

EXPOSURE TO A COMPLEX MIXTURE OF METALS ON GEOGENIC DUST:
ANALYSIS OF CLINICAL CHEMISTRY, HEMATOLOGY,
AND EPIGENETIC MARKERS

by

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DEDICATION

This thesis is dedicated to my husband.

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NOMENCLATURE

5-mC	5-Methylcytosine
AAALAC	Association for Assessment and Accreditation of Laboratory Animal Care
ALAD	δ -aminolevulinic Acid Dehydratase
ALI	Acute Lung Injury
ALKP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANID	Animal Number Identification
ANOVA	Analysis of Variance
APC	Adenomatous Polyposis Coli
ARDS	Acute Respiratory Distress Syndrome
ASC	Antibody Secreting Cell
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BAL	Bronchoalveolar Lavage
BLL	Blood Lead Level
BLM	Bureau of Land Management
bp	Base Pair
BPA	Bisphenol A
BUN	Blood Urea Nitrogen
CBN	Combination Unit
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
CPK	Creatinine Phosphokinase
CSF	Cerebral Spinal Fluid
C _t	Threshold Cycle
DHHS	Department of Health and Human Services
DIC	Disseminated Intravascular Coagulation
DMA	Dimethylarsinic Acid
DMT1	Divalent Metal Transporter 1
DNA	Deoxyribose Nucleic Acid
DNMT	DNA Methyltransferase
dsDNA	Double Stranded DNA
ED ₅₀	Effective Dose 50%
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbant Assay
EPA	Environmental Protection Agency
GFR	Glomerular Filtration Rate
HCT	Hematocrit
HD	Hemodialysis

NOMENCLATURE - CONTINUED

HGB	Hemoglobin
hMLH1	Human MutL Homolog1
HRP	Horseradish Peroxidase
i.p.	Intraperitoneal
IACUC	Institutional Animal Care and Use Committee
IARC	International Agency for Research on Cancer
ICC	Indian Childhood Cirrhosis
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
IDT	Integrated DNA Technologies
Ig	Immunoglobulin
IHEC	International Human Epigenome Consortium
iNOS	Inducible Nitric Oxide Synthase
IOM	Institute of Medicine
IRIS	Integrated Risk Information System
LINE-1	Long Interspersed Nuclear Elements-1
LOAEL	Lowest Observed Adverse Effect Level
LPS	Lipopolysaccharide
MCH	Mean Corpuscular Hemoglobin
MCHC	Mean Corpuscular Hemoglobin Concentration
MCV	Mean Corpuscular Volume
MGMT	O-6-methylguanine-DNA methyltransferase
MMA	Monomethylarsonic Acid
SQL	Method Quantitation Limit
MRL	Minimum Risk Level
MSP	Methylation-Specific PCR
MSU	Montana State University
MVDL	Montana Veterinary Diagnostic Laboratory
NAAQS	National Ambient Air Quality Standards
NDRA	Nellis Dunes Recreational Area
NFT	Neurofibrillary Tangle
NIOSH	National Institute for Occupational Safety and Health
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
OA	Oropharyngeal Aspiration
ORV	Off-road vehicle
OSHA	Occupational Safety and Health Administration
PBBK	Physiologically Based Biokinetic
PBMC	Peripheral Blood Mononucleocyte
PCR	Polymerase Chain Reaction
PDH	Pyruvate Dehydrogenase
PEL	Permissible Exposure Limit

NOMENCLATURE - CONTINUED

PFC	Plaque Forming Cell
PM	Particulate Matter
PMN	Polymorphonuclear Leukocyte
PPB	Parts Per Billion
PPM	Parts Per Million
RBC	Red Blood Cell
RDW	Red Cell Distribution
RfC	Reference Concentration
ROS	Reactive Oxygen Species
SAM	S-adenosylmethionine
SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvate Transaminase
SOT	Solid Organ Transplant
TBA	Thiobarbituric
TBE	Tris/Borate/EDTA
TiO ₂	Titanium Dioxide
TWA	Time-weighted Average
UNLV	University of Nevada Las Vegas
WBC	White Blood Cells
WHO	World Health Organization

ABSTRACT

The Nellis Dunes Recreational Area is a popular off road vehicle site in Clark County, Nevada comprised of dust containing high levels of naturally occurring heavy metals: aluminum, arsenic, cesium, chromium, cobalt, copper, iron, lead, magnesium, manganese, strontium, uranium, vanadium, and zinc. A human health risk assessment and toxicological study to estimate the risk for those who visit the area were conducted. Dust samples from throughout the area were collected and grouped into seven “combination units” (CBN) based on composition and emissivity. Clinical chemistry and hematological changes observed in mice from a sub-acute, dose-dependent, inhalation model were analyzed. Epigenetic markers, specifically 5-methylcytosine, were evaluated in genes known to regulate antibody production and secretion to elucidate a possible mode of action for antibody suppression observed in the murine model. Clinical chemistry and/or hematological changes were observed in each CBN unit, while no change was observed in 5-methylcytosine for *PRDM1*, *PAX5*, and *XBP-1* transcription factor genes regulating the B-Cell to plasma cell differentiation pathway and antibody production. Exposing B6C3F1 mice to 100 mg/kg dust from NDRA did not have effects on the methylation of CpG islands located in promoter regions of *PRDM1* or *PAX5*. Clinical chemistries and hematology varied with the differing characterizations of each CBN unit, though not all results were dose-responsive.

INTRODUCTION

Nellis Dunes Recreational Area, Clark County, Nevada

The Nellis Dunes Recreational Area (NDRA) is a popular off-road vehicle (ORV) site in Clark County located just 6-8 km northeast of Las Vegas, Nevada that is managed by the Bureau of Land Management (BLM). The large desert area spans 36 km² with varying types of landscapes (badlands, drainages, and sand dunes) and soil composition. It has recently been brought to BLM's attention that dust from NDRA contains metals and minerals of health concern. Characterization of the dust identified particulate matter (PM) smaller than 10 microns in size along with more than 20 trace metals that could potentially affect human health, including arsenic (As) (Arita and Costa, 2009; Reichard and Puga, 2010; Ren et al., 2011; Smeester et al., 2011), chromium (Cr) (Ali et al., 2011; Kondo et al., 2006; Takahashi et al., 2005), and lead (Pb) (Bihq et al., 2011; Pilsner et al., 2009; Wright et al., 2010) which have also been shown to affect the epigenome. Arsenic was of particular concern as some areas of NDRA were found to contain concentrations of approximately 300 ppm.

A number of different topsoil compositions exist in the NDRA due to the various mineral and chemical components of the geology (McLaurin et al., 2011). Previous studies by Goossens and Buck (2009a,b) found that natural winds and off-road vehicle (ORV) riding both produced dust emissions and that the various soil surfaces react differently when and how they are driven on, such as with increased speed or driving 4-wheelers compared to motorcycles or dune buggies (Goosens et al., 2012; Goosens and

Buck 2009a,b). The NDRA was originally categorized into seventeen different map units based on soil surfaces such as sand dunes with or without vegetation, silt/clay areas, rock-covered areas, or drainages (McLaurin et al., 2011). These map units were grouped together into combination units based on mineral and chemical composition, and emissivity for the purposes of the toxicological study. CBN1 combined two map units of sand dunes, vegetative and non-vegetative. CBN2 was comprised from white and yellow silt from claystone and sandstone. CBN3 combined five map units that include various stages of desert pavement with some finer-grained sediment, sand, and silt. CBN4 was derived from gravel drainages containing sandy mixtures, silt/clay, and silt/clay crust. CBN5 was comprised of non-vegetative, yellow silt, and sand often stabilized by a silty crust. CBN6 was the thin patches of sand bordering the dune field, and CBN7 was from the fine-grained, brown, claystone and siltstone bedrock (McLaurin et al., 2011).

Problem Formulation and Hazard Identification

The NDRA is visited by an estimated 300,000 ORV driving enthusiasts each year as it is the only publically available land in southern Nevada (Goossens and Buck, 2009a). Riders and other visitors of the NDRA are exposed to the metals, minerals, and small particulate matter in the desert dust from both natural wind erosion and dust emissions from ORV activity (Goossens et al., 2012). Exposure to geogenic PM or dust comprised of particles and heavy metals has been linked to a variety of human health effects. However, very little data exist on health effects associated with dust exposure in natural settings including ORV recreational sites. Also, there is abundant knowledge

about the toxicity of exposure to individual metals, but not of complex metal mixtures as they exist in NDRA. The heavy metals bound to or comprising these fine particles of dust are easily aerosolized during ORV recreation and pose a potential health risk to those exposed (Keil and Peden-Adams, 2011) as particulate matter 10 microns (PM10) or smaller can penetrate deep into the lungs when inhaled (Morman and Plumlee, 2013). If ORV riders and/or visitors are exerting themselves during activity and increasing respiration, the deposition of inhaled particles could also increase (Schultz et al., 2000).

There is potential for non-essential metals to disrupt enzymatic or cellular mechanisms by mimicking the essential metals, e.g., the replacement of phosphates by arsenate, or manganese or strontium competing with calcium in bone mineralization. Also, ionic forms of metals often encourage the formation of reactive oxygen species that could lead to the modification of proteins or DNA and ultimately abnormal gene expression and carcinogenesis (Ballatori, 2002; Basalt, 2004). The dust from NDRA is known to contain arsenic, lead, strontium, chromium, and manganese. Known health effect following individual exposures to these metals include cancers of the lung, liver, skin, and digestive tract; reduction of bone mineralization, decreased sperm count, asthma, cough, shortness of breath, slow and clumsy movement, and behavioral changes (ATSDR, 2004, 2007, 2008, 2012; Chromium Compounds, 2013; Environmental Health and Medicine Education, 2007; Manganese Compounds, 2013). The risk of recreating at NDRA directly correlates to the amount of dust actually inhaled which can fluctuate depending on the type of dust present e.g., loose, un-compacted sandy areas produce the most dust, and partly vegetated areas are more emissive than non-vegetative areas

(Goossens et al., 2011b). The scope of this assessment was to evaluate the simulated effects of an ORV driver acquiring four inhalation exposures over a single month as an illustration the potential risk to 300,000 plus annual NDRA visitors.

Toxicity can be characterized by overt or often subtle changes in critical functions of cells or organ systems. Characterizations of these effects both in human epidemiology studies and animal models are used to establish regulatory values by organizations such as the Environmental Protection Agency. It is not uncommon for immunotoxicological data to be more sensitive to xenobiotic alterations when compared to general toxicity parameters (Keil et al., 2007, 2009; Luster et al., 1992; Peden-Adams 2007a, b). Although immunological endpoint alterations measure more subtle effects of toxicity, the relationship between dose and response is only correlative. Elucidating a mode of action is necessary for establishing causality. The plaque-forming cell (PFC) assay is one of the gold standard functional assays for assessing immunotoxicity as it is a reliable measure of immunoglobulin production. Though immunoglobulin suppression measurement is a common immunotoxicological endpoint, to our knowledge no one has looked at the suppression of transcription factors by means of methylation as a potential mechanism to explain decreases in antibody production. This toxicological profile and risk assessment of the dust at NDRA observed alterations in immune function of B6C3F1 female mice that occurred in the absence of detectable changes in the weight or cellularity of secondary lymphoid organs. Dose-responsively decreased IgM antibody production by splenic B-cells in the PFC assay was detected, with the lowest observed adverse effect level measured in the 0.1 mg/kg dose group. Assessing sensitive immunotoxic endpoints

at low dose exposure levels allows detection of subtle effects altering immune function before the manifestation of overt toxicity. Flow cytometric detection of B-cell populations in secondary lymphoid tissue was measured in each dose group using surface protein marker B220. Dose responsively decreased IgM production and lack of significant changes in the number of mature B-cells led to the investigation of decreased antibody production due to an interruption in the plasma cell differentiation pathway.

Epigenetics is an organization of small molecules and proteins that manage aspects of transcription and translation thereby impacting gene expression without altering the genome. Many cellular processes are managed by epigenetic mechanisms: cell function; maintenance and regulation of chromatin organization; cellular differentiation; and gene regulation. Environmental exposures can interfere with these normal mechanisms leading to disease. This has important implications in toxicology as the field uncovers connections between adult onset disease and pathogenesis due to changes in normal epigenetic processes relative to environmental exposures. Therefore, my objective was to understand a possible mode of action for antibody suppression by epigenetic mechanisms due to inhalation exposure from dust collected at the Nellis Dunes Recreation Area.

