxides of nitrogen and sulfur, benzene, formaldehyde, polycyclic aromatic hydrocarbons, free radicals, aldehydes, volatile organic compounds, chlorinated dioxins, and endotoxin (Kurmi et al. 2012).

Fine particulate matter having an aerodynamic diameter <2.5 μm ($PM_{2.5}$) has been suggested to serve as the best exposure metric for wood smoke because it tends to be among the most elevated pollutants in wood smoke emissions (Naeher et al. 2007). Source apportionment studies from several communities in the northern Rocky Mountain region have demonstrated that residential wood smoke contributes 56% to 77% of the measured wintertime ambient $PM_{2.5}$ (Ward and Lange 2010). Firewood is not only an abundant source of fuel in this geographical region of the United States, but for some residents of rural Montana, this may be one of the only choices for a heating source because the infrastructure for gas heat does not exist in their community.

The Importance of Indoor $PM_{2.5}$

A large majority of the studies, which have evaluated the impact of $PM_{2.5}$ on children's respiratory health outcomes, have been focused on urban sources of exposure.

Because immunological responses are likely specific to the source, size and composition of the inhaled PM (Miyata and van Eeden 2011), there is a need to evaluate health outcomes in geographical locations where elevated ambient, non-urban sources of $PM_{2.5}$ occur. Additionally, consideration should be given to the fact that people are spending more and more of their time indoors (Klepeis et al. 2001). Indoor sources of biomass generated PM have been linked to poor health outcomes in developed and developing countries (Sood 2012; Pope et al. 2010). The World Health Organization (WHO) estimates that nearly half of the 2 million premature deaths annually attributed to solid fuel indoor air pollution exposure are from cases of pneumonia in children less than 5 years of age.

In the United States, it is estimated that the number of susceptible individuals (elderly and children were considered susceptible) who are exposed to residential indoor wood smoke is approximately 5 million people (Noonan et al. 2015). Noonan et al. note that this may be an underestimation because they did not include individuals with chronic diseases or infiltration of ambient wood smoke into houses without wood stoves. Our laboratory has shown that significant reductions in ambient pollution can be achieved following a large-scale wood stove change in a western Montana community, and these reductions were correlated with better respiratory health outcomes in children (Noonan et al. 2012a). By contrast, improvements in indoor air quality following stove technology upgrades has been shown to be highly variable (Noonan et al 2012)

The Potential Epigenetic Link Between PM and Asthma

Extensive data supports the relationship between ambient PM and asthma morbidity. Follow-up of cohorts or panels of asthmatic patients have demonstrated that elevations in PM_{10} and $PM_{2.5}$ concentrations are associated with increases in severe asthma

attacks, asthma symptoms, asthma medication usage, and hospital emergency department visits for asthma and upper respiratory infections (Slaughter et al. 2003; Ostro et al. 2001; McConnell et al. 2003; Yu et al. 2000; Wong et al. 1999; Thompson et al. 2001; Lin et al. 2002; Jaffe et al. 2003; Norris et al. 1999; Sheppard et al. 1999; Lipsett et al. 1997; English et al. 1998; Peel et al. 2005; Schwartz et al. 1993). While it is accepted that the development of asthma is a consequence of both genetic susceptibility and environmental exposure (Holloway et al. 2010), the biological mechanism underlying development and exacerbation are still poorly understood.

There is a need for biomarkers of exposures in the context of household air pollution exposure. Epigenetic marks such as DNA methylation could serve in this role (Rylance et al. 2013). Although gestation and early infancy are sensitive time periods, epigenetic changes can occur throughout life (Miller and Ho 2008). A small number of studies in adults have evaluated short-term $PM_{2.5}$ exposure and global measures of DNA methylation in LINE-1 and Alu repeated elements. A study of elderly subjects found that exposure to traffic-derived $PM_{2.5}$ and black carbon was associated with decreased LINE-1, but not Alu methylation (Madrigano et al. 2011; Baccarelli et al. 2009). This effect of LINE-1 hypomethylation was observed for $PM_{2.5}$ and black carbon exposures on a sub-chronic scale (Baccarelli et al. 2009). When considering a more chronic scale, the effect was evident for black carbon only (Madrigano et al. 2011). A Belgium study of non-smoking adults found that several measures of air pollution, including $PM_{2.5}$, were associated with global methylation, which was analyzed by HPLC (De Prins et al. 2013). In this study, only summer time $PM_{2.5}$ was significantly associated with global methylation and men had more hypomethylation than women.

Global methylation can be informative in the context of respiratory health, but to elucidate a mechanism, gene specific analysis is key to understanding which immunological pathways are being affected. In a population of elderly men within the Normative Aging Study, sub-chronic exposure to air pollutants including non-traffic $PM_{2.5}$ were evaluated for associations with methylation changes in genes related to lung inflammation and immunity (Lepeule et al. 2014). The researchers found that slope estimates for air pollutants were higher for participants with below median levels of methylation at several CpG locations for two out of nine airway inflammation related genes.

Throughout the last decade, candidate gene and genome-wide association studies have shown that genetic variation of several cytokines and transcriptional factors relevant to the T-helper 1/ T-helper 2 (Th1/Th2) balance are associated with asthma (Vercelli 2008; Barnes 2011; Beghe et al. 2003; Moffatt et al. 2010; Kabesch et al. 2006; Robinson 2010). For the current study, interferon gamma (IFNγ) was chosen for its involvement in the asthma pathway as a Th1 cytokine. A review of the Th1/Th2 balance, the current literature on IFNγ, and its potential role in asthma can be found in the main introduction. Briefly, several studies have shown that IFNγ plays a key role in the counter-regulation of the Th2 polarization. Modulation of the methylation profile in the promoter region of IFNγ has been shown to affect T cell polarization and/or differentiation in humans and two CpG sites have been specifically chosen (IFNγ CpG -54 and -186) because they potentially play a role in asthma.

Intervention Strategies

In the United States, the prevalence of childhood asthma has increased over the last twenty years (Kay 2001) and the annual financial burden has inflated to over 2 billion

dollars (Landrigan, 2002). There has been a call for efficient low-cost strategies to reduce exposures that exacerbate asthma symptoms and improve the quality of life for asthmatics, especially childhood asthmatics. Children spend approximately 90% of their time indoors (Klepeis 2001), therefore intervention strategies that reduce indoor exposures should be considered. Wood stove change outs have been used with some success but the reductions in the indoor $PM_{2.5}$ levels can be variable (Ward et al. 2008; Noonan et al. 2012b). The reduction in $PM_{2.5}$ and possible improvements in respiratory health for the residents (including children) are dependent on the proper usage and maintenance of the wood stove, which is problematic. In addition the EPA Burn Wise program suggests that the type of wood as well as the processing of the wood can have an impact on the amount of $PM_{2.5}$ that is generated inside and outside the home. Air filtration units can be used to reduce indoor $PM_{2.5}$ and have been shown to improve symptoms in allergic asthmatics (McDonald et al. 2002; Fisk 2013). Air filtration units are designed to capture asthma triggers such as pet allergens and particulate matter. Air filters have been shown to significantly reduce allergen-induced asthma attacks, improve quality of life scores in asthmatic children, and reduce medication usage in adult asthmatics (McDonald et al. 2002; Johnson et al. 2009; Eick and Richardson 2011).

METHODS

Subject Recruitment

The ARTIS study included 114 subjects from 97 homes. In homes where two asthmatic children were recruited to the ARTIS study, the child with the worst asthmatic symptoms was designated as the primary asthmatic. The subcohort in this epigenetic study included 37 primary asthmatic subjects. These subjects were given information about the buccal collection and analysis and agreed to participate. In addition to the general ARTIS informed consent procedures, children were separately assented to participate in the epigenetics study and parents signed a separate permission form.

Indoor Exposure Assessment

During each 48-hour sampling event per each of two winters, a DustTrak 8530 (TSI, Shoreview, MN) that continuously measured $PM_{2.5}$ mass was deployed within the home. The instrument was placed 3-5ft off the ground within the same room as the wood stove. The instrument was set to record at 60-second intervals and was zero calibrated prior to each sampling event. A co-located 3016IAQ particle counter (Lighthouse Worldwide Solutions, Fremont, CA, USA) continuously recorded simultaneous counts of particles in multiple size ranges (0.3-0.49, 0.5-0.99, 1.0-2.49, 2.5-4.9 and 5.0-10.0 μm). Air was sampled at 2.83 LPM and data was recorded on 60-second averages. Particle counts for the fine fraction ($PM_{2.5}$) were calculated to be the sum of the 0.3-0.49, 0.5-0.99, and 1.0-2.49 μm particle counts while the coarse fraction was calculated to be the sum of the 2.5-4.9 and 5.0-10.0 μm particle counts.

Health Outcome Measures Collected in Parent Study

The Pediatric Asthma Quality of Life Questionnaire (PAQLQ)

PAQLQ is a 23-item asthma-specific battery which provides domain scores for symptoms (10 items), activity limitation (5 items), and emotional function (8 items) (Juniper et al. 1996). The total PAQLQ score and each domain score are calculated as mean scores ranging from one to seven with seven as the optimal score. This questionnaire was directly administered to children twice per winter period. The PAQLQ has been validated as an evaluative tool to measure within subject changes over time due to treatment, and changes in this scale of 0.5 or more points can be directly translated as a clinically significant outcome (Juniper et al. 1996).

PiKo-1 Meter

Using the PiKo-1 meter (Ferraris) subjects performed a lung function test two times daily, in the morning and in the evening for a period of two weeks. These two-week periods were initiated at the beginning of each air sampling event. For each sample event the instrument records the best result for both peak expiratory flow (PEF) and forced expiratory volume in the first second (FEV_1). PEF and FEV_1 measures were converted to percent predicted values according to the child's age and height. Outcomes from these measures include average percent predicted morning PEF and FEV_1, average percent predicted evening PEF and FEV_1, and PEF variability. Daily diurnal PEF variability (dPFV) was calculated as evening PEF minus the prior morning PEF divided by the mean of the two measures.

Global and Gene-specific Methylation Analysis

Buccal Cell Collection and DNA extraction

Buccal cells were collected, stored, and processed according to a Gentra Puregene Buccal Cell DNA Kit (Qiagen, Valencia, CA). Briefly, the cells were collected from the subject's cheek using a cytology brush (see Figure 3.1) and stored in a buffer solution at

room temperature until all samples were collected. In compliance with this protocol, all samples were processed within 24 months from the day of collection. To extract the DNA, the cells were lysed, the proteins were removed, and the DNA was washed. The quantity of the purified DNA was measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE) and then stored at -20°C. In the event that the primary sample did not yield sufficient DNA, secondary samples meant for RNA analysis were used. Pilot data confirmed that DNA could be successfully extracted using a modified protocol. At the time of collection, samples meant for RNA extraction were collected and put immediately in RNAlater solution (instead of cell lysis buffer) and placed in a -80°C freezer upon returning to the university. The modified protocol for DNA extraction involved centrifuging the sample for 20 minutes at 20,000 x g to create a cell pellet and discarding the RNAlater-containing supernatant. From this point, the Gentra Puregene Kit could be followed as written starting with the addition of the cell lysis solution.

a. b.

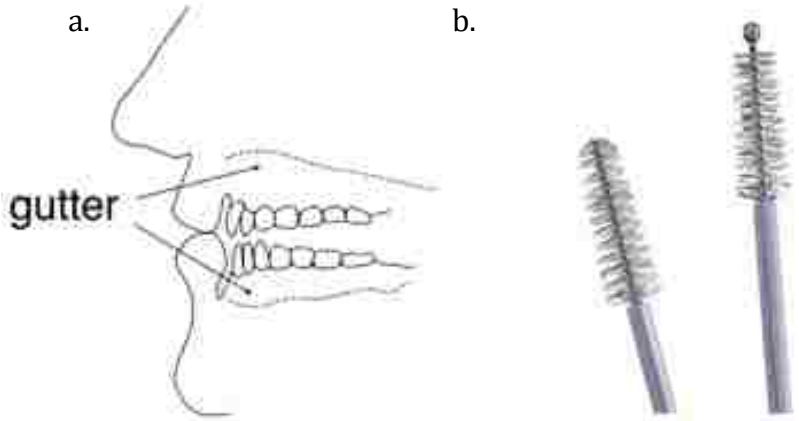

Figure 3.1. Location of buccal cell collection and brush.

The buccal cells were collected from the 4 gutters of the mouth (Figure 2.3a.). Cells were collected on a cytology brush with a gentle scraping technique (Figure 2.3b.).

Bisulfite Treatment and Methylation Analysis

DNA bisulfite treatment was carried out using the EZ DNA Methylation-Direct Kit (Zymo Research, CA, USA) according to the manufacturer's instruction. Briefly, buccal cell DNA denatured at 98°C for 10 min was incubated with modification reagents for 2.5 h at 64°C and cleaned and desulphonated. The bisulfite-modified DNA was stored at -20°C. PCR amplification and pyrosequencing steps have been described previously (Yang et al. 2004). Briefly, after sodium bisulfite conversion, the DNA was PCR amplified using a forward and reverse primer. Gel electrophoresis was used to ensure proper amplification. The PCR product was visualized using a Pyromark Q96 MD (Qiagen, Germantown, MD). Each sample was PCR amplified using previously published primer sets for LINE-1, IFNγ -54, and IFNγ -186 (see Table 3.1). Samples were run in duplicate for each assay until a %CV < 5 was achieved. Epitect (Qiagen) bisulfite treated controls, which include a methylated and unmethylated human genome sample, were used on each plate.

Gene/region	Assay	Primers	PCR Conditions
LINE-1	PCR and Pyrosequencing	F: 5'-TTTGAGTTAGGTGTGGGATATA-3'	95°C, 15 min; 44 cycles of 95°C, 30 sec; 56°C, 30 sec; 72°C, 30 sec; 72°C, 10 min; 4°C hold
		R: 5'-biotin-AAAATCAAAAAATTCCCTTTC-3'	
		S: 5'-AGTTAGGTGTGGGATATAGT-3'	
IFNλ CgG -186	PCR and Pyrosequencing	F: 5'-biotin-AGATGGTGATAGATAGGTAGGGATGATA-3'	95°C, 15 min; 45 cycles of 95°C, 30 sec; 55°C, 30 sec; 72°C, 30 sec; 72°C, 10 min; 4°C hold
		R: 5'-TCCCACCAAAATAACACAAATAAACAT -3'	
		S: 5'-AAATAAACATAATAAATCTATCTCA-3'	
IFNλ CgG -54	PCR and Pyrosequencing	F: 5'ATGTGTTGTATTTTTTTTGGTTGTTGGTAT-3'	
		R: 5'-biotin-TATCATCCCTACCTATCTATCACCATCTC-3'	
		S: 5'-ATTGAAGTTTTTTGAGGATT-3'	

Table 3.1. Primers and amplification conditions for PCR and pyrosequencing experiments

Other Covariates

Anthropometric measures were determined by trained staff using a digital scale and stadiometer. Consistent with other studies of adolescent children, BMI percentile was calculated. Age, ethnicity, and gender were captured by a questionnaire as part of the larger study design. Descriptive home characteristics data, reported by an adult resident, were captured, including size and age of home, the presence of pets and secondary sources of heating.

Statistical Analysis

Methylation percentages resulting from pyrosequencing assays for LINE-1 and IFNγ were considered first as continuous variables. To provide meaningful results, PM exposures were evaluated as a change relative to the interquartile range (IQR). $PM_{2.5}$ IQR, fine particle counts IQR, and coarse particle counts IQR were evaluated for associations for Line-1 and IFNγ methylation using linear regression.

Considering our cohort and this novel exposure setting, it is not clear from the literature whether the two IFNγ CpGs should be differentially methylated or methylated in concert. For this reason, we decided it may be prudent to evaluate the sites separately and in tandem. To evaluate the affect of the sites together we chose to evaluate them as an average (IFNγaverage) and as a ratio (Me% 54/186).

Diurnal peak flow variability was dichotomized using the cut point of 20% (Jamison and McKinley 1993). In a study comparing asthmatics to normal subjects, Jamison et al. showed that the peak flow variability of > 20% could be used as a discriminating index and this measurement was more sensitive than other measurements tested such as forced expiratory volume. For our study peak flow variability < 20% represents well

controlled asthma. PAQLQ scores were dichotomized at the median value of 5.8 (on a scale from 1-7) with higher values indicating better quality of life. Morning and evening FEV_1 were dichotomized using the cut point of 80%, which is the cut point for moderate asthma in adolescent children according to the Asthma Severity and Control Monitoring Guide.

To account for repeated measures within subject, a generalized estimating equation (GEE) normal distribution model was used to determine if associations existed between PM exposures and measures of methylation. In this model a parameter estimate of zero is the null value and values above zero indicate a positive association between PM exposures and the corresponding epigenetic marker. A GEE binomial distribution model was used to determine if associations existed between PM exposures and categorical respiratory health measures. This model estimates odds ratios with one being the null value, and odds ratio estimates greater than one suggests that the PM exposure is deleterious to the corresponding asthma outcome measures. For clarification, an odds ratio is informative about the relationship between an exposure and an outcome. More specifically, an odds ratio tells you what the odds are for an outcome in the context of an exposure compared to the odds for that outcome without the exposure. The final GEE model for both normal and binomial distribution included potential confounders (i.e. age and sex), adjusted for repeated measures, and considered the intervention.

The primary analysis for this study was a mixed model that incorporated the affect of intervention and compared pre to post measurements for log transformed PM exposures, methylation markers, and respiratory health measurements.

RESULTS

A subcohort of 37 asthmatic children from the ARTIS study participated in this study of indoor PM, respiratory health, and epigenetics. The age of the children ranged from 7 to 17 and 46% were male. The study population was 89% non-Hispanic white. The mean (sd) BMI percentile was 72.36 (23.83) and 43% (n=16) were above the 85th percentile, which is considered overweight according to the Centers for Disease Control (CDC).

With repeated measures (n=37), the 48-hour mean (sd) for indoor $PM_{2.5}$ concentration in the pre-intervention winter was 26.25 μg/m^3 (26.20) with a range of 5-106 μg/m^3. Similarly, the 48-hour mean (sd) for indoor fine particle counts and coarse particle counts were 5.5E7 (4.4E7) and 3.7E5 (3.1E5) respectively.

With repeated measures (n=37), the mean (sd) for dPFV and PAQLQ in the pre-intervention winter was 15.2% (9%) and 5.54 (1.03) respectively. Similarly, the mean (sd) for morning and evening FEV_1 was 83.69 (16.97) and 84.06 (17.68) respectively.

Buccal samples were collected 1 to 4 times for each of the 37 subjects (n=125 samples) throughout both winters. Although the study was designed to collect 4 buccal samples from each participant, less than 4 collections were made for children who were lost to follow up or due to complications and a given visit did not occur. Mean (sd) LINE-1 methylation was 65.0% (3.5) with a range of 55.5 – 74.6%. Mean (sd) IFNγ CpG -54 was 79.0% (4.9) with a range of 64.7 – 97.1%. Mean (sd) IFNγ CpG -186 was 71.0% (6.5) with a range of 49.1 – 95.9%. LINE-1 global methylation was positively associated with IFNγ CpG -54 methylation (p= <0.0001), IFNγ CpG -186 (p= 0.001), and IFNγaverage (p= <0.0001) sites, but not with Me% 54/186. Methylation at IFNγ -54 CpG and IFNγ CpG -186 tended to be positively associated, but this relationship was not

significant.

The relationship between potential confounding variables (i.e. age, and sex) and methylation and respiratory outcomes was investigated. The sex of the child was associated with both methylation measures and respiratory health outcomes. Boys relative to girls had 0.03 percentage points higher methylation at IFNγ CpG -186 (95%CI: 0.00, 0.64) and 0.13 percentage points higher IFNγaverage (95%CI: 0.02, 0.73). Boys had 74% lower odds of having below median PAQLQ (95%CI: 0.09, 0.73) and 64% lower odds of having poor morning FEV_1 (95%CI: 0.12, 1.09) relative to girls. The age of the child was also associated with methylation measures and respiratory health outcomes. For each year increase in age, methylation at IFNγ CpG -186 was 0.4 percentage points higher (95%CI: 0.2, 0.7%), IFNγaverage was 0.6 percentage points higher (95%CI: 0.4, 0.9), and Me% 54/186 was 1.0 units higher (95%CI: 1.00, 1.02). A year increase in age was associated with 20% lower odds of having high dPFV > 20% (95%CI: 0.66, 0.98) and 18% lower odds of having below median PAQLQ (95%CI: 0.66, 1.01).

In bivariate analysis of indoor PM and DNA methylation or asthma outcomes, age and sex did not appreciably influence parameter estimates except where noted below. Tables 3.4 and 3.5 present bivariate analysis results unadjusted for age and sex.

Effect of treatment on indoor PM, methylation, and health outcomes

There was a significant reduction in $PM_{2.5}$ for the filter treatment group when pre intervention was compared to post intervention measurements. The pre to post change in $PM_{2.5}$ for the filter treatment group was significantly reduced relative to the change for

the placebo treatment group (see Table 3.3). There was a similar reduction in fine particle counts for the filter treatment group when the pre treatment measurement was compared to the post treatment measurement. Pre to post change in fine particle counts for the filter treatment group was significantly reduced relative to the change for the placebo treatment group. The pre to post change in coarse particle counts was significant for the filter treatment group. The pre to post change for the filter group relative to the change for the placebo treatment group was only modestly significant for coarse particle counts because there was a trend towards a pre to post reduction in the placebo treatment group.

Considering pre to post measurements, methylation at IFNγ CpG -54 was significantly reduced in the filter and placebo treatment group. Similarly, methylation at IFNγ CpG -186 was also reduced in the filter and placebo treatment group. There was no change in LINE-1 global methylation in the filter or placebo treatment group.

When respiratory health measures were evaluated in the context of intervention, post intervention PAQLQ scores were significantly higher in the filter treatment group and this change was significant relative to the change in the placebo group. There was a nonsignificant decrease in dPFV for the filter treatment group and a nonsignificant increase in the placebo treatment group. This pre to post decrease in dPFV for the filter treatment group relative to the increase in placebo treatment group approached significance.