Thus, methylation in the promoter regions of genes encoding major transcription factors in the plasma cell differentiation pathway were investigated for alteration of methylation patterns in promoter regions which would reduce proper function and subsequent B-cell differentiation into effector plasma cells. This potential elucidation of a mode of action would strengthen the findings of decreased antibody production.

Adverse Health Effects

Metals

Aluminum (Al). Aluminum naturally occurs as the isotope ^{27}Al and is the most abundant element in the earth's crust (Goldfrank, 2011), however it is found in its trivalent state (Al^{3+}) within the body. Because it is so ubiquitous, it is readily found in the food and water supply (ATSDR, 2008a). In the United States, the average daily intake from food alone is approximately 5 to 10 mg (Yokel and McNamara, 2001).

Aluminum is poorly absorbed by both the lungs and the gastrointestinal tract, and even less likely to be absorbed across the skin (Goldfrank, 2011; Klaassen, 2013). Uptake of Al can occur passively by diffusion through paracellular pathways, or actively by utilizing transferrin and its cellular receptors (ATSDR, 2008a; Goldfrank, 2011; Harris and Messori, 2002). Gastrointestinal absorption depends on pH and the presence of citrate equating to less than 1% of dietary aluminum being absorbed (Goldfrank, 2011; Klaassen, 2013). Pulmonary absorption was determined to be 1.5-2% from increased aluminum output measured in the urine of workers exposed to fumes containing aluminum (Yokel and McNamara, 2001).

Once absorbed, aluminum travels through plasma as approximately 90% is bound to transferrin and the majority of the remaining is bound to citrate (Yokel and McNamara, 2001). It can deposit to a number of different tissues predominantly burdening the skeletal system with 50-70% depositing in the bone (Yokel and McNamara, 2001), 25% in the lungs (Ganrot, 1986) and lesser amounts to other organ

systems such as brain, kidneys, heart, and liver (Goldfrank, 2011). Al is not metabolized in humans and is mostly excreted unchanged in the urine at a rate of 4 to 12 µg per day (ATSDR, 2008a). Because aluminum's primary excretion route is through the kidneys, people with renal disease are more susceptible to aluminum toxicity.

Fibrosis is the most frequent pulmonary manifestation brought on by inhalation of aluminum containing dusts, however workers exposed to these dusts are concomitantly exposed to other chemicals and it is unsure whether or not aluminum is driving the pathology (ATSDR, 2008a). The fibrosis developed in humans could be due to the dust itself and not exclusively the aluminum exposure (Sjögren et al., 2007).

Ninety percent of aluminum found in cerebral spinal fluid (CSF) is bound to citrate (Yokel and McNamara, 2001). Workers chronically exposed to aluminum dusts or fumes have impaired neurobehavioral exams (ATSDR, 2008a), however this is not corroborated with animal studies as the neurological exams performed on animals only examined organ weight and/or histopathology without performing any functional analysis (ATSDR, 2008a). In the 1960's some "potroom" workers exhibited problems with balance, tremors, memory impairment, and loss of cognitive ability (McLaughlin et al., 1962, Rifat et al., 1990) and progressive encephalopathy was referred to as "potroom palsy." (Longstreth et al., 1985; McLaughlin et al., 1962). In the 1970's encephalopathy that had developed in renal failure patients undergoing hemodialysis (HD) was attributed to dialysis fluid contaminated with aluminum salts (Alfrey et al., 1972). HD patients were also found to have osteomalacia and microcytic hypochromic anemia that was linked to

aluminum toxicity when treatment with deferoxamine, an aluminum chelating agent, improved both conditions (Elliott et al., 1978; Ward et al., 1978).

Aluminum interferes with hemoglobin synthesis by inhibiting δ -aminolevulinic acid dehydrogenase (Abdulla et al., 1979; Meredith, 1979). Thus, the microcytic hypochromic anemia that develops does not respond to iron supplement therapy (Jeffery et al., 1996). People with aluminum toxicity can also see effects of the musculoskeletal system. It is likely that aluminum competes with other cations in the bone and therefore leads to osteopathy including osteomalacia (Griswold, 1983), however, the kinetics of other cations such as magnesium, phosphate, and calcium are not affected in patients with aluminum toxicity (Burnatowska-Hledin et al, 1983).

There is an active debate about the role of aluminum in Alzheimer's disease. Increased levels of aluminum are found in the brains of Alzheimer patients and within neurofibrillary tangles (NFTs) of experimental animals; however, it is not clear whether or not the aluminum is the cause of or is secondary to these conditions (Bondy, 2010; Klaassen, 2013; Sjogren et al., 2007). It is possible that staining methods used to drive some early studies of aluminum in Alzheimer brains actually lead to aluminum contamination of the tissue (Bondy, 2010; Makjanic et al., 1998). Additional investigation revealed that the structural and chemical composition of NFTs in Alzheimer disease are different from those in aluminum encephalopathy (WHO, 1997). Though there is no definitive evidence establishing aluminum as a cause for Alzheimer disease, there are studies suggesting links between aluminum exposure and other neurodegenerative diseases (Bondy, 2010, Kawahara, 2005).

Arsenic (As). Acute arsenic exposure is typically limited to workplace or hazardous waste areas; however, it does occur naturally in the soil with a range of < 0.1-97 µg/g across the United States, the mean being 7.2 µg/g (ATSDR, 2007a). The most likely route of exposure outside these specific circumstances is via the oral route from food and water contamination (ATSDR, 2007a). Grains and produce are the most likely dietary consumables to be contaminated with an estimated 1 to 20 µg of inorganic arsenic as well as drinking water containing an average 2 µg/L (ATSDR, 2007a).

Arsenic can exist in different forms with inorganics being responsible for the majority of arsenic-induced toxicity. Two relevant species of inorganic arsenic are pentavalent [As^{5+} or As(V)] and trivalent [As^{3+} or As(III)], of which As(III) is more toxic, however As(V) is more prevalent in the natural environment (Del Razo et al., 1990). The most common cause for chronic arsenic exposure is currently from contaminated drinking water (Goldfrank, 2011). Drinking wells in Bangladesh are contaminated with arsenic that leaches from minerals in the soil which has led to the poisoning of millions of citizens (Paul et al., 2000). The U.S. EPA lowered its maximum contaminant level in drinking water from 50 ppb to 10 ppb after finding increased risks of lung and bladder cancer associated with ingesting drinking water with the allowable limit of 50 ppb As (EPA, 2000). WHO also recommends a maximum contaminant level of 10 ppb arsenic in drinking water (WHO, 2011).

One mechanism of toxicity for As(III) is interference with the pyruvate dehydrogenase (PDH) complex. In this pathway As(III) binds the sulfhydryl groups of dihydrolipoamide preventing its conversion to lipoamide, which is a cofactor necessary

for the decarboxylation of pyruvate to eventually produce Acetyl CoA (Goldfrank, 2011). Decreased Acetyl CoA prevents the citric acid cycle from operating at its normal pace which subsequently leads to decreased ATP production. Lipoamide is also necessary for the production of succinyl CoA, which is required for amino acid production and porphyrin synthesis. Deficiencies of these physiological compounds could manifest as anemia in persons chronically exposed to arsenic (Goldfrank, 2011). Trivalent arsenic is known to interfere with glucose-6-phosphate dehydrogenase, glutathione synthetase, and glutathione reductase. These enzymes play a critical role in maintaining reactive oxygen species (ROS) and preventing oxidative damage (Aposhian and Aposhian, 1989).

Experiments in animals with the trivalent arsenical phenylarsine oxide exhibited interference with glucose transfer into cells by insulin and damage to the β -islet cells of the pancreas (Boquist et al., 1988). These interferences can lead to hypoglycemia due to a depletion of glycogen synthesis.

Though trivalent arsenic is most commonly associated with toxicity, pentavalent arsenic cannot be ignored. Fifty to seventy percent of As(V) reduces to As(III) by glutathione during its metabolism (Concha et al., 1998a; Huang and Lee, 1996; Styblo et al., 1995; Vahter, 1999; Vahter and Marafante, 1983). It also shares a similar chemical structure to phosphate and can be mistaken as such in biological pathways. It is thought that As(V) is able to use the same mechanisms as phosphate for cellular entry (Huang and Lee, 1996). The substitution of As(V) for inorganic phosphate inhibits oxidative phosphorylation and thus the production of ATP (Chen et al., 1986; Rein et al., 1979).

The absorption of arsenic is dependent on the compound size and solubility. It can be absorbed across the lungs and mucosal sites, and by the small intestines in the gastrointestinal tract (Goldfrank, 2011). Arsenic can be absorbed dermally; however this exposure only constitutes a risk with chronic, and not acute, scenarios (Goldfrank, 2011). The risk of toxicity due to dermal exposure does change if the skin is not intact.

As₂O₃ given to eight patients in a single 10 mg intravenous dose was determined to have an average α elimination half-life of 0.89 hours and a β elimination half-life of 12.13 hours (Shen et al., 1997); however a study that administered humans an intravenous dose of ⁷⁴As, a radioisotope, exhibited three phases of clearance. The first phase showed a half-life of 1-2 hours with > 90% thought to clear from the plasma after 2-3 hours due to renal elimination and redistribution to the tissues. A second phase was determined to be from 3 hours to 7 days with a half-life of 30 hours, and a third phase after 10 days estimated the half-life to be 300 hours (Mealy et al., 1959). Other studies credit urinary excretion for the elimination of 30-60% of arsenic absorbed via inhalation (Holland et al., 1959; Pinto et al., 1976; Vahter et al., 1986).

Once absorbed, the primary sites for distribution tend to be liver, kidney, muscle, and skin (Goldfrank, 2011); however, arsenic will also quickly distribute to the central nervous system. Mealy et al., (1959) found that 0.3% of ⁷⁴As distributed to brain tissue biopsy samples within the first hour after administration.

In women who are pregnant, arsenic has been shown to cross the placenta and accumulate in the fetus (Lugo et al., 1969), and two separate studies measured low levels

of arsenic in the breast milk of mothers exposed to roughly 200 µg/L in their drinking water (Concha et al., 1998b; Samanta et al., 2007).

Metabolism of arsenic occurs by the addition of methyl groups taken primarily from *S*-adenosylmethionine (SAM) to form either monomethylarsonic acid (MMA) or dimethylarsinic acid (DMA) when one or two methyl groups are added. This methylation mostly takes place in the liver and to a smaller extent in the kidneys, lungs, and the testes in males (Goldfrank, 2011). The addition of methyl groups polarizes the molecule making it more water soluble and able to be excreted in the urine, however the methylated intermediates may prove more toxic than the arsenate or arsenite parent molecules (Petrick et al., 2000, 2001; Vahter M, 2000).

Arsenic is a well-known disruptor of gene expression (Andrew et al., 2008; Bourdonnay et al., 2009; Somji et al., 2011; Su et al., 2006). Andrew et al. (2008) alone found changes in expression of 259 genes after exposure to varying levels of arsenic in drinking water. Hypermethylation of CpG islands, global hypomethylation, and histone modifications have been induced by arsenic indicating that some modes of action for As toxicity are epigenetically driven (Arita and Costa, 2009; Reichard and Puga, 2010; Ren et al., 2011; Smeester et al., 2011). As previously mentioned, the metabolism of arsenic reduces As (V) to As (III) then requires the addition of methyl groups for polarization of the molecule, making it more water soluble, and facilitating its excretion in the urine. This methylation process utilizes SAM as the methyl donor, which is also the methyl source required for DNA methylation. It is thought that this resource competition could affect proper methylation of the epigenome in instances of increased exposure to arsenic.

Arsenic has also been associated with dose dependent reduction of DNA methyltransferase (DNMT) activity, the enzyme that catalyzes methylation of cytosine nucleobases, which correlates with studies of broad mechanisms of epigenetic alterations and observations of global hypomethylation (Ahlborn et al., 2008; Cui et al., 2006; Reichard et al., 2007; Zhao et al., 1997). Arsenic has been known to induce hypo and hypermethylation in both *in vitro* and *in vivo* experiments (Davis et al, 2000; Martinez et al., 2011; Mass and Wang, 1997; Okoji et al. 2002; Xie et al., 2004; Zhao et al., 1997; Zhong and Mass, 2001), as well as in observations of human populations (Majumdar et al., 2010; Pilsner et al., 2007,2009). In this study, the only metal species characterized in the NDRA dust was arsenic and all samples were primarily As(V).