Indoor PM and methylation measurements

Indoor $PM_{2.5}$ was associated with IFNγ CpG -54 methylation, Me% 54/186, and LINE-1 methylation, however the relationship with LINE-1 methylation was no longer significant

after adjusting for age and sex. An interquartile range increase in $PM_{2.5}$ was associated with a 0.46 percentage point increase in IFNγ CpG -54 methylation (95%CI: 0.19, 1.14) and a 0.97 unit increase in Me% 54/186 (95%CI: 0.93, 1.01). Indoor fine particle counts were associated with IFNγ CpG -54 methylation, IFNγ CpG -186 methylation, and with IFNγaverage, however these relationships were no longer significant when adjusting for age and sex. Indoor coarse particle counts were associated with IFNγ CpG -54 methylation, IFNγ CpG -186 methylation, and with IFNγaverage and after adjusting for age and sex these relationships remained significant. An interquartile range increase in coarse particle counts was associated with a 1.55 percentage point increase in IFNγ CpG -54 methylation (95%CI: 1.18, 2.04), a 1.55 percentage point increase in IFNγ CpG -186 (95%CI: 1.04, 2.31), and a 1.56 percentage point increase in IFNγaverage (95%CI: 1.16, 2.08).

Indoor PM and respiratory health outcomes

Indoor $PM_{2.5}$, fine particle counts, and coarse particle counts were associated with poor PAQLQ and these relationships were robust even after adjusting for age and sex. An interquartile range increase in $PM_{2.5}$ was associated with 72% greater odds of having poor quality of life (95%CI: 0.95, 3.12). Similarly, an interquartile range increase in fine fraction particle counts was associated with 69% greater odds of having poor quality of life (95%CI: 1.15, 2.48). An interquartile range increase in coarse fraction particle counts was associated with 27% greater odds of having below median PAQLQ (95%CI: 0.99, 1.64).

Methylation measurements and respiratory health outcomes

IFNγ CpG -186 methylation and Me% 54/186 were associated with dPFV and these relationships remained significant after adjusting for age and sex. A percentage point

increase in IFNγ CpG -186 methylation was associated with 7% greater odds of having dPFV above 20% (95%CI: 1.01, 1.14). A unit increase in the Me% 54/186 was associated with 99% reduced odds of having dPFV above 20% (95%CI: 0.00, 0.59). IFNγ CpG -186 methylation and Me% 54/186 were associated with PAQLQ but only the relationship between IFNγ CpG -186 and PAQLQ remained significant after adjusting for age and sex. A percentage point increase in IFNγ CpG -186 methylation was associated with 5% greater odds of having poor PAQLQ (95%CI: 1.00, 1.11).

	N	Mean	SD	Min	Max
Age	37	12.81	2.53	7	17
Gender					
Male	17 (46%)				
Female	20 (54%)				
Ethnicity					
Non-Hispanic White	33 (89%)				
BMI percentile	37*	72.36	23.83	6.79	99.46
dPFV (%)	37*	15.2	9.0	3.8	42.4
Morning FEV_1 (%)	37*	83.69	16.97	23.79	111.85
Evening FEV_1 (%)	37*	84.06	17.68	15.63	108.28
PAQLQ	37*	5.54	1.03	3.23	7.00
Indoor $PM_{2.5}$ ($\mu g/m^3$)	37*	25.41	34.41	0.70	199.79
Fine Fraction Particle Counts	37*	5.5E7	4.4E7	5.3E6	1.7E9
Coarse Fraction Particle Counts	37*	3.7E5	3.1E5	5.0E4	1.3E6
IFNγ -54	37*	79.03	4.94	64.74	97.09
IFNγ -186	37*	70.96	6.51	49.05	95.91
IFNγ average	37*	74.99	4.55	64.50	96.50
IFNγ ratio	37*	1.12	0.12	0.76	1.85
LINE-1	37*	65.04	3.46	55.49	74.59

* includes repeated measures within the pre-intervention winter

Table 3.2. Population characteristics. Definitions: BMI, body mass index; dPFV, diurnal peak flow variability; FEV_1, forced expiratory volume in one second; PAQLQ, pediatric quality of life questionnaire.

	Tx3 (Filter)				Tx1 (Placebo)				Tx3 Change relative to Tx1 Change	
	Pre Tx	Post Tx	Change	p	Pre Tx	Post Tx	Change	p	Estimate (CI)	p
Log PM2.5	2.93 (0.91)	1.67 (1.08)	-1.08	< 0.0001	2.87 (0.68)	3.18 (1.17)	0.35	0.03	-1.44(-1.86, -1.02)	< 0.0001
Log Fine Counts	17.44 (0.82)	15.70 (2.06)	-1.71	< 0.0001	17.67 (0.64)	17.80 (0.86)	0.12	0.7	-1.83 (-2.68, 0.97)	< 0.0002
Log Coarse Counts	12.57 (0.61)	11.05(2.37)	-1.54	< 0.0001	12.55 (0.87)	11.98 (0.50)	-0.52	0.14	-1.03 (-1.96, 0.09)	0.03
IFNγ -54	80.52 (4.16)	77.89 (3.03)	-2.79	0.0005	79.63 (2.91)	76.81 (2.43)	-2.98	0.0009	0.19 (-2.00, 2.39)	0.86
IFNγ -186	72.82 (4.03)	68.48 (5.28)	-4.39	< 0.0001	72.81 (4.37)	69.48 (4.46)	-3.13	0.0003	-1.25 (-3.33, 0.83)	0.23
IFNγ average	76.67 (3.01)	73.19 (3.35)	-3.65	< 0.0001	76.22 (2.57)	73.14 (2.83)	-3.11	< 0.0001	-0.55 (-2.22, 1.13)	0.51
IFN ratio	1.11 (0.08)	1.15 (0.11)	0.04	0.01	1.10 (0.08)	1.11 (0.07)	0	0.76	0.03 (-0.01, 0.08)	0.14
LINE-1	64.70 (2.96)	64.92 (3.06)	0.01	0.98	65.12 (2.26)	64.94 (2.29)	-0.32	0.47	0.33 (-0.88, 1.55)	0.58
dPFV	15.3 (9)	14.7 (7)	-0.70	0.26	15.0 (8)	16.0 (7)	0.02	0.16	-0.03 (-0.07, 0.00)	0.07
PAQLQ	5.46 (1.04)	5.88 (0.68)	0.45	0.0002	5.66 (1.02)	5.51 (0.89)	0.11	0.37	0.33 (0.01, 0.66)	0.05
Morning FEV1	86.09 (13.62)	81.61 (21.53)	0.24	0.92	80.64 (20.34)	86.62 (11.08)	2.61	0.32	-2.37 (-9.49, 4.76)	0.5
Evening FEV1	86.88 (12.98)	83.72 (22.70)	0.7	0.77	80.48 (22.05)	87.11 (13.20)	1.91	0.48	-1.20 9-8.43, 6.02)	0.73

Table 3.3. Effect if treatment.

Effect of treatment on indoor PM, methylation measurements and respiratory health outcomes. The pre and post treatment (tx) columns are given as an exponentiated estimate (sd). The change column is the change from the respective pre to post measurements. The Change in filter treatment group relative to placebo treatment group is given as an estimate (95% confidence interval). Definitions: dPFV, diurnal peak flow variability; PAQLQ, pediatric quality of life questionnaire; FEV_1, forced expiratory volume in the first second.

	IFNγ -54	p	IFNγ -186	p	IFNγ average	p	IFNγ ratio	p	LINE-1	p
Log PM2.5 IQR	0.46 (0.19, 1.14)	0.10	3.29 (0.62, 17.50)	0.16	1.14 (0.43, 3.04)	0.80	0.97 (0.93, 1.01)	0.10	0.50 (0.21, 1.19)	0.12
Log Fine Count IQR	1.46 (0.94, 2.27)	0.09	1.99 (0.97, 4.12)	0.06	1.76 (1.11, 2.78)	0.02	1.00 (0.98, 1.01)	0.55	0.83 (0.56, 1.24)	0.36
Log Coarse Count IQR	1.55 (91.18, 2.04)	0.002	1.55 (1.04, 2.31)	0.03	1.56 (1.16, 2.08)	0.003	1.00 (0.98, 1.01)	0.55	0.83 (0.56, 1.24)	0.36

Table 3.4. Bivariate analysis of indoor PM and methylation measurements. Data from pre and post winters were used in this analysis. Unadjusted results from the GEE normal model are given as percentage point change followed by 95% CI. IQR is interquartile range.

	dPFV 1 is > 20%	p	PAQLQ 1 is < 5.85	p	Morning FEV1 1 is < 80%	p	Evening FEV1 1 is < 80%	p
log PM2.5 IQR	1.41 (0.77, 2.58)	0.27	1.72 (0.95, 3.12)	0.07	1.13 (0.68, 1.86)	0.65	0.85 (0.48, 1.50)	0.57
log fine count IQR	1.32 (0.91, 1.93)	0.15	1.69 (1.15, 2.48)	0.008	0.94 (0.68, 1.30)	0.71	0.89 (0.69, 1.15)	0.36
log coarse count IQR	1.18 (0.89, 1.56)	0.25	1.27 (0.99, 1.64)	0.06	1.02 (0.88, 1.19)	0.77	0.99 (0.88, 1.12)	0.92
IFN -54	1.01 (0.95, 1.07)	0.85	1.00 (0.93, 1.07)	0.95	1.03 (0.97, 1.09)	0.37	1.01 (0.95, 1.07)	0.78
IFN -186	1.07 (1.01, 1.14)	0.03	1.05 (1.00, 1.11)	0.05	1.03 (0.96, 1.10)	0.42	1.04 (0.97, 1.11)	0.32
IFN average	1.06 (0.98, 1.13)	0.13	1.05 (0.97, 1.13)	0.24	1.04 (0.95, 1.14)	0.35	1.04 (0.95, 1.13)	0.39
IFN ratio	0.01 (0.00, 0.59)	0.03	0.07 (0.00, 0.93)	0.04	0.73 (0.06, 9.60)	0.81	0.31 (0.01, 9.27)	0.5
LINE-1	1.06 (0.97, 1.17)	0.21	1.01 (0.89, 1.14)	0.86	1.02 (0.93, 1.11)	0.70	0.99 (0.87, 1.13)	0.93

Table 3.5. Bivariate analysis of indoor PM or methylation measurements and respiratory health outcomes. Data from pre and post winters were used in this analysis. Unadjusted results from the GEE binomial model are presented as odds ratio followed by 95% confidence interval. Definitions: IQR, interquartile range; dPFV, diurnal peak flow variability; PAQLQ, pediatric quality of life questionnaire; FEV_1, forced expiratory volume in one second.

DISCUSSION

In this cohort of asthmatic children, wintertime measurements of $PM_{2.5}$, fine particle counts, and coarse particle counts were associated with poor quality of life. With the introduction of a filter treatment in the home, we found that these indoor PM measurements were significantly reduced relative to the placebo treatment. Additionally, we found that PAQLQ improved and dPFV decreased with the introduction of filter treatment relative to placebo treatment. Interestingly, methylation at both of the IFNγ CpG sites that were measured was reduced for the filter treatment group and the placebo treatment group. Coarse particle counts were positively associated with methylation for both of these sites and our results suggest that there was an unexpected reduction of coarse particles in the placebo filter group. Visual inspections of the placebo filter in the home confirm this observation, as the placebo filters still trapped dust and other PM on the porous filter media. We also hypothesize that the reduction IFNγ promoter methylation may have to do with the subjects increase in age. The epigenome is known to change over time, however, it is difficult to speculate if these changes occur on the scale of a single year.

In bivariate analysis, IFNγ CPG -186 was associated with a marker of poor respiratory health and with PAQLQ. The literature suggests that increased methylation at both CpG -54 and -186 of IFNγ is associated with suppression of IFNγ, but these studies did not evaluate buccal cell DNA. Suppression of IFNγ would shift the balance from Th1 to Th2 and this is consistent with observations of poor respiratory health. However, to completely investigate the functionality of epigenetic modulation at these CpG sites, RNA expression data is necessary. RNA expression analysis was outside the scope of this project and this is a major limitation for the interpretation of the methylation results.

Using a crude mediation analysis, we were able to evaluate whether PM exposures were affecting PAQLQ through an epigenetic pathway (data not shown). Essentially, single methylation variables were added to the GEE binomial model to see if the effect of a PM variable on PAQLQ was appreciably influenced. The results of this analysis did not suggest that methylation at the chosen CpG sites were important in this context.

In this cohort we found some interesting results regarding the age and sex of the asthmatic children. Boys had higher methylation at IFNγ CpG -186 and IFNγaverage compared to girls. Boys also had better PAQLQ and FEV_1 compared to girls. The findings with respect to sex appear to be contradictory if the literature on methylation of the IFNγ gene is correct. We would expect more methylation to be associated with poor respiratory health. We found similar contradictory results for age. Older children had more IFNγ methylation and better respiratory health. The finding that methylation increases at IFNγ CpG -186 with age is also intriguing because it suggest that the reduction in methylation at this site that we saw in response to treatment may not be due to the passing of time.

It is important to remember that the literature informing our interpretation of these data were not conducted in the same cellular matrix and the subjects were not asthmatic. The findings from this novel application of epigenetic biomarkers within the context of an indoor PM intervention study with asthmatic participants warrant further investigation.

CONCLUSION

The filter intervention in the ARTIS study successfully reduced wintertime measurements of indoor PM in the homes of childhood asthmatics and these reductions were associated with better respiratory health outcomes. Global methylation measured with LINE-1 was not associated with respiratory health measures or PM exposure suggesting that this epigenetic marker may not be a sensitive marker for exposures in asthmatic children. Methylation at CpG sites within the promoter of IFNγ was associated with indoor PM measurements and respiratory health measures, however we did not find that PM was affecting respiratory health through this epigenetic pathway. Further investigation into RNA expression of IFNγ and other genes related to the Th1/Th2 balance will help to clearly define the interplay between indoor PM exposure and immunoregulation among asthmatic children..

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CHAPTER 4

Evaluating the effect of ambient particulate pollution on DNA methylation in Alaskan sled dogs: Potential applications for a sentinel model of human health

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Abstract

Exposure to ambient particulate matter (PM) is known to be associated with increased morbidity and mortality in human populations. During the winter months in Fairbanks, Alaska, severe temperature inversions lead to elevated concentrations of ambient PM smaller than 2.5 microns ($PM_{2.5}$). Sled dogs represent an easily accessible environmentally exposed population that may yield findings informative for human health risk assessment.

In this pilot study, we evaluated whether ambient PM was associated with markers of global methylation in sled dogs. Kennels were strategically recruited to provide a wide $PM_{2.5}$ exposure gradient for the Fairbanks area. Continuous monitoring of ambient $PM_{2.5}$ was conducted at each kennel during the winter of 2012/13 using a DustTrak 8530. Dogs received a physical examination and assessment of standard hematology and clinical chemistries. Global methylation was determined using the LUminometric Methylation Assay (LUMA) and 5-Methycytosine (5-mC) Quantification.

Three sled dog kennels (n ~30 dogs/kennel) were evaluated and sampled. The average $PM_{2.5}$ concentrations measured for kennels A, B, and C were 90 $\mu g/m^3$, 48 $\mu g/m^3$, 16 $\mu g/m^3$ ($p < 0.0001$), respectively. The average (standard deviation) global methylation percentage for each kennel measured by LUMA was 76.22 (1.85), 76.52 (1.82), and 76.72 (2.26), respectively. The average (standard deviation) global methylation percentage for each kennel measured by 5-mC was 0.16 (0.04), 0.15 (0.04), and 0.15 (0.05), respectively. There was no statistically significant difference between the three kennels and their average global methylation percentage either by LUMA or 5-mC.

In this study we evaluated global methylation using LUMA and 5-mC and found no differences between kennels, though exposure to ambient $PM_{2.5}$ was significantly different between kennels. As more information becomes available regarding immunologically-related canine genes and functionally active promoter subunits, the utility of this surrogate could increase.

INTRODUCTION

Ambient particulate matter (PM) is known to be associated with increased morbidity and mortality in many exposed human populations, with these adverse effects particularly evident among individuals with pre-existing respiratory or cardiovascular conditions (Sacks et al. 2011). However, the mechanisms whereby PM causes adverse health outcomes in the general population including those with pre-existing conditions remain unclear.

The inflammatory response generated from human exposure to airborne PM is largely determined by source and size of the particles (Kocbach et al. 2008). Source apportionment studies show that the Fairbanks airshed is dominated by wood smoke PM (Ward et al. 2012), which is at least partially due to the low cost and availability of wood relative to other popular fuel sources such as heating oil or natural gas. During the cold winter months in Fairbanks, severe temperature inversions (characterized by stagnant air) and extreme low temperatures lead to significant PM exposures for extended periods of time. It is not uncommon to see 24-hour average concentrations of ambient PM smaller than 2.5 microns ($PM_{2.5}$) reach 50 – 60 $\mu g/m^3$ for several consecutive days, significantly above the Environmental Protection Agency's (EPA) 24-hour National Ambient Air Quality Standard (NAAQS) for $PM_{2.5}$ (35 $\mu g/m^3$). These elevated wintertime $PM_{2.5}$ concentrations have resulted in Fairbanks having some of the highest measured $PM_{2.5}$ concentrations throughout the entire EPA monitoring network, and a designation of nonattainment for $PM_{2.5}$.

There is considerable evidence suggesting that traffic related pollution is a risk factor for poor respiratory and cardiovascular outcomes (Ruckerl et al. 2011). Previously, we have demonstrated that elevated ambient $PM_{2.5}$ concentrations due to residential wood smoke

was associated with respiratory conditions and symptoms (Noonan et al. 2012). Because immunological responses are likely specific to the source, size and composition of the inhaled PM (Miyata and van Eeden 2011), there is a need to evaluate health outcomes in geographical locations where high ambient $PM_{2.5}$ occur.

There are often practical, logistical, and ethical considerations that make human community-based studies challenging. Additionally, humans often have practices that are potentially confounding such as tobacco use, legal and illegal use of drugs, varying transportation and movement. In such cases, there is utility in establishing a sentinel model for human health that could reduce the likelihood of potential confounding from these factors (Reif 2011). Groups such as the Morris Animal Foundation have highlighted the utility of the domestic dog as a biomedical model for researching the genetic and epigenetic etiologies of canine cancers (Morris Animal Foundation). Domestic dogs have been used previously to study the public health effects of air pollution both in lieu of human studies (Calderon-Garciduenas et al. 2001) and in parallel (Calderon-Garciduenas et al. 2008).

This study represents a novel application of sled dogs as sentinel animals for health impacts associated with exposure to ambient air pollution. The long winters and pristine mushing topography makes Fairbanks ideal for such a study. For example, Fairbanks is home to a large potential cohort of thousands of sled dogs, with these working domestic animals clustered in large groups of up to 100 per kennel. The relatively place-bound subjects, the spatial distribution of kennels, and the elevated but varied concentrations of ambient $PM_{2.5}$ provide an ideal opportunity to accurately assess ambient air exposures in this setting.

Uniform diets, structured exercise routines, and multigenerational pedigrees add elements that are similar to experimental animal studies and a clear advantage over human subjects with high variability in these and other factors. Considering body size, life span, and behavior, which can all impact disease pathology, a dog may serve as a more biologically relevant model for human health compared to mice or rats. Dogs have also shared a living environment with humans longer than any other domestic animal and very likely also shared pathogens, which may have equally shaped the way both species' immune systems defend against disease (Boyko 2011).

Epigenetics play an important role in developmental processes, cell proliferation, and the maintenance of genome stability (Jones and Baylin 2007). Global DNA hypomethylation is one type of epigenetic alteration and is hypothesized to play a role early in carcinogenesis by activating oncogenes and/or genomic instability (Feinberg 2004). Recently, epigenetic modifications such as DNA hypo- or hyper-methylation have been shown to occur in response to environmental exposures, and both human and animal studies suggest that such changes are relevant to risk for chronic disease. In particular, studies have found that exposure to air pollution can affect methylation patterns at the global (Madrigano et al. 2011; Baccarelli et al. 2009; Tarantini et al. 2009) and gene-specific levels (Liu et al. 2008; Salam et al. 2012; Nadeau et al. 2010). The current body of literature clearly indicates a role for methylation pattern changes in genes responsible for the regulation of the T-effector pathway, T-regulatory pathway, and airway inflammation (Lovinsky-Desir and Miller 2012). Deciphering the epigenetic landscape of exposed populations could lead to potential treatments because of the reversible nature of epigenetic markers (Wright 2013).

In this manuscript, we for the first time evaluate global methylation in Alaskan sled dogs in order to determine the effect of exposure to ambient PM (composed predominantly of residential wood smoke) on global DNA methylation.

METHODS

Kennel Recruitment

Data from both stationary and mobile $PM_{2.5}$ air monitors were used to identify kennels located in "high", "medium", and "low" $PM_{2.5}$ areas around the city of Fairbanks. Ultimately three kennels were strategically recruited with the intention of yielding a wide $PM_{2.5}$ exposure gradient across sample locations. To be considered for inclusion, the kennel had to have greater than 30 sled dogs on site and access to an electrical outlet. Kennel A was an urban kennel located inside the Fairbanks EPA $PM_{2.5}$ designated non-attainment area, Kennel B was an urban kennel located approximately 15 miles Southwest of Fairbanks, and Kennel C was a rural kennel located approximately 50 miles Southeast of Fairbanks. The sled dog owners were contacted and given information about the study. Those owners who agreed to participate were asked to sign a consent form. This study was approved by the University of Montana (UM) and University of Alaska Fairbanks (UAF) Institutional Animal Care ad Use Committee (IACUC #283615-4) before any onsite work was conducted.

$PM_{2.5}$ Exposure Characterization

During the winter of 2012/13, a DustTrak 8530 (TSI, Shoreview, MN) that continuously measured $PM_{2.5}$ mass was deployed to each of the three kennels. The DustTrak was housed in an insulated and heated enclosure to protect it from the extreme winter temperatures. While sampling, this monitor was placed approximately 1 meter off of the ground within 15 meters from the dog yard. The sled dogs in this study were all housed in very close proximity to each other at their respective kennel. The DustTrak was on a 60-second recording interval, and was zero calibrated prior to each sampling event. Field staff collected $PM_{2.5}$ mass data at each kennel from November 21, 2012 through February 27, 2013.

Veterinary Examination, Hematology and Clinical Chemistry

Following the winter sampling period, field staff and a licensed veterinarian visited each dog kennel to conduct a standard physical examination and collect three blood samples from all study animals. Serum samples, collected in serum separator tubes, were used for comprehensive clinical chemistry panels to analyze general health markers. Whole blood samples were collected for hematocrit and complete blood counts. Whole blood and serum samples were processed and analyzed at the UAF Animal Resource Center clinical laboratory and the Wildlife Toxicology Laboratory (WTL). Blood samples for DNA analysis were collected in a PreAnalytix DNeasy blood tube (Qiagen, Germantown, MD) and shipped to the University of Montana for processing.