Inhalation exposure manifests with toxicity of the respiratory system and has been shown to modulate immune function (Aranyi et al., 1985; Holson et al., 1999). Observations in humans and animals have found altered lymphocyte activation (Biswas et al., 2008; Cho et al., 2012; Conde et al., 2007; Gonsebatt et al., 1994; Martin-Chouly et al., 2011; Morzadec et al., 2012; Ostrosky-Wegman, 1991; Soto-Pena and Vega, 2008), increased cytokines indicative of inflammation in humans and human cell lines (Ahmed et al., 2011; Hernandez-Castro, 2009; Lemarie et al., 2006; Soto-Pena, 2006; Wu et al., 2003), decreased expression of CD69 in humans, human cells, and mice (Andrew et al., 2008; Conde et al., 2007; Tenorio and Saavedra, 2005), decreased TNF- α in humans and animals (Argos et al., 2006; Biswas et al., 2008; Hermann and Kim, 2005; Kozul et al., 2009; Lantz et al., 1994; Salgado-Bustamante et al., 2010), increased CD14 in humans and human cell lines (Hernandez-Castro, 2009; Lamarie et al., 2006; Sakurai et al., 2006;

Wu et al., 2003;), decreased IL-1 β in humans and animals (Andrew et al., 2008; Argos et al., 2006; Kozul et al., 2009a,b; Mattingly et al., 2009), and decrease MHC class II expression in humans and animals (Andrew et al., 2008; Sikorski et al., 1991).

Arsenic is a well-established carcinogen known to cause skin, liver, bladder, and lung cancer. Inorganic arsenic has been deemed carcinogenic by the Department of Health and Human Services (DHHS), the International Agency for Research on Cancer (IARC), and the Environmental Protection Agency (EPA) (ATSDR, 2007a).

Cesium (Cs). ¹³³Cesium is non-radioactive and occurs naturally in soil at an average concentration of 1 ppm (ATSDR, 2004b). Literature regarding the health effects of inhaled cesium is almost non-existent, however it is known that cesium compounds are able to solubilize in water and can therefore be absorbed via ingestion, dermal exposure, and through inhalation (Klaassen, 2013). A Physiologically based biokinetic (PBBK) model analyzed by Leggett et al. (2003) calculated Cs to distribute primarily to liver, kidneys, muscle tissue, and the GI tract. Cs can cross the placenta in pregnancy and has also been found in breast milk of lactating women (Klaassen, 2013). Cs is excreted in the urine (Klaassen, 2013).

Stable cesium has been recorded as a gastrointestinal and ocular irritant in high-dose exposures (ATSDR, 2004b) and has also been associated with increased cardiac arrhythmia and QT wavelength prolongation following an ingestion of cesium salts (Bangh et al., 2001; Dalal et al., 2004; Harik et al., 2002; Saliba et al., 2001).

Chromium (Cr). Chromium is naturally occurring in soil, rocks, plants, and animals, and exists primarily in its trivalent [Cr(III) or Cr^{3+}] or hexavalent [Cr(VI) or Cr^{6+}] forms. Cr(III) plays an important role in glucose metabolism (IOM, Food and Nutrition Board, 2006), most likely when it is in the form of chromodulin. The Institute of Medicine (IOM) has deemed Cr(III) as an essential nutrient and recommends a daily intake of 20-45 μg a day, though the concept of essentiality is debated among scientists since there is no disease associated with the deficiency of trivalent chromium (ATSDR, 2012a).

The absorption of chromium is dependent on its valence state. Cr(VI) compounds occur most often in a tetrahedral conformation which allows the molecule to utilize the same nonspecific transport systems into the cell as other tetrahedral compounds, such as phosphate and sulfate (Arslan et al., 1987; Costa, 1997). Two to ten percent of hexavalent chromium is absorbed, which is higher than the absorption of trivalent chromium. Only about 0.5-2% of Cr(III) makes it into peripheral circulation (Klaassen, 2013), this lower absorption rate is most likely due to its octahedral conformation which diffuses slowly across cell membranes (Cohen, 2006). Once absorbed, hexavalent chromate rapidly reduces to a trivalent form in cells of the gastrointestinal tract or red blood cells (Goldfrank, 2011). This reduction process can cause reactive intermediates such as DNA and protein adducts, and free radical formation (Aitio et al., 1984; ATSDR, 2012a; Proctor et al., 2002; Shrivastava et al., 2002). A case study examining a patient who ingested a lethal dose of potassium dichromate (a hexavalent form of chromium) found that >80% of the oral dose reduced to Cr(III) in the blood (Iserson et al., 1983). In

RBCs Cr(III) can then bind intracellular proteins or hemoglobin, and is therefore stored within the cell for the duration of the RBCs lifespan (ATSDR, 2012a). Because of this rapid reduction from hexavalent to trivalent chromium, Cr(III) contributes almost the entire body burden of chromium (Goldfrank, 2011). Chromium distributes throughout the body and can be found mostly in kidneys and liver as it readily binds glutathione and ascorbate (ATSDR, 2012a; Costa, 1997), and to a lesser extent the marrow, lungs, lymph nodes, spleen, and testes (Costa, 1997). Renal elimination is the primary mechanism for excretion of chromium. Paustenbach et al. (1996) parenterally administered Cr(VI) and measured 80% to have been eliminated in the urine as Cr(III), and 2-20% eliminated in feces (Paustenbach et al., 1996).

Exposures to hexavalent chromium primarily take place in an occupational setting by inhalation. The identification of chromium in the tissue, serum, and urine of those exposed in the workplace suggests that chromium can be absorbed across the lungs (Cavalleri and Minoia, 1985; Gylseth et al., 1977; Kiilunen et al., 1983; Mancuso, 1997; Minoia and Cavalleri, 1988; Randall and Gibson, 1987; Tossavainen et al., 1980). Symptoms manifesting in workers who work in an occupational setting containing airborne chromium ranged from asthma, pulmonary distress, sneezing, rhinorrhea, labored breathing, nasal septum ulceration (Gomes, 1972; Kleinfeld and Rosso, 1965; Lee and Goh, 1988; Lieberman, 1941; Mancuso, 1951; Meyers, 1950; Novey et al., 1983).

Occupational exposure data provide conflicting conclusions about hematological effects due to chromium exposure; however, Mancuso (1951) observed an increase in

monocytes and eosinophils with an overall leukocytosis in 97 workers exposed to an insoluble chromite ore (trivalent chromium) and soluble sodium chromate and dichromate (hexavalent forms of chromium).

Acute inhalation exposure to hexavalent chromium can cause mild lung irritation, hyperplasia, accumulation of macrophages, and impaired lung function (ATSDR, 2012a). Rats exposed to 0.025mg Cr(VI)/m³ in the form of potassium dichromate had increased lymphocytes in lung fluid obtained by bronchoalveolar lavage (BAL) when exposed for either 28 or 90 days (ATSDR, 2012a). Due to the oxidative nature of Cr(VI) and its reduction within the red blood cell, intravascular hemolysis or disseminated intravascular coagulation (DIC) could potentially develop (Goldfrank, 2011). Acute toxicity could also cause tubular necrosis in the kidneys which could lead to renal failure (Wedeen, 1991).

In a study assessing 15 men who were exposed to a number of compounds including hexavalent chromium in the form of lead chromate some lymphocyte populations were reduced. CD4⁺ T-cells, B-cells, and natural killer cells decreased by 30-50% compared to controls, though, serum IgA, IgG, and IgM were not significantly different (Boscolo et al., 1997). Mignini et al. (2009) examined employees working in hide, shoe, and leather industries and did not find any significant differences in lymphocyte subpopulations. IL-12 levels were found to be elevated in lipopolysaccharide (LPS) stimulated peripheral blood mononucleocytes (PBMC) of workers exposed to chromium (Katiyar et al., 2009); however, Mignini et al. (2009) saw a significant decrease of IL-12 levels in those who work with hide, leather, and shoes. Katiyar et al. (2009) also saw significantly increased levels of interferon- γ in both stimulated and

unstimulated PBMCs from chromium workers. This contrasts with Mignini et al. (2009) who found no changes in interferon- γ levels. They did, however, measure increased IL-2 and IL-6.

Hexavalent chromium has been established as a known human carcinogen by the National Toxicology Program (NTP, 2011a). Occupational exposure to airborne chromium is associated with increased risk of lung cancer (IARC, 1990). Trivalent forms of chromium are generally considered to be non-genotoxic (Klaassen, 2013). Cr(VI) compounds can generate ROS, inhibit DNA replication, and prevent protein synthesis. It is also known to interfere with p53 function, cell cycle arrest, DNA repair, and apoptosis (Costa and Klein, 2006; O'Brien et al., 2003). Because inhaled hexavalent chromium can be absorbed across the lungs and enter systemic circulation, it can cause mutagenesis in systems throughout the body such as bone, stomach, prostate, kidney, bladder, and hematopoietic system (Costa, 1997).

In humans occupationally exposed to Cr via inhalation, methylation of the promoter region for genes: human MutL Homolog1 (hMLH1), p16INK4a, O-6-methylguanine-DNA methyltransferase (MGMT), and Adenomatous Polyposis Coli (APC), were found in those who also suffered from lung cancer (Ali et al., 2011; Kondo et al., 2006; Takahashi et al., 2005). Cheng et al. (2002, 2004a,b) observed alterations in the epigenome of offspring whose fathers were exposed to Cr(III). Follow up work observed methylation in the spacer promoter region of 45S rRNA in sperm cells, suggesting that the offspring would potentially be at an increased risk of tumor development (Shiao et al., 2005, 2011).

Cobalt (Co). Cobalt is a necessary micronutrient as it is the ion in vitamin B₁₂, also known as cyanocobalamin. Vitamin B₁₂ is acquired from the diet, particularly from seafood, pork, chicken and eggs. Pernicious anemia can be caused by a B₁₂ vitamin deficiency, though, over exposure to Co is associated with toxicity.

The bioavailability of cobalt depends on the compound and is highly varied ranging from five to forty five percent for oral exposure and roughly thirty percent for inhalation (Lison et al., 1994). Once absorbed the majority of Co is rapidly excreted in the urine and then to a lesser extent the feces (Lison et al., 1994). Some of cobalt's toxicity can be attributed to its binding of sulfhydryl groups and its Co²⁺ form competing with divalent cations in normal biological processes. Co²⁺ is thought to displace Mg²⁺ during RNA synthesis interfering with protein and enzyme genesis. It also utilizes the Ca²⁺ uptake mechanisms of the red blood cell, but is not excreted via Ca²⁺ pumps and can accumulate in the cytosol of RBCs (Simonsen et al., 2012). Co²⁺ binds to albumin in the serum to facilitate dissemination and it will accumulate in soft tissues such as pancreas, kidney, heart, liver, and to a smaller extent the musculoskeletal system (Simonsen et al., 2012).

Co has been used as a therapeutic agent for a number of years as it stimulates the production of erythropoietin, appreciably without toxic effects (Simonsen et al., 2012). The mechanism for this stimulation is not entirely understood, but patients undergoing CoCl₂ therapy saw an increase in RBC count, hemoglobin, and hematocrit (Chan, 2011).

Acute exposure manifests in in the cardiovascular system, endocrine system, hematologic system, and a few instances of gastrointestinal distress and some nervous

system involvement have been observed in humans undergoing CoCl_2 therapy (Chan, 2011; Schirmacher, 1967). A sudden increase in cardiomyopathies in 1966 was traced back to CoSO_4 used in a few select breweries as a foam stabilizer for beer. Post mortem examination of the decedents found Co concentrations ten times higher in cardiac tissue than in control subjects (Chan, 2011). It is noted that each decedent qualified as malnourished and the observed cardiomyopathy was similar to that seen in infantile malnutrition and chronic alcoholism (Chan, 2011). Also, the exposure to those drinking the CoSO_4 in beer was far less than doses of CoCl_2 used in therapy for RBC disorders; therefore it is thought that Co induced cardiomyopathy requires concurrent nutritional deficiency (Kesteloot et al., 1968; Morin and Daniel, 1967).

Thyroid hormone disruption has been observed in acute and chronic exposures to cobalt. Patients receiving Co treatment for sickle cell anemia experienced varying thyroid disorders including goiter and hypothyroidism (Gross et al., 1955; Klinck, 1955; Kriss, 1955). There is also occupational data showing workers from a cobalt refinery with low T_3 concentrations from exposure to Co metals, oxides, and salts via inhalation (Swennen, 1993).