Global Methylation Analysis

DNA was extracted using a PreAnalytix DNeasy Kit (Qiagen, Germantown, MD). Concentration of DNA was assessed using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE).

LUminometric Methylation Assay (LUMA)

Global methylation was determined using LUMA (Karimi et al. 2006). The assay was run in duplicate using 500ng of total DNA with samples analyzed in a randomized order to reduce the effect of inter-plate variation. Each plate included 21 DNA samples, as well as positive, negative, and water controls. The assay was visualized using a Pyromark Q96 MD (Qiagen, Germantown, MD). All samples used in the final analysis had an intra-assay coefficient of variation of ≤ 5%. Lambda DNA (NEB, Ipswich, MA) was used as a negative control, and was methylated using CpG methyltransferase (NEB, Ipswich, MA)

and a New England Biolabs optimized protocol, which was then used as a positive control.

5-Methylcytosine (5-mC) Quantification

Global DNA methylation levels also were evaluated using the ELISA-based Methylflash™ Methylated DNA Quantification Kit (Colorimetric) (Epigentek, NY, USA). The Kit measures the 5-mC content as a percentage of the total cytosine in the DNA sample. The assay was performed in duplicate according to manufacturer's instructions with 100ng of total DNA. The 5-mC in DNA was detected using capture and detection antibodies and then quantified colorimetrically by reading the absorbance in a microplate spectrophotometer. Absolute quantification standard curves were obtained by plotting the concentration of the positive control (methylated polynucleotide containing 50% of 5-methylcytosine (5-mC)) supplied with the assay kit against the optical density (OD) at 450nm. The assay was validated by calibrating the different amounts of 5-mC DNA in a commercially available standard (CpGenome™ 5-mC) (EMD Millipore, Billerica, USA). The percentage of 5-mC in genomic DNA was calculated using the following formula: 5-mC % = $\frac{\text{Sample OD} - \text{Negative Control OD}}{2* \times \text{Slope} \times \text{Input DNA amount}} \times 100\%$

* 2 is a factor to normalize 5-mC in the positive control to 100%, as the positive control contains only 50% of 5-mC

Statistical Analysis

Descriptive factors including dog sex, age, and weight were compared between kennels using analysis of variance or chi-square tests. Clinical chemistry and hematology variables were evaluated as pairwise comparisons relative to the lowest exposure group, Kennel C. Ambient $PM_{2.5}$ concentrations at each kennel site were summarized for the

total monitoring periods and by the two-week period prior to the day of data collection for dogs at the kennels. Differences in mean ambient $PM_{2.5}$ concentrations and mean global methylation percentage between kennel sites were evaluated by analysis of variance, followed by post-hoc t-tests when appropriate. Pearson correlations were used to compare global methylation percentage and the 35 clinical chemistry variables across and within kennels.

RESULTS

In total, 87 sled dogs participated in this study, including 29 from Kennel A, 30 from Kennel B, and 28 from Kennel C. Two UAF veterinarians, who were blinded to the kennel designation, evaluated the clinical chemistry data in an effort to identify if animals should be removed based on health characteristics. Following this evaluation, the veterinarians advised that four animals should be excluded. An additional four animals were excluded because they had more than five clinical chemistry values that were greater than two standard deviations away from the mean. Therefore a population of 75 sled dogs was used in the following analysis. The weight and sex of the excluded animals were not significantly different from the included population of sled dogs ($p = 0.94$ and 0.38 respectively), however, the dogs that were removed from the study were more likely to be older compared to those included in the study ($p= 0.07$). With respect to kennel designation there was a significant difference in age and weight. Dogs in Kennel C were significantly younger than dog in the other kennels ($p= 0.01$), and dogs in kennel A weighed significantly less than dogs in the other kennels ($p= 0.01$; table 4.1.).

Characteristics [a]	Kennel A (n=27)	Kennel B (n=25)	Kennel C (n=23)
Age, years	6.1 (3.2)	6.0 (3.3)	3.6 (2.8)
Weight (lbs)	49.6 (6.3)	55.5 (7.4)	52.6 (6.6)
Sex			
Female	17 (63)	13 (52)	10 (43)
Male	10 (37)	12 (48)	13 (57)
Pregnant [b]	2 (12)	1 (8)	0

[a] mean (sd) or n (%)
[b] n (% female)

Table 4.1. Population characteristics.

Ambient $PM_{2.5}$ Concentrations at the Kennels

$PM_{2.5}$ data were collected at each kennel from November 21, 2012 through February 27, 2013. Although samples were collected at each site the majority of the time, due to logistic issues including weather and power outages, each kennel was sampled for a subset of the total study period (see table 4.2.). The average $PM_{2.5}$ concentration (sd) measured during the study period for each site was, 90 (15) $\mu g/m^3$ for Kennel A, 48 (22) $\mu g/m^3$ for Kennel B, and 16 (8) $\mu g/m^3$ for Kennel C ($p< 0.0001$). For the 2-week period prior to the dog sampling and examination at each kennel, the measured average $PM_{2.5}$ concentration (sd) was 46 (32) $\mu g/m^3$ for Kennel A, 42 (32) $\mu g/m^3$ for Kennel B, and 9 (4) $\mu g/m^3$ for Kennel C ($p< 0.001$).

The average daily temperature for the winter period was -20°C (max 0°C, min -37°C) and the relative humidity was 79% (max 100%, min 60%) (Fairbanks North Star Borough ; Western Regional Climate Center). From January 27, 2013 to February 27, 2013, the average daily temperature was -18°C (max -12°C, min -37°C) and the relative humidity was 81% (max 93%, min 62%), (Fairbanks North Star Borough ; Western Regional Climate Center).

	Kennel A	Kennel B	Kennel C	p value
Sample Days	78	59	46	
Percentage of Day Above 35μg/m^3	69	49	4	
Percentage of Day Above 100μg/m^3	35	12	0	
Average $PM_{2.5}$ (μg/m^3)				
Nov 21 - Feb 27 mean (sd)	90 (15)	48 (22)	16 (8)	< 0.0001
2 weeks prior to exam mean (sd)	46 (32)	42 (32)	9 (4)	< 0.001

Table 4.2. Ambient $PM_{2.5}$ Collection Data

Global Methylation

DNA was extracted and analyzed for 83 dogs out of the original population of 87. Figure 4.1. shows the percent global methylation (%gMe) for the dogs included in this study (n=75). The average %gMe (SD) was 76.22 (1.85) for Kennel A, 76.52 (1.82) for Kennel B, and 76.72 (2.26) for Kennel C (p= 0.66). While the $PM_{2.5}$ exposure of Kennels A and B were different over the course of the study, there was no difference in exposure in the two-week period prior to blood draw. If Kennels A and B are combined, there is no difference between the average %gMe of Kennel A+B relative to Kennel C measured by LUMA or 5-mC (data not shown). Global methylation was not associated with age, weight, or sex. The average (standard deviation) global methylation percentage for each kennel measured by 5-mC was 0.16 (0.04) for Kennel A, 0.15 (0.04) for Kennel B, and 0.15 (0.05) for Kennel C (p= 0.24). The methylation percentages from the LUMA and 5-mC assay were not significantly correlated. The above findings were not influenced by the exclusion criteria based on aberrant veterinary examinations and outlying clinical chemistry values. Global methylation values among the excluded dogs (n=8) were similar to the other dogs. Inclusion of all dogs in the analyses did not result in any statistical differences in mean global methylation by kennel or other descriptive characteristics (data not shown).

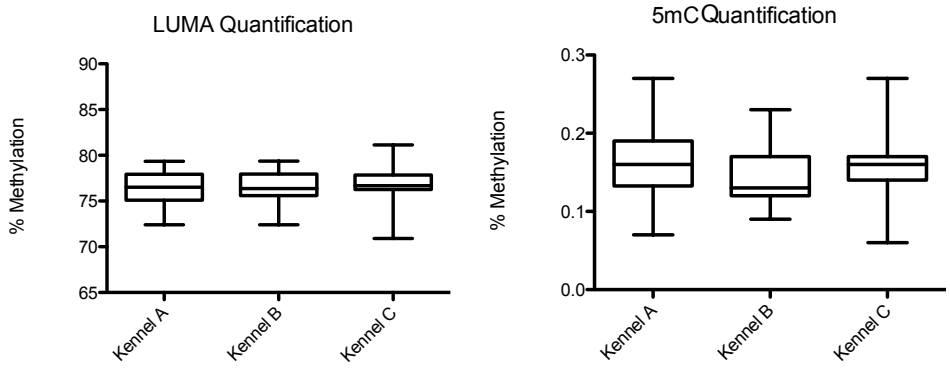

Figure 4.1. Global DNA methylation in sled dogs.
Box plots showing the % global methylation for the three kennels quantified by LUMA (p= 0.66) and 5mC (p= 0.24)

Standard Hematology and Clinical Chemistry

A total of 35 parameters were evaluated (see table 4.3. and 4.4). Sodium (Na) (p= 0.01), glucose (GLU) (p= 0.05), and granulocytes (GRAN) (p= 0.05), were significantly correlated and monocyte percentage (MPR) (p= 0.06) and white blood cell count (WBC) (p= 0.07) were marginally correlated with global methylation values analyzed by LUMA. White blood cell counts (WBC) (p= 0.004) and mid-sized cells (MID) (p= 0.01) were significantly correlated with global methylation values analyzed by 5-mC.

Variable [a]	Kennel A (n=27)	Kennel B (n=25)	Kennel C (n=23)
Na	146.14 (1.23)[b]	146.48 (1.66)	147.13 (1.82)
K	4.47 (0.34)	4.31 (0.22)	4.41 (0.32)
Cl	110.00 (2.76)	104.88 (3.66)[b]	109.22 (3.19)
ALP	44.58 (17.57)[b]	44.60 (41.41)	29.93 (11.98)
ALT	63.62 (46.50)	49.36 (29.23)	47.73 (21.24)
BUN	17.19 (4.14)	21.48 (4.32)	25.08 (19.66)
Ca	9.15 (0.44)	9.52 (0.45)	9.89 (4.56)
CRE	0.44 (0.09)	0.50 (0.09)	2.00 (4.71)
GGT	14.88 (2.47)	15.28 (3.80)	19.09 (17.81)
GLU	97.54 (6.51)	110.52 (11.15)[b]	94.12 (25.69)
TP	5.87 (0.39)	6.04 (0.37)	5.63 (1.16)
TBIL	0.22 (0.15)	0.22 (0.17)	1.60 (6.85)
ALB	3.62 (0.23)[b]	3.87 (0.26)	3.91 (0.30)
TCHO	246.92 (74.13)	225.48 (74.92)	219.13 (58.07)
IP	4.05 (0.56)	3.84 (0.59)	15.94 (59.09)
GLOB	2.25 (0.34)[b]	2.17 (0.32)[b]	1.96 (0.30)
NaKRatio	32.89 (2.33)	34.08 (1.87)	33.57 (2.50)

[a] mean (sd)
[b] $p < 0.05$ compared to Kennel C

Abbreviations: Na: Sodium, K: Potassium, CL: Chloride, ALP: Alkaline Phosphatase, ALT: Alanine Transferase, BUN: Blood Urea Nitrogen, Ca: Calcium, CRE: Creatinine, GGT: Gamma-Glutamyl Transpeptidase, GLU: Glucose, TP: Total Protein, TBIL: Total Bilirubin, ALB: Albumin, TCHO: Total Cholesterol, IP: Phosphorus, GLOB: Globulins, NaKRatio: Sodium Potassium Ratio

Table 4.3. Clinical chemistry values.
Selected clinical chemistry mean (sd) values for subjects at the time of sample collection.

Variable [a]	Kennel A	Kennel B	Kennel C
	(n=27)	(n=25)	(n=23)
WBC	7.52 (1.96)	8.98 (2.59)	7.79 (1.97)
LYMF	1.20 (0.61) [b]	1.62 (0.62)	1.56 (0.54)
MID	0.76 (0.22) [b]	0.77 (0.25) [b]	0.60 (0.12)
GRAN	5.56 (1.62)	6.58 (2.39)	5.63 (1.75)
LPR	16.55 (7.44) [b]	19.29 (6.56)	20.90 (6.56)
MPR	9.36 (1.45) [b]	7.99 (1.93)	7.54 (1.83)
GPR	74.09 (7.82)	72.72 (6.95)	71.56 (6.99)
HCT	44.26 (5.02) [b]	46.04 (4.74)	47.98 (4.18)
MCV	64.48 (1.84)	61.31 (2.99) [b]	63.31 (2.31)
RDWR	22.97 (1.08) [b]	23.69 (1.25) [b]	24.63 (1.80)
RBC	6.86 (0.75) [b]	7.51 (0.70)	7.60 (0.84)
HGB	16.02 (1.70) [b]	16.68 (1.69)	17.54 (1.46)
MCHC	36.23 (0.55) [b]	36.25 (0.51)	36.57 (0.64)
MCH	23.37 (0.67)	22.22 (1.14) [b]	23.14 (0.93)
PLT	343.48 (92.54) [b]	364.28 (107.51) [b]	279.13 (62.89)
MPV	8.75 (0.76)	8.68 (0.69)	9.14 (0.98)
TRBC	12.69 (0.06) [b]	12.68 (0.05) [b]	12.53 (0.05)
TWBC	12.88 (0.04) [b]	12.82 (0.04) [b]	12.66 (0.05)

[a] mean (sd)
[b] $p < 0.05$ compared to Kennel C

Abbreviations (units): WBC: White Blood Cells ($10^3/\mu l$), LYMF: Lymphocytes ($10^3/\mu l$), MID: Mid-sized Cells ($10^3/\mu l$), GRAN: Granulocytes ($10^3/\mu l$), LPR: Lymphocyte Percentage (%), MPR: Monocyte Percentage (%), GPR: Granulocyte Percentage (%), HCT: Hematocrit (%), MCV: Red Blood Cell Count Derivative (fl), RDWR: Red Blood Cell Count Derivative (%), MCHC: Red Blood Cell Count Derivative (g/dl), MCH: Red Blood Cell Count Derivatives (pg), RBC: Red Blood Cells ($10^6/\mu l$), HGB: Hemoglobin (g/dl), PLT: Platelets ($10^3/\mu l$), MPV: Platelet Count Derivative (fl), TRBC: Red Blood Cell Count Time (sec), TWBC: White Blood Cell Count Time (sec)

Table 4.4. Hematology values.
Selected hematology mean (sd) values for subjects at the time of sample collection.

DISCUSSION

In this study, two approaches were used to summarize genome-wide DNA methylation. Our results suggest that global methylation is not associated with elevated levels of ambient $PM_{2.5}$ compared to relatively low levels when using sled dogs as sentinels. It is suggested that hypomethylation of the genome does occur with age in animals (Dolinoy and Faulk 2012). However, age was not associated with global methylation in this study. We had no a priori hypotheses with respect to clinical chemistries and global methylation. Thus, these finding should be interpreted with caution and perhaps warrant further investigation.

Global methylation quantified by LUMA for dogs appears to be consistent with recent work published by Head et al., which suggests that mammals have intermediate global methylation (Head et al. 2014). This publication found that fish had higher global methylation relative to mammals, while birds had lower.

There have been recent publications that suggest some genes may be up regulated in response to hypomethylation (Spruijt and Vermeulen 2014), but it is generally accepted that promoter region hypermethylation of a gene suppresses protein production and global hypomethylation causes genome instability. Several genes in the T-helper 2 (Th2) pathway have been found to be susceptible to methylation (Lovinsky-Desir and Miller 2012). It is hypothesized that there is interplay between environmental factors such as $PM_{2.5}$ and respiratory phenotypes, which may be mediated by epigenetic factors such as DNA methylation (Zhang et al. 2014). A recent genome wide study investigated 19,000 genes in 141 subjects for the Normative Aging Study and concluded that airborne particulate matter was associated with the methylation pattern of genes involved in the asthma pathway (Sofer et al. 2013). The literature regarding the

temporal nature and sustainability of these epigenetic changes *in vivo* is sparse (Lovinsky-Desir and Miller 2012). It is possible that the time course from exposure to the resulting epigenetic change ranges from minutes to months and is gene specific.

Two exposure sampling time frames are described in Figure 4.2, which shows the total study period and the two-week period leading up to the physical exam and blood collection. Two weeks is an arbitrary cut-point, but it does draw attention to the idea that proximity of the clinical examination to an air pollution event should be considered. For example, differential methylation patterns of the iNOS gene, which is involved in asthma and other obstructive pulmonary conditions, have been found in welders after an 8-hour shift (Kile et al. 2013). The authors used Line-1 and Alu as proxies for global methylation but did not find differences relative to exposure. However, short-term exposure was associated with epigenetic changes to the iNOS gene promoter region.

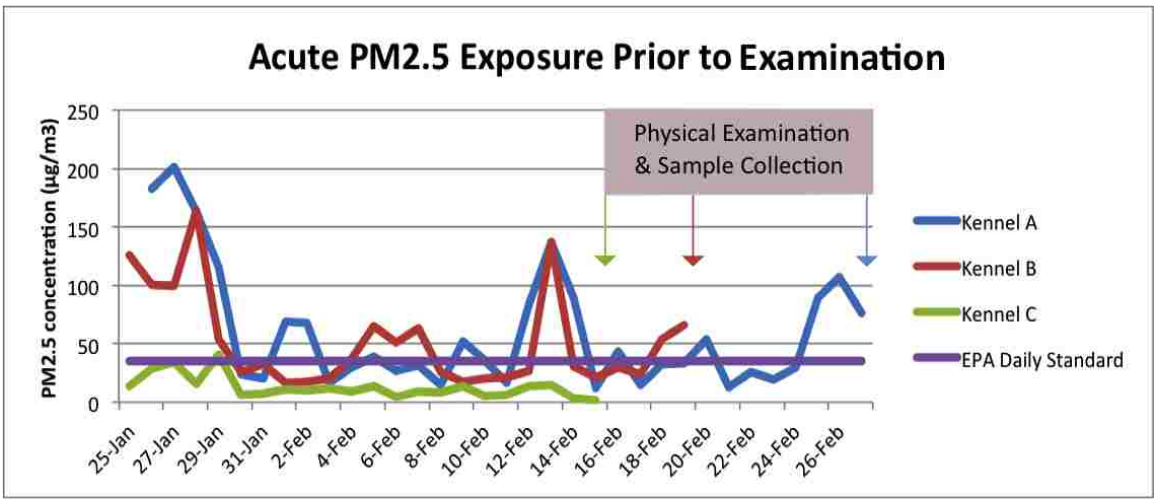

Figure 4.2. Acute $PM_{2.5}$ exposure prior to examination.
$PM_{2.5}$ was measured at three sled dog kennels over the 2012/13 winter. Kennel A, Kennel B, and Kennel C were sampled for 78, 59, and 46 days respectively. This figure shows a subset of these days representing the acute exposure levels that were measured leading up to the sample collections.

In this study LUMA and 5mC were used to detect differential global methylation profiles between three kennels using samples collected at a single visit. Future studies could consider broadening the focus to include gene-specific methylation profiles and conduct more frequent blood collections, including a pre-winter blood collection, to increase the likelihood of capturing $PM_{2.5}$ event-related changes in global and or gene-specific methylation. As more epigenetic information becomes available for the canine genome, genes responsible for the production of immuno-regulatory cytokines and methylation-related enzymes should be evaluated.

Human cell lineages by nature are epigenetically distinct and therefore data from studies that utilize whole blood samples should be evaluated with caution (Reinius et al. 2012). As strategies for partitioning cell populations are developed, the domestic dog model could be used to inform and validate these new methods.

CONCLUSION

In this study we identified $PM_{2.5}$ micro climates in and around the Fairbanks non-attainment area for $PM_{2.5}$. However, when global methylation was evaluated using LUMA and 5-mC, we found no differences between kennels. Several hematology and clinical chemistry variables were correlated with both LUMA and 5-mC, but these results are difficult to interpret and warrant further investigation.

The number of funded epigenetic studies has increased exponentially over the last twenty years. However, only a small percentage of those studies focused on environmental exposures (Burris and Baccarelli 2014). There is increasing interest in animal models for human diseases with environmental exposure and epigenetic components. With improvements in gene and whole-genome techniques, these sentinels can be valuable tools for approaches that are not feasible in humans (Dolinoy and Faulk 2012). Additional research to identify and characterize canine repetitive elements and specific genes that have human homologues will increase the utility of the domestic dog as a surrogate for environmental exposures and impacts on human health.

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Conclusion

In a cohort of asthmatic children living in homes that heat with wood stoves in western Montana, we found that respiratory health was associated with several diet variables. Most of the diet variables that were positively associated with good respiratory health would be considered part of a prudent diet. This suggests that for asthmatics, eating a non-Western diet (i.e. one with less sugar and fat and more fruits, vegetables, and omega 3s) may help with controlling asthma symptoms and exacerbations. This is a positive finding that adds to the overall goal of the ARTIS study. As observed in the larger ARTIS study, the households investigated in this sub-study supported the filter intervention was effective in reducing indoor air pollution and improving respiratory health.

The novelty of this project was the addition of global and gene specific DNA methylation as a target for potential biomarkers. We hypothesized that environmental exposures (i.e. indoor PM and dietary nutrients) may be influencing respiratory health through an epigenetic mechanism. In our evaluation of this hypothesis, we did not find data to suggest this to be correct in the case of LINE-1, IFNγ CpG -54, or -186 methylation. Interestingly we did find that both indoor PM and some dietary factors were associated with methylation patterns. Despite the overall repeated measures association between indoor $PM_{2.5}$ concentrations and lower methylation at the two IFNγ CpG sites, we were unable to demonstrate a change in DNA methylation with respect to filter intervention despite the strong exposure reduction efficacy. Further, from winter one to winter two, there was a reduction in methylation at both IFNγ CpG sites, but not LINE-1, regardless of whether the child lived in a filter intervention or placebo home. At this point, we have not identified the cause of this reduction, but it does not appear to be directly related to

the reductions in indoor PM because there were not significant reductions of indoor PM in the placebo group.

The secular trend in lower average percent DNA methylation across winters also does not appear to be associated with dietary changes despite an overall relationship between some dietary factors and DNA methylation. In our initial evaluation of FFQ results regarding consistency between winter one and winter two for this cohort the results suggest that intake of selected nutrients was similar year to year, though this question may warrant further investigation. Thus, the year-to-year changes in DNA methylation markers are unlikely to be due to corresponding year-to-year changes in indoor PM or diet. Our findings in the sled dog study also fail to support a direct relationship between PM exposure and DNA methylation, although this study is not directly parallel to the child asthma study and did not include gene specific CpG markers.