Cobalt is known to cause deleterious effects to the pulmonary system under conditions of chronic exposure, namely cobalt hypersensitivity-induced asthma and “hard metal disease” (Chan, 2011).

Carcinogenicity of cobalt has not been extensively tested in humans and current carcinogenic standings are based on animal studies. It is considered to be possibly carcinogenic to humans (group 2B) by the International Agency for Research on Cancer

(IARC) and is reasonably anticipated to be carcinogenic in humans by the National Toxicology Program (NTP) (IARC, 1991; NTP, 2011b).

Copper (Cu). Average daily intake of copper in the United States is 0.9-1.7 mg in adults (Chambers et al., 2010; Ervin et al., 2004).

Copper is primarily absorbed through the GI tract in the small intestine. Once it has reached circulation, it binds to plasma proteins such as ceruloplasmin and to a lesser extent albumin which then carry it to the liver. In the liver, copper binds to metalloproteins such as methionine for storage and later use in the genesis of a number of enzymes, or it is excreted in the bile and eliminated in the feces (Goldfrank, 2011; Stern, 2010).

Copper toxicity in the U.S. is not common in healthy adults, but excess consumption of copper can be hazardous to individuals with liver or kidney diseases, or those who have an inborne error of copper metabolism such as Wilson's disease, Indian Childhood Cirrhosis (ICC), or Hereditary Aceruloplasminemia (Goldfrank, 2011; Klaassen, 2013).

Copper has been recorded as a respiratory irritant in those who work with copper containing dust. Symptoms included coughing, thoracic pain, sneezing, and runny nose (Askergren and Mellgren 1975; Suciu et al, 1981). Cu has also been implicated as a the cause of "vineyard sprayer's lung," an occupational disease observed in workers who used an antifungal agent containing up to 2.5% copper sulfate (Pimentel and Marquez, 1969). This disease was often associated with copper containing granulomas, fibrohyaline

nodules, and desquamation of macrophages in the alveoli (Pimentel and Marquez, 1969; Plamenac et al., 1985).

Drummond et al. (1986) observed reduced ability to clear a pulmonary infection with *Klebsiella pneumoniae* in mice given a single high dose of CuSO_4 (3.3 mg/m^3) and those given multiple exposures to $0.12\text{-}0.13 \text{ mg/m}^3$ over a 10 day period.

Not enough data exists to adequately assess the carcinogenicity of copper. It is not classified by the EPA (Group D), and the pesticide copper 8-hydroxyquinoline is considered unclassifiable to carcinogenicity by the IARC (ATSDR, 2004a; EPA-IRIS, 2015).

Iron (Fe). Iron is an abundant transition metal that is essential for hemoglobin synthesis and proper erythropoiesis. It is also important in the formation of myoglobin, cytochromes, and metallo-flavoprotein enzymes (Klassaan, 2013; Goldfrank, 2011). Iron has two oxidation states Fe^{2+} and Fe^{3+} . The reduction and oxidation of iron throughout its metabolic process can cause oxidative stress (Reissman and Coleman, 1955; Robotham et al., 1974; Klaassen, 2013), which is thought to play a role in the development of atherosclerosis and ischemic heart disease in cases of excessive exposure (Alpert, 2004). There is evidence that mortality due to cardiovascular disease is secondary to hepatic iron overload (Gujja et al., 2010; Yuan and Li, 2003).

When ingested in excess, iron initially affects epithelium of the gastrointestinal tract and causes nausea, vomiting, abdominal pain, and diarrhea. Once absorbed into the bloodstream, the iron will bind transferrin and can be distributed throughout the body (Goldfrank, 2011). An examination of 11 patients who expired due to oral overdose of

iron showed accumulation in the stomach, liver, small bowel, heart, lungs, kidney, and brain (Pestaner et al., 1999). Excessive amounts of iron ions can disrupt oxidative phosphorylation causing a buildup of hydrogen ions in the person's serum causing metabolic acidosis (Goldfrank, 2011).

Data regarding inhalation toxicity of iron are limited; however, cases of pneumoconiosis, Black Lung Disease, have been observed in occupational settings where workers are exposed to iron oxide fumes or dust (Doherty et al., 2004). OSHA has set a permissible exposure limit (PEL) of TWA 10 mg/m³ and NIOSH's PEL is TWA 5 mg/m³ (<http://www.cdc.gov/niosh/npg/npgd0344.html> date: January 16, 2015). Most cases of acute iron toxicity are from ingesting excessive amounts of iron containing dietary supplements, frequent blood transfusions, or from hemochromatosis, a genetic disease associated with altered iron metabolism and excessive storage of iron in the tissues (Papanikolaou and Pantopoulos, 2005; Weinberg, 2010; Doig, 2012).

The body does not have an efficient mechanism for excreting iron, instead iron absorption is moderated by the hormone hepcidine based on the demands of erythropoiesis (Papanikolaou and Pantopoulos, 2005), but the primary route for iron that is excreted is in the feces (Klaassen, 2013).

Huang (2003) reviewed animal, cell culture, and epidemiological studies and argues that evidence exists to potentially implicate iron as a carcinogen, however, literature from IARC, ATSDR, EPA, and NTP regarding iron carcinogenicity could not be found.

Lead (Pb). Lead is primarily absorbed in the gut and via inhalation, with minimal amounts absorbed dermally. In the gut, lead is absorbed in adults at roughly three to ten percent of what is orally ingested, whereas children can absorb as much as 50% (ATSDR, 2007b). Airborne lead has been reported a minor source of exposure (ATSDR, 2007b). However, submicron particles of inorganic lead can absorb into the blood stream via inhalation and within an hour after inhalation exposure, 50% of absorbed lead was detected in the liver (James et al., 1994).

Systemic distribution of lead does not depend on the route of absorption. Ninety nine percent of lead in circulation is bound to intracellular erythrocyte proteins with the remainder bound and distributed by albumin and other plasma proteins (ATSDR, 2007b). In adults, 93% of the body burden sequesters in bones, and in children, 73% in the skeletal system. This primary storage site is known to increase in concentration over time, with more accumulation in the regions that are actively growing and remodeling at the time of exposure. A large pool of lead in bone can contribute to blood lead levels even after exposure has passed due to a relatively slow turnover with bone metabolism. This can also cause maternal to fetal transfer of lead as mothers resorb bone for the production of the fetal skeleton.

Though most lead ends up in the skeletal system, it is initially deposited into soft tissues where it is associated with a number of adverse effects: genotoxicity, hepatotoxicity, nephrotoxicity, hematotoxicity, cardiovascular effects, gastrointestinal effects, musculoskeletal effects, immunological and lymphoreticular effects, reproductive and developmental effects, and effects of the endocrine system (ATSDR, 2007b). Lead is

also known to affect the CNS, causing epilepsy, mental retardation, optic neuropathy, and learning disabilities in children with higher blood lead levels (ATSDR, 2007b; Bellinger, 2005; Goyer, 1990; Laraque and Trasande, 2005). The classic manifestation of adult lead toxicity is peripheral neuropathy (Lindgren et al., 1996) even though lead affects virtually every neurotransmitter system in the brain. Along with distribution to bone matrices and CNS, lead can be found in the bone marrow, hair, kidneys, and in the cardiovascular and immune systems.

Metabolism of inorganic lead includes the formation of complexes with a variety of ligands, both protein and non-protein. Albumin and non-protein sulfhydryls are the major extracellular ligands, and the major intracellular ligand is ALAD. The intracellular metabolism of lead interrupts heme biosynthesis within red blood cells and can cause a microcytic, hypochromic anemia (ATSDR, 2007b). Organic lead compounds metabolize in the liver via oxidative dealkylation catalyzed by the cytochrome P450 pathway. The half-life of lead in systemic circulation is about thirty days in stark contrast to a half-life of twenty years in bone. Circulating lead is primarily excreted through glomerular filtration though one third utilizes the biliary tract. Sweat, saliva, hair, nails, and breast milk also provide some minor routes of excretion (ATSDR, 2007b), while roughly 20% of inhaled lead will be exhaled, never absorbed, and excreted by yet another mechanism (Heard et al., 1979).

Alterations in immune function, including changes in T-cell subpopulations, T-cell mitogen response, and a reduction in chemotaxis of neutrophils, have been observed in some lead workers having blood lead levels (BLLs) from 30-70 $\mu\text{g/dL}$ (ATSDR,

2007b). Ewers et al. (1982) found that lead workers having a median BLL of 59 µg/dL exhibited significant suppression of IgM when compared to the control group (median BLL of 11.7 µg/dL). Decreased IgM populations correlated with those who experienced increased susceptibility to colds and influenza per year. Other occupational studies have found decreased populations of CD3⁺ and CD4⁺ cells, and decreased C3 and C4 complement levels in workers having a mean BLL of 74.8 µg/dL (Basaran and Ündeger, 2000; Fischbein et al., 1993; Ündeger et al., 1996). A study by Sata et al. (1998) examined cell populations in lead workers with a mean BLL of 19 µg/dL and found significantly decreased populations of memory T-cells, though none of the subjects showed signs of having a current infection. An association between BLLs and increased serum IgE levels in children has also been shown. These findings combined with results from rodent studies have led some to suggest that lead-exposed children are at risk for asthma development (ATSDR, 2007b).

Lead is known to cause a large array of deleterious health effects in humans. A study of non-human primates exposed to Pb exhibited globally decreased levels of both DNMTs and the methylation binding protein MeCP2 (Bihic et al., 2011). Pilsner et al., (2009) observed that DNA methylation in cord blood inversely correlated to prenatal Pb exposure, suggesting that the fetal epigenome can be influenced by the maternal Pb burden. An association between DNA methylation of long interspersed nuclear elements-1 (LINE-1) and Pb exposure was observed by Wright et al. (2010). It is suggested that this relationship could potentially be used as a biomarker for historical lead exposure in humans.

OSHA's permissible exposure limit for workplace air is $50 \mu\text{g}/\text{m}^3$ for an eight-hour workday. Those exposed to airborne lead in concentrations of $30 \mu\text{g}/\text{m}^3$, which is the OSHA action level, for more than thirty days annually, should monitor his or her blood lead levels (NIOSH, 2012). The US EPA has set a regulatory value of $0.15 \mu\text{g}/\text{m}^3$ for lead in the ambient air (the NAAQS for lead). The U.S. EPA monitors ambient air lead concentrations in select sites in the U.S. Quarterly average airborne concentrations of lead are not to exceed $1.5 \mu\text{g}/\text{m}^3$ (www.epa.gov/air/oaqps/greenbk/inte.html, accessed July 2014). National airborne lead concentrations have decreased dramatically since 1980, when lead was eliminated from gasoline (www.epa.gov/air/lead; accessed July 2014). No inhalation RfC is available in the U.S. Environmental Protection Agency's (US-EPA) Integrated Risk Information System (IRIS) database.

DHHS considers Pb to be reasonably carcinogenic based on animals studies, though human data is limited. EPA designates it as a probable human carcinogen, and the IARC classifies inorganic lead as probably carcinogenic to humans (ATSDR, 2007b).

Magnesium (Mg). Magnesium is an essential nutrient derived from meat, seafood, grains, nuts, and green vegetables in the diet, and can also be found in hard drinking water (Hartwig, 2001; Klaassen, 2013). Ingested Mg is absorbed mostly in the small intestine though the amount is dependent on the homeostatic need (e.g., 75% of Mg may be absorbed in a person with a low Mg diet, and only 25% absorbed if a person eats a rich Mg diet) and potentially by the presence of other dietary items such as fiber or calcium (Brink and Beynen, 1992; Hartwig, 2001), as both inhibit Mg absorption (Klaassen, 2013). However, Fine et al. (1991) did not find dietary concentrations of Ca to interfere

with appropriate Mg absorption.

Once in circulation Mg primarily distributes to bone and muscle but will also deposit to other organ systems with < 1% remaining in extracellular fluid (Klaassen, 2013). Magnesium cycles through the kidneys where 95% is resorbed in the glomeruli and remains in circulation (Klaassen, 2013). It is a necessary cofactor for genomic stability, DNA replication and repair, protein synthesis, and other fundamental cellular processes (Anastassopoulou and Theophanides, 2002; Hartwig, 2001; Herroeder et al., 2011).

Magnesium does not typically cause toxicity in humans, though numerous adverse health outcomes are associated with Mg deficiency. However, it can be toxic to individuals with decreased renal function who simultaneously ingest increased volumes of magnesium, such as in antacids or other magnesium containing pharmaceuticals (Klaassen, 2013; Smilkstein et al, 1988). Toxicity can manifest as lethargy, nausea, increased thirst, bradycardia, hypotension, and even death if serum concentrations increase to > 15 mEq/L (Goldfrank, 2011; Herroeder et al., 2011). Inhaled magnesium oxide has been associated with metal fume fever in industrial workers (Klaassen, 2013), and with increases in respiratory tract tumors when used as a carrier molecule for benzo(a)pyrene intratracheal instillation in hamsters (Stenback et al., 1975).

Manganese (Mn). Manganese is naturally occurring in the earth's crust and is incorporated into the atmosphere at an average concentration of $0.02 \mu\text{g}/\text{m}^3$ across the United States; however, ambient levels can measure from 0.22 - $0.3 \mu\text{g}/\text{m}^3$ near industries that mine or work with manganese compounds (ATSDR, 2012b). Mn exists in the food

and water supply, and can therefore be acquired through diet. It can readily be absorbed from fruits, vegetables, legumes, grains, nuts, and tea (Goldfrank, 2011; Klaassen, 2013). It is an essential nutrient and requires a daily intake of at least 2.3 mg for men and 1.8mg for women, though the average is 0.7-10.9 mg/day (ATSDR, 2012b; FNB/IOM, 2001). It is utilized in the synthesis of hexokinase, glutamine synthase, xanthine oxidase, and superoxide dismutase (Aschner and Aschner, 2005; Barceloux, 1999a). A MRL of 0.3 $\mu\text{g}/\text{m}^3$ has been established for manganese (Roels et al., 1992).

Inhalation exposure to manganese primarily occurs in mines, smelters, or other occupational settings that involve welding, making steel and iron alloys, batteries, glass, electrical coils, and producing potassium permanganate (KMnO_4) (Klaassen, 2013).

Dietary exposure is managed by regulation of gut absorption and hepatobiliary excretion (Anderson et al., 1999; Aschner and Aschner, 2005; Aschner et al., 2005; Roth, 2006); however, several animal studies have observed that manganese inhaled through the nose can be transferred across olfactory or trigeminal presynaptic nerves that essentially deliver the metal directly to the brain (Brenneman et al. 2000; Dorman et al. 2001, 2002; Elder et al. 2006; Fechter et al. 2002; Henriksson et al. 1999; Lewis et al. 2005; Normandin et al. 2004; Thompson et al. 2011; Tjälve and Henriksson 1999; Tjälve et al. 1996; Vitarella et al. 2000). Manganese and iron cations can both utilize divalent metal transporter 1 (DMT1) which may be the major route by which it crosses the blood brain barrier from circulation (Aschner, 2006; Crossgrove and Zheng, 2004; Gunshin, 1997; Thomson, 1971). Because of the competition between Fe^{2+} and Mn^{2+} , increased Mn absorption occurs in the presence of iron-deficiency anemia with dietary absorption

effectively doubling in those anemic individuals (Barceloux, 1999a, Finley, 1999; Mena, 1969). Mn also exhibits an inverse relationship with Ca^{2+} levels and gastrointestinal absorption (Barceloux, 1999a; Freeland-Graves, 1991).

Roughly five times greater amounts of Mn are found in erythrocytes than in plasma (Schroeder and Nason, 1971), in the form of metmanganoglobin (Goldfrank, 2011; Moffat and Loe, 1976); however, Mn will bind serum proteins in the liquid fraction of blood (β -globulin, γ -globulin, transmanganin, albumin, and transferrin) (Aschner and Gannon, 1994; Barceloux, 1999a; Klaassen, 2013). Deposition of Mn in tissues occur at highest concentrations in the liver, pancreas, kidneys, and pituitary gland, all tissues with high mitochondria stores (Barceloux, 1999a; Klaassen, 2013; Sumino et al., 1975). Mn can cross the placenta though accumulation in the fetus does not seem to occur (Wilson, 1991). It is found in breast milk and infant formulas, and absorption from breast milk is ten times greater than that of formula (Stastny et al., 1984; Davidsson et al., 1989).

Effects of manganese exposure via inhalation manifest in the central nervous system. High dose exposures, $>1\text{-}5\text{ mg/m}^3$, over relatively long periods of time in occupational settings cause a progressive neurological disorder referred to as “manganism” or “manganese madness.” Initial symptoms include delayed reaction time, reduced hand-eye coordination, headache, insomnia, muscle cramping, memory loss, and changes in behavior and emotional stability (ATSDR, 2012b; Guilarte, 2010; Klaassen, 2013), but as the disease progresses patients develop a Parkinson’s like disease due to damage of dopaminergic neurons (ATSDR, 2012b; Klaassen, 2013). At this stage individuals can exhibit dystonia, hypokinesia, tumors, difficulty with speech, lowered

libido, and a “cock-walk” gait in their movement (Aschner et al., 2005; ATSDR, 2012b; Crossgrove and Zheng, 2004; Klaassen, 2013). Though “manganism” and “manganese madness” are associated with overt exposure to Mn, symptoms of neurotoxicity have been experienced in workers exposed to $< 1 \text{ mg/m}^3$ (Klaassen, 2013; Rodier, 1955).

Pulmonary effects can also be seen in workers exposed to ambient manganese, specifically manganese oxides MnO_2 and Mn_3O_4 , however since Mn in the air is accompanied by particulate matter, it cannot be ruled that Mn is the causal fraction (ATSDR, 2012b). There have also been some incidences of altered cardiovascular functions in humans and animals with oral toxicity of Mn, but cardiovascular effects have not been noted in scenarios of manganese inhalation (ATSDR, 2012b; Jiang and Zheng, 2005).

There is no evidence that manganese is carcinogenic. It has been published as not classifiable as to human carcinogenicity in the US-EPA’s IRIS (<http://www.epa.gov/iris/subst/0373.htm>, accessed March 10, 2015).

Strontium (Sr). Strontium is a naturally occurring element on the earth’s crust and primarily exists compounded with other elements. It does have a stable form of Sr^{2+} along with radioisotopes ^{84}Sr , ^{86}Sr , ^{87}Sr , ^{88}Sr , and ^{90}Sr . The radioisotopes all have the same biological behavior and therefore affect the body similarly regardless of isotope (ATSDR, 2004b). Sr can be found in soil and water, and therefore in the food supply, which is the primary route of exposure (ATSDR, 2004b). It can be obtained from fish and livestock, and dairy products, leafy vegetables, and grains. The average concentration of Sr in air levels across the United States is 20ng/m^3 though higher concentrations will

accumulate in areas with coal-burning plants that have smoke stack emissions (ATSDR, 2004b).

Absorption of Sr is dependent on the solubility. If the compound is water soluble, it will readily be absorbed across the lungs and into circulation. Non-water soluble Sr containing compounds will stay in the lungs and can cause localized pulmonary effects such as pneumonitis and fibrosis as exhibited in beagles by Snipes et al. (1979) (ATSDR, 2004b; Snipes et al., 1979). Only small portions of Sr are absorbed through the intestines in adults, but children are more susceptible to gut absorption and are therefore more likely to experience adverse health affect from ingesting higher amounts of Sr (ATSDR, 2004b). Strontium and calcium are closely related in their behavior and can sometimes be interchanged in physiological processes. Therefore, a large percentage of strontium will accumulate in bones and teeth. It can bind bone surfaces in adults, but may be used in the genesis of bone formation in children who are actively growing (ATSDR, 2004b). Because it accumulates in bone, strontium can take a long time to be eliminated from the body and will slowly be released into circulation as bone cells participate in resorption (ATSDR, 2004b). Sr that is absorbed and not sequestered in bone is eliminated through urine and sweat, and feces (ATSDR, 2004b). Diets rich in calcium and protein will inhibit excess strontium absorption from the gut (ATSDR, 2004b). Radioisotopes of strontium present physiological damage associated with radiation exposure which can cause bone and soft tissue injury over time. ⁸⁹Sr is used as a chemotherapeutic agent to eliminate cancerous cells in the bone marrow as it targets rapidly dividing cells. This will interrupt hematopoiesis inducing pancytopenia in peripheral circulation and the marrow.

Ironically, excessive oral exposure to radioactive strontium in the Techa River population, an area in Eastern Europe, led to a higher incidence of leukemias and non-leukemic hematopoietic malignancies (Krestinina et al., 2013).

Limited data exist regarding toxicity of strontium in humans. Some cases of rickets have been recorded in Turkish children exposed to high levels of Sr in their diet as well as some incidence of osteomalacia development in hemodialysis patients exposed to Sr in their dialysis water. Radiostrontium is classified as carcinogenic to humans by the IARC, though cases of carcinogenesis have only been established with oral exposure (ATSDR, 2004b).

Uranium (U). Uranium is a radioactive element that occurs in rock, soil, and air (ATSDR, 2013). It exists as ^{234}U , ^{235}U , and ^{238}U in the environment and has chemical forms, U^{4+} and U^{6+} , that will compound other elements (Keith et al., 2007; Klaassen, 2013). Both chemical and radioactive forms of U behave the same under physiological conditions, as does depleted uranium even though it contains 40% less radioactivity than ^{235}U (ATSDR, 2013; Craft et al., 2004). Because it is ubiquitous, exposure through air and the food and water supply is continuous. Daily intake from food is 0.9-1.5 μg and 0.001-0.01 μg from sources such as scallops, onion, potato, parsley, table salt, beef and poultry (ATSDR, 2013; Keith et al., 2007). Food contamination of uranium most likely is caused by residual dirt as bioconcentration of the element is not observed in higher trophic levels (Keith et al., 2007). Uranium exposure from water sources vary throughout the country. It can make it into the water supply through dissolution or erosion of rock

and soil, nuclear energy waste, mining, and use of uranium containing fertilizers (Keith et al., 2007; WHO, 2012).

The uranyl ion is the hexavalent form of uranium bound to an oxygen creating UO^{2+} . This ion will compound with other chemicals to become water soluble. It is the form most often found in aquatic life, birds, plants, and human body fluids (Keith, 2007; Klaassen, 2013; Sheppard et al., 2005).

Absorption of uranium is low and depends on the bioavailability of the compound. Across the gut ranges from 0.1-6% and the lungs depend on the size and solubility of the particle. It was estimated that 1-5% of uranium penetrated into the alveolar region of the lung in individuals exposed to high levels of uranium in their workplace air, indicating that the burden to the lungs is quite low (Keith et al., 2007). Insoluble compounds may stay in pulmonary tissue and cause localized effects (ATSDR, 2013; Jiang and Aschner, 2006; Keith et al., 2007).

Upon absorption uranium will bind citrate, bicarbonate, or plasma proteins in the bloodstream, distribute to tissues throughout the body, and preferentially deposit to bone, liver, and kidney (ADSTR, 2013; Keith et al., 2007; Klaassen, 2013). Two thirds of uranium will be excreted in the urine after 24 hours, however it can remain in bone for roughly 1.5 years (ATSDR, 2013; Craft et al., 2004). Uranium has been shown to cross the placenta in animal studies after parenteral administration and it has also been observed in human breast milk (ATSDR, 2013; Keith et al., 2007; Wappelhorst et al., 2002).

The primary mechanism of toxicity for uranium is the kidney, more specifically the proximal convoluted tubule and the glomerulus, but this level of damage requires acute, high-level exposure (Lu and Zhao, 1990; Pavlakis et al., 1996). Long term occupational exposures of lower concentrations do not cause increased incidence of urogenital or renal diseases, or other significant signs of kidney damage (ATSDR, 2013; Keith et al., 2007).

Limited immunotoxicological data exist regarding uranium exposure. An *in vitro* study by Wan et al. (2006) observed increased apoptosis and necrosis in mouse peritoneal macrophages and splenic CD4+ T-cells after 24 hour exposure to $\geq 100 \mu\text{M}$ and $\geq 500 \mu\text{M}$ of depleted uranium, respectively. However, epidemiological data of workers from uranium mines and other occupational settings where uranium exists in the atmosphere do not show increased incidence of death or disease from effects to the immune system (Checkoway et al., 1988; Keane and Polednak, 1983; NIOSH, 1987; Polednak and Frome, 1981).

Because uranium deposits in bone, there is potential for chronic exposure to interfere with proper bone growth, the development of osteoporosis, and higher risk of bone cancer (Jiang and Aschner, 2006; Klaassen, 2013). Much of the literature regarding uranium does not consider it a neurotoxin, however Jiang and Aschner (2006) argue this needs to be readdressed as there is a growing body of evidence that uranium can impact the nervous system of animals (Briner and Murray, 2005; Barber et al., 2005; Lemercier et al, 2003; Pellmar et al., 1999a,b).