The change in DNA methylation markers could be associated with year-to-year changes in an unmeasured factor that was not captured by the study design. For example, it is conceivable that all the children in our study were exposed to ambient PM from wildfires between winter assessments and this exposure could have influenced the IFNγ CpG methylation profile. The overall ARTIS study was less susceptible to this type of secular effect because the study populations were widely distributed geographically, whereas the sub-study was limited to the western Montana study households.

One other possibility could be an effect of a laboratory process. For example, there was a year between the collection of these samples and the samples from both winter one and winter two were not processed until the study's end. This is also unlikely because

Qiagen has validated the protocol and the reagents for use up to 24 months from collection to processing. Further, DNA degradation is not likely to lead to demethylation, rather it would lead to fragmentation. Within the pyrosequencing protocol, there are QA/QC measures that ensure data collected from fragmented DNA is not used and requires the sample to be repeated. In this situation, our protocol would be to extract DNA from a sample stored in RNAlater, which was stored at -80°C.

Global methylation was affected by diet but not by indoor PM in aims 1 and 2, respectively. In the cases where LINE-1 methylation was significantly different when comparing tertiles of nutrients, the actual difference in LINE-1 methylation were relatively small. However, it is difficult to translate percentage point difference in LINE-1 methylation to clinical significance. It is interesting to note the difference in the range of global methylation between asthmatic children and sled dogs. For LINE-1 methylation measurements in asthmatic children there was a range of approximately 20 percentage points and for LUMA in sled dogs there was a range of approximately 10 percentage points. These two assays are only proxies for global methylation and they interrogate the epigenome in two very different ways. That being the case, if we consider the two assays to be comparable for the sake of this argument, why should the data from the human cohort be so much more variable than the canine cohort? As it was noted in the introduction, regarding sled dogs, there is much less variability in diet, exercise, age, breeding (in some cases), and exposure. The sled dogs included in the final analysis were also all considered healthy after a veterinary examination and this is in contrast to a cohort of children who have a disease characterized by chronic inflammation.

In the rapidly evolving field of epigenetics, the use and understanding of global methylation analysis has changed in the short period of time from our design of the

ARTIS epigenetics sub-study to the present. After a thorough literature review and consulting leaders in the field of epigenetics in the context of childhood asthma, LINE-1 was the proxy measurement of choice for global methylation in humans. Global hypomethyation in the context of cancer epigenetics is indicative of genome instability and a hallmark of the disease. The epigenetic field has been molded by the findings from the cancer field and this likely was a driver for the fact that global methylation was part of nearly every epigenetic study that was published in the early 2000's. Presently, this measurement has gone out of favor in most published papers because the field is moving from descriptive to mechanistic studies. Global methylation is difficult to interpret because it represents changes that occur throughout the entire epigenome, which encompasses gene regions that may become hypomethylated in response to a given exposure and others that become hypermethylated. There could be substantial epigenetic changes at specific loci that occur in response to an exposure, but if overall hypo/hyper methylation changes are averaged across the genome as with global methylation markers, the result of such analysis may fail to elucidate epigenetic associations with exposure factors of interest. This could explain why LUMA measurements were similar for three groups of dogs that were exposed to variable levels of $PM_{2.5}$.

In addition to the ideals regarding the usefulness of global methylation analysis, there were also recent findings in the epigenetic research community that required us to abandon our initial intention to include the IL-4 promoter CpG markers in our Aim 1 and Aim 2 studies. Several groups had published data in the murine model that suggested there were functionally relevant CpGs or CpG islands in the promoter region of IL-4 that were important for respiratory health. Rachel Miller and others investigated several of these IL-4 CpGs in humans, and they were not found to be associated with urban PM

and PAH exposure and respiratory health. Recent studies have further suggested that IL-4 may be controlled by micro-RNAs and not by DNA methylation. These findings suggest that with regard to IL-4 promoter methylation analysis, our resources would be better used on a different strategy.

The original purpose of including IL-4 CpG sites in the methylation analysis was to investigate the Th1/Th2 balance concept. At this point, we have IFNγ data, which is only half of that story. There are other cytokines that are important in the Th2 pathway such as IL-5 and IL-13. Our lab has taken steps to evaluate the function of several CpGs in the promoter region of these additional Th2 cytokines. In the future, archived samples could be used to evaluate how methylation as promoter sites for Th2 cytokines such as IL-5 and IL-13 are affected by dietary and particulate exposures in our asthmatic child cohort.

This dissertation has focused mostly on epigenetic responses to environmental exposures, which were measured in adolescence (humans and dogs) and adulthood (dogs). We have also conducted preliminary studies focused on perinatal exposure windows and these results will hopefully be used to inform study design for future work focused on Developmental Origins of Health and Disease (DOHaD). This specialized field is interested in elucidating the early causes of diseases that occur later in life, for example cancer or obesity. The results from Aim 1 regarding the ability of diet to influence the epigenome are very intriguing. While these effects are possibly important in the ability of the asthmatic child to control their asthma in this stage of life, it is conceivable that asthma status and nutritional status may have an affect on their progeny. This has grand implications for the future of our species. We are exposed to more chemicals now than any other time in history. The airshed in some countries has

reached toxic levels for pollutants known to be harmful for health. Humans are ubiquitously exposed to foodstuffs. Through these and other exposures, we may be changing the trajectory for our species' health into the future. Decisions that we make as individuals and as a community that affect the environment including the food we consume and the air we breathe are possibly impacting our grandchildren. Therefore findings from epigenetic research that help to inform personal choices as well as public policy have the capacity to improve health for people worldwide today and for generations to come.

APPENDIX

Comprehensive Methods

Bisulfite Treatment Protocol

Note -First perform nanodrop to determine concentration of DNA

Use Zymo Research EZ DNA Methylation-Direct Kit (D5021)
When Opening New Box:

- Prepare CT Conversion Reagent (enough for 9 samples)
  - Add 790 uL Solubilization Buffer
  - Add 300 uL Dilution Buffer
  - Vortex for 10 minutes
  - Add 160 uL Reaction Buffer
  - Vortex additional minute
- Prepare M-Wash Buffer
  - Add 96-mL of 100% EtOH
- Will not usually use Proteinase K, if needed, see protocol that came with Zymo Kit

Bisulfite Conversion of DNA

1) Make a 500 ng DNA/20 uL dilution (w/ autoclaved ddH2O) in 0.2 uL PCR tubes
   - See Excel Spreadsheet for conversion
2) Add 130 uL of CT Conversion Reagent to each
3) Place tubes directly in Thermocycler
4) Run Program "bisulfite" – takes ~3.5 hours
5) In an IC-column (looks like Eppie w/ filter) that is placed in a collection tube add:
   - 600 uL M-binding buffer
   - 150 uL Sample + CT Conversion Reagent
   - Close cap, invert gently several times being careful to hold on to both the IC-column and collection tube

Centrifuge in 5430R at 11,000 rcf for 30 seconds, discard flow-through
6) Add 100 uL M-Wash Buffer
Centrifuge in 5430R at 11,000 rcf for 30 seconds, discard flow-through
7) Add 200 uL M-Desulphonation Buffer
Let stand at room temp for 15 minutes
Centrifuge in 5430R at 11,000 rcf for 30 seconds, discard flow-through
8) Add 200 uL M-Wash Buffer
Centrifuge in 5430R at 11,000 rcf for 30 seconds, discard flow-through
Repeat Step 8
9) Place IC column into 1.5 mL Eppendorf tube (label lid)
10) Add 10 uL M-Elution Buffer **Get pipette tip as close to filter as possible before dispensing elution buffer**
Centrifuge in 5430R at 11,000 rcf for 30 seconds **Do NOT discard flow-through**
Repeat Step 10
11) Final Centrifuge in 5430R at 11,000 rcf for 30 seconds
12) Sample is contained in 1.5 mL Eppendorf and is ready for PCR, or can be stored at 4° C

****All reagents can be stored at room temperature with exception of:

- CT Conversion Reagent – Store at -20° C
- Final Product – Store at 4° C
- Proteinase K prepared solution – Store at -20° C

PCR Protocol

Use PyroMark PCR Kit (in -20° C Freezer)
* Notes Before Starting:
- One primer must be biotinylated (biotin label) at the 5' end for sequencing
- Do not use Q-solution or additional $MgCl_2$
- Perform calculations for primer to have a final concentration of 0.2 uM
  - Example: LINE-1: 40.2nmoles/40.2 uL H2O = 1 nm/uL= 1mM
  - Stock: 1 mM
  - Working: (1000 uM)*(x uL) = (0.2 uM)*(25 uL)
  - x=.005 uL which is an unrealistic amount of primer to add,
    ∴ make a 1/100 dilution
    1 uL stock/99 uL ddH2O for a 10 uM Working solution
    ∴ Add 0.5 uL primer to 25 uL for 0.2 uM final concentration

1) Remove box from freezer and allow reagents to thaw
2) Vortex each component briefly to ensure they are mixed
3) Create a Reaction Mix Stock for X+2* Samples as follows:

Component	Volume per Reaction (uL)	Volume * (X+2) (uL)	Final Concentration
PCR Master Mix	12.5	12.5*_____	1X
CoralLoad Conc, 10X	2.5	2.5*_____	1X
Forward Primer	0.5	0.5*_____	0.2 uM
Reverse Primer	0.5	0.5*_____	0.2 uM
RNase-free H2O	8.0	8.0*_____	
Total:	24	24* (X+2)	

*Make enough for 2 extra samples to ensure there is enough mix for all the samples
4) In PCR tubes add:
- 24 uL of Reaction Mix
- 1 uL of sample (product from bisulfite reaction)

5) Place directly in thermocycler (into smaller holes)
6) Run program LINE-1 (takes ~2.5 hrs)
7) Remove sample. Ready for pyrosequencing, gel electrophoresis, or store at -20° C

Gel Electrophoresis – To test amplification

5X TBE Buffer Stock Recipe
- 27 g Tris-Base
- 13.75 g Boric Acid
- 10 mL 0.5 M EDTA, pH 8.0
- Mix in 500 mL graduated cylinder with stir bar, bring to ~450 mL with ddH2O
- Once dissolved bring to 500 mL with ddH2O, add to glass container, label.

0.5X TBE Buffer Recipe

- 100 mL 5X TBE Buffer Stock
- 900 mL ddH2O
- Mix in 1000 mL Erlenmeyer Flask
- Label & cover with parafilm

1) Make a 3% gel by adding 1.5 g of SeaKem Agarose to 50 mL of 0.5X TBE buffer in 100 mL Erlenmeyer flask
    - Microwave @ 40% for ~1:15 minutes, with a paper towel in the top of the flask
    - Stir gently, careful to not create bubbles
2) Once cool (can touch flask to skin), add 1.5 uL EtBr (10mg/ml)
Stir gently.
    - ***EtBr is a carcinogen. Use caution and ALWAYS wear gloves***
3) While cooling, set up gel rig, make sure gel plate is tight in holder
4) Slowly poor agarose solution into gel plate, add comb
    - Can use pipette tip to move bubbles to bottom of gel
    - Wash 100 mL Erlenmeyer flask immediately
5) Allow to solidify, ~ 10 minutes
6) While gel is setting up, prepare well solutions as follows:

Component	Volume
Sample or Ladder	5 uL
ddH2O	5 uL
6X Loading Dye	2 uL
Total:	12 uL

* Prepared volumes by tightly applying parafilm to eppy-tube holder to make shallow wells
7) Once gel is solid, turn so that the well are near the black (Run to Red)
8) Add 0.5X TBE buffer to fill line
9) Load wells, from right to left, starting with the ladder
10) Run at 100 V for ~20 minutes or until bands have migrated about 1 inch.
    - Start at 75 V for 1 min, then 85 V for 1 min, then 100V for 20 min.
11) Place gel on clear tray, with wells towards the back.
12) Take picture with imager, make sure setting is set to “Ethidium Bromide”
13) Copy to flash drive, open on desktop with Windows Image and Fax Viewer & Save as a JPEG with format: DD.MM.YYYYSample NameInitials
Email to yourself and to PI if necessary.
14) Dispose of gel in BioHazard bin.