Uranium is not considered carcinogenic by the NTP, IARC, or the EPA. There are

data to showing increased incidence of lung cancer in miners, however it is thought that the cancer is most likely caused by subsequent exposure to radon, diesel exhaust, or silica than by the uranium itself (ATSDR, 2013; Keith et al., 2007).

Vanadium (V). Vanadium is (V) is an element that is naturally occurring and widely distributed on the earth's crust with an average concentration of 100mg/kg (ATSDR, 2012c). It has a number of valence states with V^{+4} and V^{+5} being of toxicological relevance. The most common route of exposure is via ingestion from daily food and water intake, approximately 0.002 mg per day, but inhalation of vanadium is also a possible route of exposure. Human and animal research has shown that the primary targets of toxicity resulting from oral exposure are the hematological system, the developing organism and the gastrointestinal tract, and the respiratory system after inhalation exposure (ATSDR, 2012c).

Intratracheal studies in rodents suggest high rates of absorption through the lungs followed by rapid pulmonary clearance (NTP, 2002), though the amount absorbed across the lung is not known in humans. Once in systemic circulation, vanadium can reversibly bind transferrin and can also be taken up by erythrocytes. Schroeder et al. (1963) observed V in lung and intestinal tissue from humans post mortem, in 52% and 16% of cases, respectively. In rats, radiolabeled vanadium was detectible in all organs with the exception of the brain, 15 minutes after 0.36 mg/kg dose via acute intratracheal administration. Highest concentrations in this rodent model were found in lungs, followed by the heart and kidneys (Oberg et al., 1978).

Vanadium circulates as V^{+5} in blood, but is typically found as V^{+4} in tissues. Its redox cycle is dependent on the availability of reductive and oxidative compounds (Byczkowski and Kulkarni, 1998). The excretion of vanadium observed by Oberg et al. (1978) was found primarily in the urine of rats and to a lesser extent, the feces.

Acute inhalation studies in humans have only found vanadium to cause a persistent cough in a small number of subjects when exposed to vanadium pentoxide dust for 8 hours (Zenz and Berg, 1976), or when acutely exposed to 0.6 mg V/m^3 (ATSDR, 2012c). Studies evaluating health effects of occupational inhalation exposure conclude that the respiratory system is the primary target organ. Inhalation studies in mice and rats found that both acute exposure (0.56 mg V/m^3) and chronic exposure (0.28 V/m^3) induced lung lesions, fibrosis, and inflammation (ATSDR, 2012c). Additional symptoms from exposures longer than two days included hyperplasia in nasal goblet cells and the larynx. Vanadium pentoxide concentrations $\geq 4.5 \text{ mg/m}^3$ trigger respiratory distress in laboratory animals (ATSDR, 2012c).

There have been no studies assessing carcinogenicity in humans; however, there is some evidence of lung carcinomas developing in chronically exposed rodents. Vanadium pentoxide has been classified as a 2B carcinogen by the IARC; however the US EPA and DHHS have not given it any classification for carcinogenicity (ATSDR, 2012c Chronic inhalation RfC is currently in draft form with the US EPA and confirmed at this time (US EPA IRIS 2011; <http://www.epa.gov/iris/subst/0125.htm> accessed March 3, 2015).

Consumption of ≥ 14 mg vanadium in humans results in nausea, cramps and diarrhea, although most studies found that people developed a tolerance to vanadium after a week or two. Both oral and inhalation studies of exposure to vanadium compounds in rats found a variety of hematological effects, such as increases in reticulocytes and decreases in hemoglobin (ATSDR, 2012c).

Studies of the developmental effects of vanadium exposure have been conducted in laboratory animals. Pups born to mothers exposed to ≥ 2.1 mg V/kg/day showed reduced growth; higher exposures caused weight loss in the mothers, and a variety of malformations or death in the pups (ATSDR, 2012c).

Zinc (Zn). Like many other metals zinc is naturally occurring in the earth's crust and is therefore ubiquitous, however it is typically found as zinc oxide or other compound forms and does not occur as elemental zinc in nature (ATSDR, 2005; Sandstead and Au, 2007). It can be found in the food and water supply and is acquired into the diet through grains, legumes, nuts, seafood, meat, and dairy products (Barceloux, 1999b). The average daily intake of zinc from food in humans is 5.2-16.2 mg and diet is the primary route of exposure for the general population (ATSDR, 2005). Zinc is a necessary nutrient as it plays various critical roles in human physiology. A deficiency of Zn can cause spontaneous abortions, premature labor, and developmental delays in unborn children, and can also cause abnormal neuropsychological function, fatigue, dermatitis, poor wound healing, hypogonadism, and lowered immune function (ATSDR, 2005; Cai et al., 2005; Cousins et al., 2006; Prasad, 2004; Sandstead and Au, 2007). The recommended dietary allowance for men is 11 mg/day, 8 mg/day in women, 12 mg/day

for nursing or pregnant women, 3 mg in children younger than three years, and 8 mg for children from 4-13 years of age (ATSDR, 2005; Yates et al., 2001).

Inhalation is a concerning exposure route for those working with zinc in occupations such as mining, smelting, welding, and galvanizing. Acute inhalation exposure to Zn, generally in the range of 77-660 mg/m³, can cause “metal fume fever,” a condition characterized by fever, nausea, chills, malaise, dyspnea, cough, chest pain, reduced lung volume, and leukocytosis (ATSDR, 2005; Goldfrank, 2011; Klaassen, 2013). Acute exposures to lower concentrations of airborne zinc and zinc oxide, 8-12 mg/m³ and 14-45 mg/m³ respectively, do not induce metal fume fever (ATSDR, 2005). One account of mortality caused by inhaled zinc chloride occurred during World War II. Seventy individuals trapped in a tunnel were exposed to 33,000 mg/m³ ambient zinc from a smoke bomb. Ten of them died within four days as a result of this exposure (Evans, 1945). Other accounts of morbidity associated with exposure to zinc chloride from smoke bombs include lacrimation, rhinitis, dyspnea, stridor, retrosternal chest pain, upper respiratory tract inflammation, acute lung injury (ALI), and acute respiratory distress syndrome (ARDS) (Hjortso et al., 1988; Homma et al., 1992; Matarese and Matthews, 1986). Nasal inhalation of zinc sulfate in animals and zinc gluconate in humans lead to both transient and permanent anosmia (Jafek et al., 2004; McBride et al., 2003).

Absorption of zinc in the gut is regulated homeostatically and influenced by the presence of calcium (Wood and Zheng, 1997). Absorption of inhaled zinc compounds in humans and animals is not well understood and data is limited. Levels of zinc in workers exposed to zinc oxide were found to be elevated in blood and urine samples (Hamdi,

1969), and Gordon et al. (1992) observed lung retention values of 19.8% in guinea pigs, 11.5% in rats, and 4.7% in rabbits after nose-only inhalation exposure to 3.5-9.1 mg/m³ of 0.17 µm particles of zinc oxide.

Zinc is found in leukocytes and platelets, bound to albumin and α₂-macroglobulin in plasma, and also bound to carbonic anhydrase in erythrocytes (Bentley and Grubb, 1991; Giroux et al., 1976; Ohno et al., 1985; Wastney et al., 1986). Predominant tissues of zinc accumulation are in muscle, bone, liver, GI tract, kidneys, skin, lungs, brain, heart, and pancreas (Bentley and Grubb, 1991; Drinker and Drinker, 1928; He et al., 1991; Llobet et al., 1988; Wastney et al., 1986).

Increased zinc absorption will induce metallothionein to counter zinc overload, however a number of metals will bind metallothionein with a greater affinity. Greater zinc absorption can therefore lead to increased copper elimination which can subsequently lead to anemia (Fiske et al., 1994; Yates et al., 2001). Renal excretion accounts for some of zinc's elimination, but it is primarily excreted in the feces (Hamdi, 1969; Milne et al., 1983; Sandstead and Au, 1997).

Proper amounts of zinc are necessary for normal immune function, however some exposures have been observed to negatively impact the immune system. Correlations between acute exposure to 77-153 mg/m³ zinc oxide in welders and activated T-cells and subpopulations after 20 hours of exposure, as well as increases of neutrophils, macrophages, and lymphocytes in bronchoalveolar lavage fluid (Blanc et al., 1991). Stacey (1986) observed inhibition of antibody dependent T-cell mediated cytotoxicity and spontaneous cell mediated cytotoxicity at concentrations of 100 and 250 µM in vitro.

A study of eleven healthy adult men who ingested 150 mg of zinc sulfate twice a day for six weeks showed significant increases in plasma zinc levels accompanied by reduced lymphocyte stimulation to phytohemagglutinin. Numbers of lymphocytes and lymphocyte subpopulations were normal, but the chemotactic response of neutrophils was decreased as was phagocytosis (Chandra, 1984).

Studies of both oral and inhalation exposure in humans and animals have failed to show increase in cancer incidence for chronic exposure. Therefore, the EPA has classified zinc and zinc compounds as a group D carcinogen, not classifiable as to human carcinogenicity (ATSDR, 2005).

Particulate Matter

Epidemiologic studies have illustrated a relationship between urban particulate matter and human health effects (Boezen et al., 1998; Dockery et al., 1994; Schwartz et al., 1993), but an information gap still exists regarding the hazard of exposure to inhaled geogenic or geanthropogenic particulate matter. Dust is complex mixture of organic matter and minerals that can vary in chemical composition and size. Particulate matter can be “ultrafine” ($<0.1 \mu\text{M}$), the $2.5 \mu\text{M}$ fraction referred to as “fine,” and PM₁₀ fraction as “coarse,” though each fraction is associated with varying degrees of adverse health effects (Englert, 2004).

Size, solubility, and specific surface area are critical factors in deposition and absorption of particulate matter in the lungs (Figure 1). Particles 5-10 μM in diameter are typically retained in the upper respiratory tract, or nasopharynx region, and cleared by

sneezing, wiping, or blowing the nose. Particles that are soluble may dissolve in mucous lining of nasal passages and may be absorbed across epithelial cells into the blood stream

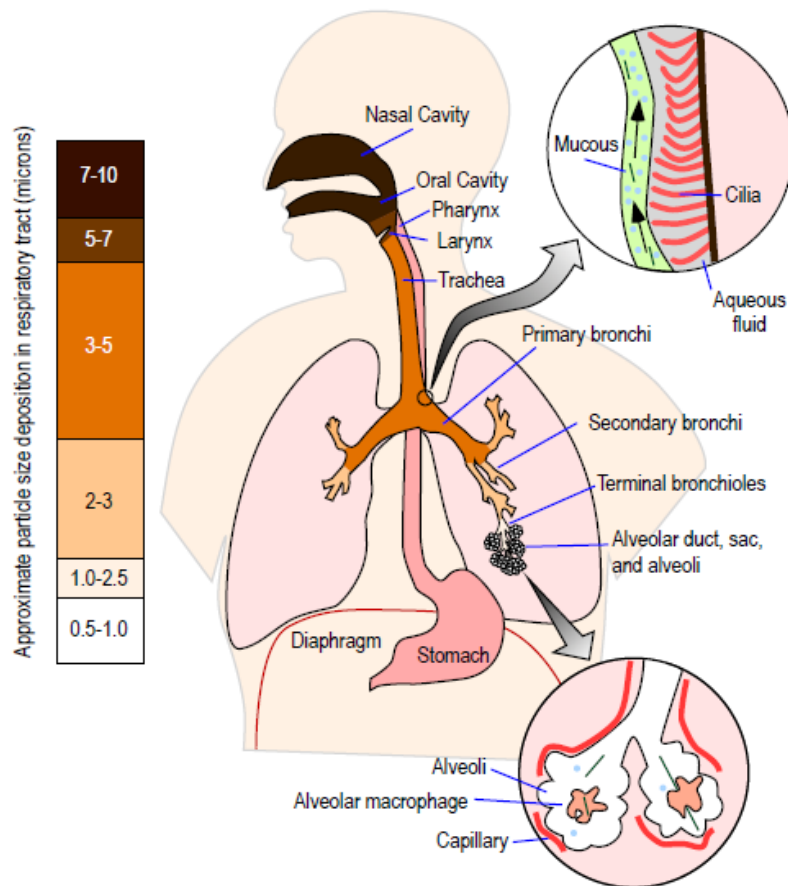


Figure 1. Illustration of the respiratory system. Legend indicates particulate matter size and color code that corresponds with deposition throughout the pulmonary system. Image from (Morman and Plumlee, 2013) modified from (Newman et al. 2001).

(Leikauf, 2013). Finer particles will deposit in the tracheobronchiolar regions of the respiratory tract. This compartment contains the larynx and terminal bronchioles that consist of ciliated cells and a functional mucous lining. This mucociliary system creates a mechanism that can carry particles from the lower airways up to the larynx where they

can be swallowed or cleared into the lymphatic system. Coughing could also propel particulate matter from lower recesses of the lungs to the upper respiratory tract for clearance (Leikauf, 2013; Morman and Plumlee, 2013). Ultrafine particles are most likely to deposit in the alveoli where they can absorb into systemic circulation or are phagocytized by resident macrophages and cleared through the lymphatics system (Warheit, 1988).

The average daily amount of breaths taken by adults is between 10,000-20,000 L depending on activity level. If the atmosphere contains a low particle burden of $10 \mu\text{g}/\text{m}^3$ the daily mass of inhaled particles is roughly $100 \mu\text{g}$ (Gilmour and Koren, 2000). If breaths are taken through the nose, the nasal passage acts as a filter and excludes or eliminates larger particles. However, if breathing occurs through the mouth, less filtration takes place, and more and larger particulate matter can access the respiratory tract (Morman and Plumlee, 2013). Labored breathing from exercise or physical exertion can increase ventilation rate and breathing through the mouth which potentially increases deposition of respirable particles (Schultz et al, 2000).

Dust from the NDRA poses risk to those exposed because it contains particulate matter smaller than ten microns, which when inhaled can penetrate deeper recesses of the lungs (Morman and Plumlee, 2013), and because it also contains mineral trace metals within its composition or has these metals adsorbed to the dust particle. A growing body of evidence shows that exposure to particulate matter in air pollution can be associated with a number of health effects such as increased risk of stroke or death, or particularly by exacerbating symptoms of existing cardiovascular and respiratory illness (Beelen et

al., 2008; Dockery et al., 1993; Lee, 2006; Pope et al., 1995; Ruckerl et al., 2011; Samet et al., 2000; Samoli et al., 2008). It often contains sulfates, nitrates, ammonia, and sodium chloride in addition to organic air pollutants (WHO, 2014). Because air pollution is so ubiquitous, the World Health Organization (WHO) has stated that PM affects more people than any other pollutant and estimates that 3.7 million worldwide premature deaths were attributed to ambient air pollution in urban and rural areas (WHO, 2014). Most information regarding toxicity from PM exposure comes from occupational or urban settings. In the great smog of December 1952 in London PM₁₀ concentrations measured approximately 3,000 $\mu\text{g}/\text{m}^3$. An increase of hospital admissions, cases of pneumonia, and mortality were observed during December and into February 1953. Pope et al. (2002) observed increased risk of cardiopulmonary and lung cancer mortality with increased exposure to PM_{2.5} by monitoring long-term exposure to 1.2 million people living in metropolitan areas throughout the United States.

A study of electric furnace steel plant workers exposed to ambient PM investigated levels of DNA methylation of Alu, LINE1, and iNOS in serum samples. An initial serum sample was collected from workers who had two days off work to serve as a baseline sample. Samples were collected again after a 3-day working period. PM₁₀ levels from the work environment correlated to levels of DNA demethylation of Alu and LINE1, though there was not much variation between the methylation observed in the baseline sample and the samples collected after three days of work. This suggests that PM has a sustained effect on methylation despite the two-day interim. Demethylation in

the iNOS promoter was also observed after three consecutive days of work (Tarantini et al., 2009).

Not only is particulate matter exposure correlated with increased pathogenesis, but dust containing inorganic metals causes health effects independent from those caused by exposure to the mass of the particle. Ghio (2004) reviewed air quality data from Utah Valley during August 1986 to September 1987 when a local steel mill was closed due to a labor dispute. The mill was known to contribute more than 80% of the industrial related PM in the valley during operation and therefore provided a unique opportunity to observe immunotoxicological endpoints in healthy individuals with and without influence of the steel mill. Operation of the mill contributed increased concentrations of iron, copper, zinc, lead, nickel, and vanadium into the valley air. The data showed increased oxidative stress based on thiobarbituric acid (TBA) reactive products of deoxyribose, increased IL-6 and IL-8, increased percentage of PMNs in BAL fluid, and inflammation of the lungs in 1986 and 1988 during the time the mill was in operation compared to 1987 while it was closed. Particularly telling were the lung inflammation results as 500 µg aqueous extracts of particulate matter were administered directly into the lungs of health volunteers and BAL fluid collected 24 hours later (Ghio, 2004). The disparate results between control and treatment groups in this study and observations from Franklin et al., (2008) regarding increased rates of mortality related to aluminum, arsenic, sulfate, silicon, and nickel containing 2.5 micron particulate matter provide insight into the need for assessing effects of the metals comprising ambient air particles and not just the effects from the particulate matter itself.

Although these data are compelling for the investigation into particulate matter during this assessment, they provide little insight into anticipated health risks posed by geogenic matter in a recreational setting.

NDRA Toxicological Study Design

B6C3F1 female mice were exposed to doses of 0.01, 0.1, 1, 10, and 100 mg geological sample/kg/day in phosphate-buffered saline once a week over a four week period. The dose was administered via OA allowing direct delivery to the lung which mimics inhalation exposure. This technique permits administration of a quantifiable dose and avoids exposure to the skin and pelt like other inhalation exposure techniques. Also, as the median particle diameter given in the dose response trials was less than 5 μ m in diameter, it represents fine particles that can travel deeper into the lung and provide a greater estimation of health risk. To understand if the health effect observed in animals was due to the metals on the particle, or from the particles themselves, a particulate matter experiment was designed using additional mice that were exposed to levels of titanium dioxide (TiO₂) comparable to the dust exposure. TiO₂ is a biologically inert substance that acts as a control for the dust particles to determine whether reactions to the dust particles are independent of metal interactions. Five days prior to the end of the dosing period, animals were given intraperitoneal injections of sheep red blood cells, T-cell-dependent antigens used to stimulate the IgM antibody response. At the end of the dosing period, peripheral blood was collected via retro-orbital bleed, and the animals were euthanized by CO₂ asphyxiation. Lung, thymus, kidney, brain, and heart weights

were measured; lungs and brains were preserved in formalin for histopathology and neurotoxicological examination. Organs of the immune system were immediately processed for immunotoxicological assessments. Spleen cells were used in the PFC assay which measured the IgM response to injected sheep red blood cells.

Data from these results are represented as mean ($\pm$ standard error of the mean). A one-way ANOVA was run on IgM antibody production, body and organ weights, clinical chemistries and hematological differences by dose. When ANOVA indicated a statistically significant dose effect within operation, individual post hoc comparisons using a t-test were run as appropriate. The effective dose for 50% of the mouse population (ED_{50}), NOAELs, and LOAELs were established based on these data.

Titanium Dioxide

TiO₂ was used as a particle positive control. It is small particulate matter roughly 45 microns in size and is considered neutral as it does not have any metals adsorbed to its surface. This particle control was administered to mice by the same delivery method and protocol as the NDRA dust samples. No significant change in IgM production from the 0 mg/kg mice to the dosed TiO₂ mice groups demonstrates that the decreased immunoglobulin production observed in our mice groups exposed to NDRA dust can be contributed to the metal make-up of the dust and not the particulate matter itself.

Clinical Chemistry & Hematology

For hematology and clinical chemistry endpoints, blood from anesthetized animals was collected into a microtainer tube containing EDTA, which kept the blood from coagulating. Once collected, samples were sent overnight to the Montana Veterinary Diagnostic Laboratory (MVDL) in Bozeman, MT, for hematology and clinical chemistry analysis. Hematology parameters included: white blood cells (WBC; $10^9/L$), red blood cells (RBC; $10^9/L$), hemoglobin (HGB; g/dl), hematocrit (HCT; %), mean corpuscular volume (MCV; fl), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; %), red cell distribution (RDW; %), platelet count ($10^9/L$), neutrophils (%), neutrophils ($10^9/L$), lymphocytes (%), lymphocytes ($10^9/L$), monocytes (%), monocytes ($10^9/L$), eosinophils (%), eosinophils ($10^9/L$). Clinical chemistry parameters included: creatine phosphokinase (CPK; IU/I), aspartate aminotransferase/serum glutamic oxaloacetic transaminase (AST/SGOT; IU/I), alanine aminotransferase/serum glutamic pyruvate transaminase (ALT/SGPT; IU/I), alkaline phosphatase (ALKP; IU/I), glucose (mg/dl), cholesterol (mg/dl), total protein (g/dl), albumin (g/dl), globulin (g/dl), phosphate (mg/dl), blood urea nitrogen (BUN; mg/dl), creatinine (mg/dl), and total bilirubin (mg/dl). Hematologies were run on all samples; however clinical chemistries performed were dependent on the volume of sample provided and were only run when sufficient volume was available. Hematology and clinical chemistry analyses were not performed in duplicate or triplicate as were other assays.

For determination of blood metal and metalloid concentrations, blood from anesthetized animals was collected into a microtainer tube containing heparin, which kept the blood from coagulating. All blood tubes were weighed before and after collection of blood to determine the weight of each blood sample. Once collected, samples were frozen at -80 °C and then shipped to the Laboratory Services Bureau of the Montana Department of Public Health and Human Services for analysis of metal and metalloid concentrations. Metals/metalloids included: arsenic, cadmium, chromium, lead, magnesium, manganese, molybdenum, nickel, strontium, vanadium, and zinc. Blood metal/metalloid concentrations were not performed in duplicate or triplicate as were other assays.

PFC Assay and Lymphocyte Populations

Toxicity can be characterized by overt or often subtle changes in critical functions of cells or organ systems. Characterizations of these effects both in human epidemiology studies and animal models are used to establish regulatory values by organizations such as the Environmental Protection Agency. While immunological endpoint alterations measure more subtle effects of toxicity (Keil et al., 2007, 2009; Luster et al., 1992; Peden-Adams et al., 2007a, b), the relationship between dose and response is only correlative. Elucidating a mode of action is necessary for establishing causality. The PFC assay is one of the gold standard functional assays for assessing immunotoxicity as it is a reliable measure of immunoglobulin production. Though measuring antibody suppression is a common immunotoxicological endpoint, to our knowledge no one has looked at the suppression of transcription factors by means of methylation as a potential mechanism to

explain decreases in antibody production. Our toxicological profile and risk assessment of the dust at NDRA observed alterations in immune function of B6C3F1 female mice that occurred in the absence of detectable changes in the weight or cellularity of secondary lymphoid organs. By the PFC assay, our preliminary data shows dose-responsively decreased IgM antibody production by splenic B-cells in our dose groups when compared to control mice, with our lowest observed adverse effect level measured in the 0.01 mg/kg dose group (Figures 2-3). By assessing sensitive immunotoxic endpoints at low dose exposure levels, we were able to detect subtle effects of altered immune function before the manifestation of overt toxicity. Identifying causality by determining a mode of action in addition to these findings would strengthen our assessment of risk for human exposure.

From another immunotoxicological endpoint assessment, we identified B-cell populations in secondary lymphoid tissue by measuring the surface protein marker B220 in each dose group, which when analyzed by flow cytometry, showed no significant changes from the control (Figures 6-9). Taken together, these findings lead us to believe that the decreased antibody production is due to an interruption in the plasma cell differentiation pathway. Thus, to elucidate a mode of action of the observed effects, in this proposal we intend to explore whether the major transcription factors of the plasma cell differentiation pathway have been suppressed via methylation of their promoter regions, reducing their proper function, and subsequently altering normal B-cell differentiation into effector plasma cells.