Pyrosequencing Protocol

To Do Before Starting:

- Make sure all reagents are to room temperature before beginning.
  - Binding Buffer, Annealing Buffer, Sepharose Beads, etc.
  - Remove PCR product from -20 Freezer
- Turn on Hot Plate to 80° C
- Turn on PyroMark Q96 MD, and start-up computer

1) Perform calculations for sequencing primer.

- Example: LINE-1 Sequencing primer, 39.2 nmoles/39.2 uL = 1 nm/uL = 1 mM = 1000 uM
- Working: Want 10 uM 1/100 dilution
  - Add 5 uL of stock to 495 uL autoclaved ddH2O
- Annealing: Want 0.3 uM in 12 uL/sample Annealing buffer
  - Make up in 1.5 mL eppie
  - 12 uL/sample * (4+2) samples = 72 uL
  - (0.3 uM)*(72 uL) = (10 uM)(x uL)
  - x= 2.16 uL of 10 uM Working + 69.84 uL of Qiagen Annealing Buffer

2) Prepare plate set up with template below

ASSAY_____DATE_____

3) Turn on PyroMark, Heat plate (80° C), and PC (no password, just hit enter)

Setting up Run on Computer

1) Double Click on PyroMark CpG software
2) If assay is not already set up:

File → New assay, or click on the Double Helix icon

3) Enter your sequence to analyze

- All methylation sites should be represented by a Y
- Click on "Generate Dispensation Order" or type in your own

4) Save the Entry under "Examples" on harddrive

- C:
  - Program Files → Qiagen → PyroMark CpG → "Ex

5) File → New Run, or click on the Play button

6) Select Instrument parameters (MD or HS(A)Code0005CDT)
7) Select a set of wells, from plate set up above), and drag assay into the group to populate the entire group
8) Enter Sample ID into each well (either manually or by importing from an excel spreadsheet saved as .txt or .csv, right click, plate setup, and insert sample layout file)
9) Tools → Volume information, print or record
10) Save the run to hard drive in Example Files folder
11) Computer is ready for the sample. Go to Preparing Plate for Pyrosequencing.

Preparing Plate for Pyrosequencing

1) Prepare the Vacuum Work Station

1	EtOH (70%)	110 mL
2	Denaturation Solution(NaOH)	90 mL
3	Wash Buffer, 1x	110 mL
4	MilliQ Water	110 mL
Park	MilliQ Water	180 mL

2) Prepare the PyroMaster Mix in 1.5 mL eppy, vortex

Component	Volume	Volume X+2
Sepharose Beads	2 uL	2* (X+2) uL
Binding Buffer	40 uL	40* (X+2) uL
RNase-Free H2O	28 uL	28* (X+2) uL

*Note: Gently shake sepharose beads until completely dissolved
3) Vortex Mix right before use. In a 96 well shallow plate add 70 uL of Master mix & 10 uL of PCR product for a total of 80 uL/well
4)Place on shaker (secure to plate with adhesive plate cover) for 10 minutes @ 1400 RPM at room temperature
5) Meanwhile, on PyroMark Plate (opaque, white plate), add 12 uL of 0.3 uM Annealing Sequencing Primer to each well.
6) Placed PyroMark Plate on worktable (bottom right) – slide in
7) Remove PCR tubes from shaker, place on a plate on worktable (bottom left)
*Note: Beads will settle at the bottom so do not let sit for more than 1 minute before starting the vacuum. If it has been more than 1 minute, place back on shaker
8) Turn vacuum on, turn on vacuum switch, rinse filter probes in the Park position by "sucking" up the milliQ water.
9) Carefully lowered filter probes into PCR tubes, held for 15 seconds

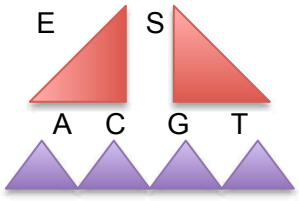
- Made sure to get to VERY bottom of the tubes, and rotate right, left, back, forward in order to get all of the beads
- Take care to keep filter probes in same orientation throughout!!

10) Placed probes into EtOH for 5 seconds
11) Placed probes into Denaturation Solution for 5 seconds
12) Placed probes into 1X Wash Buffer for 10 seconds
13) Raise the tool beyond 90° vertical for 5 seconds to drain liquid from the filter
14) Hold filter over (NOT IN) PyroMark Plate, turn off vacuum switch, lower probes into plate in SAME orientation as PCR plate. Shake filter tips gently to release beads into the wells of PyroMark Plate.
15) Place filter probes in MilliQ H2O, turn vacuum on and wash. Wash again in the MilliQ water in the Park position. Turn vacuum off.

15) Place PyroMark plate on heating block (80°C), cover with Qiagen block, for 2 minutes
16) Let cool at room temperature for at least 5 minutes. Cover with parafilm, so well contents don't evaporate.
17) Plate ready to read, but first set up tip holder and perform drip test.

Setting Up Tip Holder & Drip Test

1) Obtain CDT capillary tubes from black box in drawer under PyroMark and Nucleotides from CDT Reagent box in 4 degree fridge
    - Remove rubber ends off of tips, save to put back on when finished
2) Placed capillary tubes in order as shown in image below:



3) Added the volumes determined from the software (Tools – Volume Info)
    - Make sure to pipette as close to the bottom corners of the tips as possible.
4) Place Tip holder inside PyroMark
    - Open the smaller top lid and place holder with orange label facing you.
5) Performed Drip Test:
    - Open Lid with "open lid" icon on software
    - Place blank plate in holder (PyroMark Plate with tape covering the wells)
    - Close Lid with "close lid" icon on software
    - Click on icon, wait a few seconds.
    - When complete, Open lid, Remove plate and look for 6 small drops in the middle of the plate in this pattern:
        - * *
        - * * * *
    - Wipe off plate with KimWipe and place back on top of PyroMark
6) If drip test is not successful, make sure that the capillary tube tips are not clogged.

7) If drip test is successful, you are ready for your run!!
Open Lid & Place plate in, close lid
8) Click the large Play icon.

Analyzing the Run:

1) Pop-up says Run Finished! Click OK
2) Select all of the wells ran, Click on "Analyze Selected"

Clean-Up

1) Clean up the vacuum workstation:
    - Empty troughs into sink with copious quantities of water
    - Rinse troughs with DI water
    - Place on workstation upside down to dry
2) Clean up after run:
    - Remove plate and throw away

- “Milk” the capillary tubes, place plastic tip protectors back on tubes
- Place all reagents back in storage (mostly in the fridge)

Luminometric Methylation Assay (LUMA)

Global DNA methylation was also determined using the LUminometric Methylation Assay (LUMA), which is based on the ability of two isoschizomers (MspI and HpaII) to digest sequences differentially depending on the methylation status of the CpG site contained within the sequence as described in Karimi et al. (2006). In brief, 300 ng of genomic DNA were cleaved at 37°C for 4 hours and 80°C for 20 minutes with HpaII/EcoRI or MspI/EcoRI in two separate 20µl reactions containing 2 µl of Tango buffer (Thermo Scientific, Waltham, MA) and 5 units of each restriction enzyme (NEB, Ipswich, MA). 15µl of annealing buffer (20 mM TRIS-acetate, 2 mM Mg-acetate pH 7.6) were mixed with the digested samples and placed in a PyroMark Q96 MD system. The dispensation order used for sequencing was: GTGTCACATGTGTG. For calculations, the peak heights of dispensations 9 and 10 were used. Samples with peaks lower than 3, the cut-off value for DNA quality, were discarded. Percentage of DNA methylation was expressed as [1-(HpaII/EcoRIΣG/ΣT)/ (MspI/ EcoRIΣG/ΣT)]*100. The samples were analyzed in technical duplicates and each plate included a positive, negative, and water control. Lambda DNA (NEB) was used as a negative control and methylated Lambda DNA using M.SssI methyltransferase was then used as a positive control. All samples used in the final analysis had an intra-assay coefficient of variation of ≤ 5%.

Buccal Cell Collection Protocol

Subjects: subjects will include the consented asthmatic children and when applicable their consented non-asthmatic sibling between the ages of 7 and 17. Mark the DNA/RNA tube with the appropriate "subject ID". (For example: 101- asthmatic child #1, 201-asthmatic child #2, 801- non-asthmatic sibling)

Supplies needed for each subject:
1- Blue DNA Tube
3- Red RNA Tubes
4- Cytology brushes

General Supplies:
Scissors
Alcohol wipes
Gloves
Cooler/ice pack
Binder with labels

Explanation: Explain the process to the participant as follows: "As part of this study, we are going to collect cheek swabs from you today. I will need you to rinse your mouth out with some water (tap water is fine). The collection brush (figure 1) is very soft and should not hurt your cheek at all, but if it does we can stop at any time. I am going to unwrap the brush and place it inside your mouth (figure 2), spinning it and moving it back, forth for 15-20 seconds. Then, I will remove the brush from your mouth and place it in this tube, cut off the shaft, and close the tube. We will do this a total of 4 times, one brush per gutter (figure 2). Do you have any questions before we begin?"

Labels: Label the 3 RED tubes with "RNA" labels and the 1 BLUE tubes with "DNA" labels. Place the tubes in the transport box and remember to keep the box in an upright position so the buffer does not leak. Transport samples in a cooler with an ice pack. Place "RNA" buccal samples in a -80 degree freezer within two hours of collection. "DNA" samples are safe at room temperature. Mark the storage time on the sample collection record log in the buccal cell binder.

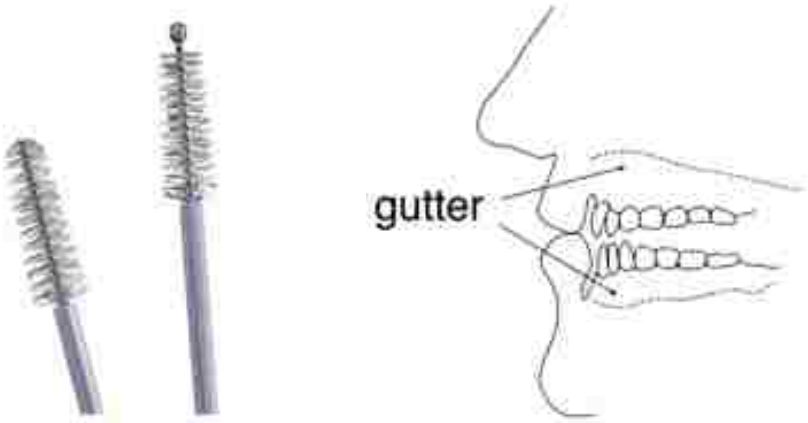


Figure A.1. Location of buccal collection and brush.
Buccal collection was conducted using a cytology brush (pictured on the left). Buccal cells were collected from the gutters (pictured on the right) of the participant's mouth.