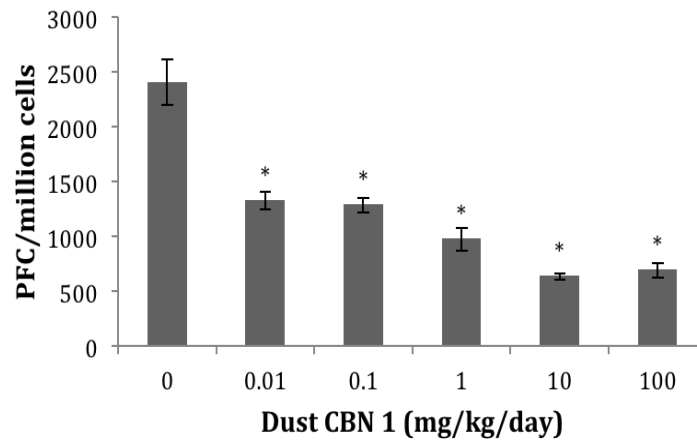


Figure 2. Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN1. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

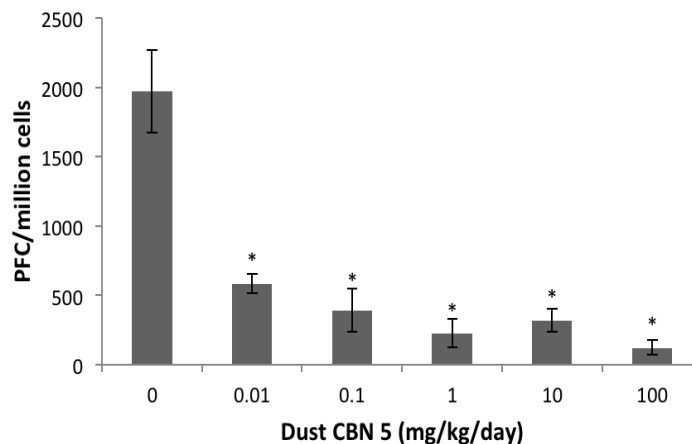


Figure 3. Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN5. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

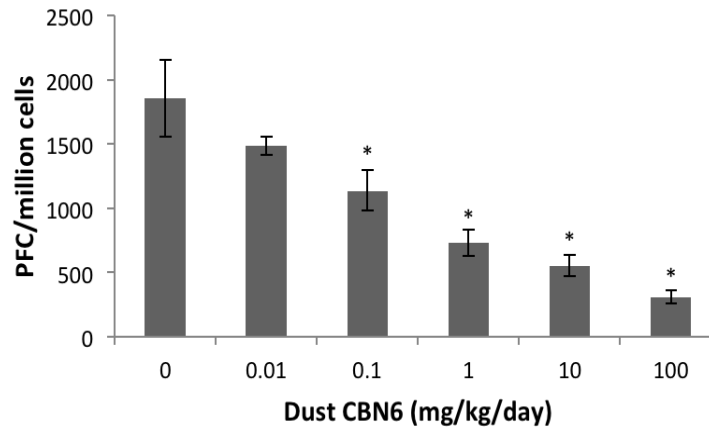


Figure 4. Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN6. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

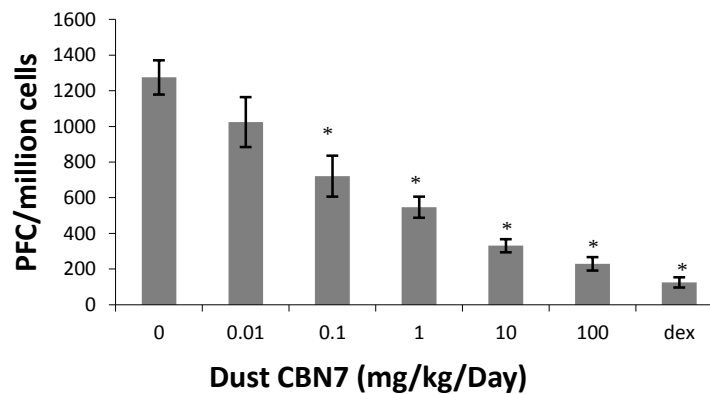


Figure 5. Sheep red blood cell-specific-IgM antibody production in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN7. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

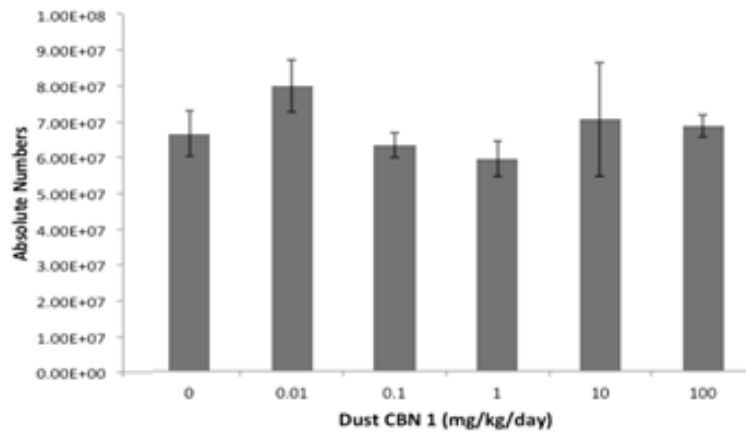


Figure 6. Splenic B-cell lymphocytes (B220) in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN1. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

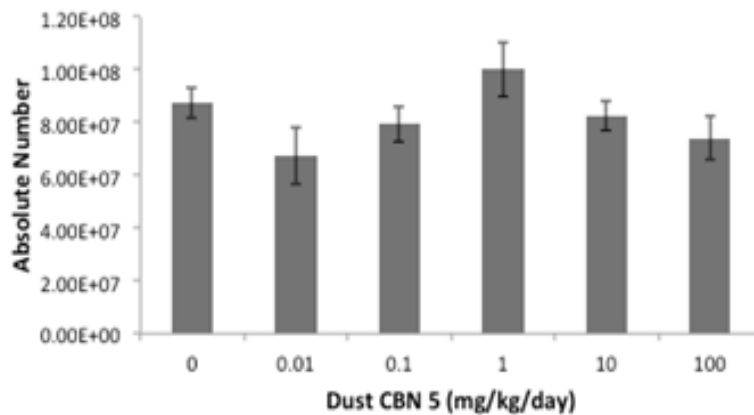


Figure 7. Splenic B-cell lymphocytes (B220) in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN5. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

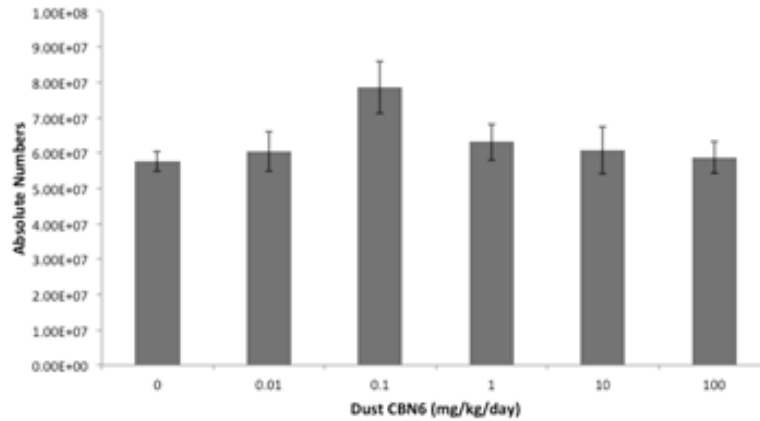


Figure 8. Splenic B-cell lymphocytes (B220) in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN6. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

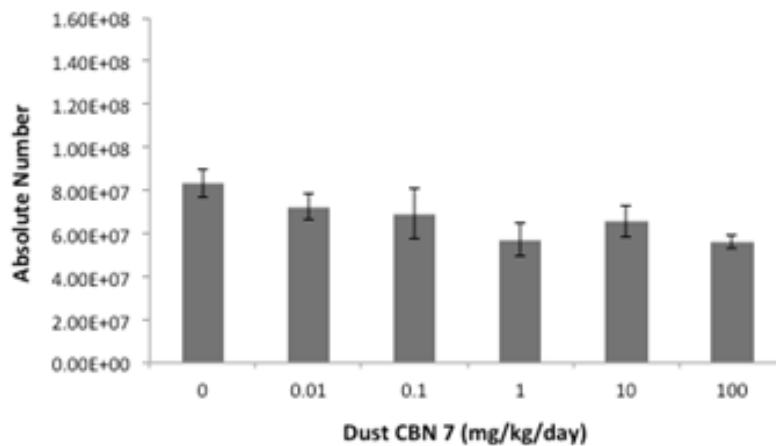


Figure 9. Splenic B-cell lymphocytes (B220) in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA combination unit CBN7. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of three trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$).

Epigenetic Marker Analysis

The concept of epigenetics was coined by C.H. Waddington in 1942 as a way to explain tissue differentiation during embryogenesis; however, its implications in disease did not emerge until the 1980's when loss of DNA methylation was observed to be associated with colorectal cancer (Feinberg and Vogelstein, 1983). The recognition of exogenous factors impacting disease led investigators to explore the relationship of DNA modifications and exposure to environmental agents on adult onset pathology. Exposure to environmental factors such as tobacco smoke, phthalates, BPA, alcohol, and of our particular interest, metals, have been associated with alterations of the epigenome (Aguilera et al., 2010; Arita and Costa, 2009; Breton et al., 2009; Dolinoy et al., 2007; Shukla et al., 2008; Sozzie et al., 1997; Yanagawa et al., 2002).

Many cellular processes are managed by epigenetic mechanisms including cell tissue function, maintenance and regulation of chromatin organization, cellular differentiation, and the regulation of gene expression. Environmental exposures can interfere with these mechanisms leading to altered gene expression and potential pathogenesis. Exploring epigenetic alterations induced by xenobiotics has important implications in toxicology as more connections between environmental exposures and disease pathogenesis are linked to epigenetic processes. Investigations of such processes may elucidate mechanisms of toxicity on a molecular level (Aguilera et al., 2010; Arita and Costa, 2009; Sulentic and Kaminski, 2011; Szyf, 2007). Our toxicological profile demonstrated a dose-responsive decrease in IgM antibody production in B6C3F1 female mice following sub-acute exposure via oropharyngeal aspiration to dust deposits from the

NDRA. Our objective is to understand a possible mode of action for this IgM immunosuppression. To evaluate this objective, we examined methylation patterns in key promoter regions of genes encoding transcription factors known to be critical in B-cell to plasma cell differentiation and antibody secretion.

DNA methylation is one of the epigenetic regulators of gene expression. Methylation of the cytosine in cytosine-guanine dinucleotides, found in gene promoter regions, provides conformational changes to the DNA and can sterically hinder transcription factor binding, thereby inhibiting gene expression (Ooi et al., 2009; Weaver et al., 2005). We identified key transcription factors and examined methylation patterning in transcription factor genes associated with B-cell to plasma cell differentiation.

Plasma Cell Differentiation Pathway

The plasma cell differentiation pathway is complex and our current understanding is incomplete. It is known that there is a delicate relationship between transcription factors actively maintaining B-cell phenotype and those that promote antibody secreting cell (ASC) differentiation (Shapiro-Shelef and Calame, 2005). The relationship between the two sets of transcription factors is antagonistic and they counterbalance the activation of this pathway (Shapiro-Shelef and Calame, 2005). PAX5, BLIMP-1 (*PRDM1*), and XBP-1 are critical to this regulation, among others.

PAX5 and Bcl6 are necessary for B-cell identity and prevent mature B-cells from differentiating while they are actively expressed. It is not until they are both down regulated that differentiation into an effector plasma cell can occur (Danbara et al., 2001;

Nutt et al., 2011). BLIMP-1, encoded by the *PRDM1* gene, acts as a repressor and its involvement is necessary for B-cells to fully and properly differentiate (Shapiro-Shelef et al., 2003; Turner et al., 1994); however, it is not required for the initiation of the pathway (Kallies et al., 2007). BLIMP-1 down regulates Bcl6 (Cimmino et al., 2008) and PAX5 (Lin et al., 2002) eliminating B-cell identity and allowing up regulation of XBP-1. XBP-1 was originally thought to be essential for effector cell differentiation and immunoglobulin secretion (Reimold et al., 2001); however, XBP-1 knock out mice were observed to have differentiated ASCs, but the cells were unable to secrete immunoglobulin (Nutt et al., 2011). This suggests the requirement of XBP-1 for antibody secretion (Li et al., 2013; Reimold et al., 2001; Schebesta et al., 2002; Underhill et al., 2003). We hypothesized that B6C3F1 female mice exposed to 100 mg/kg/dust will have altered methylation of the genes encoding transcription factors BLIMP-1, PAX5, and XBP-1 compared to control mice.

We anticipated decreased methylation, allowing increased transcription, in the promoter region of the gene encoding the PAX5 transcription factor as PAX5 is inhibitory towards downstream transcription factors that promote differentiation into plasma cells and Ig production. An increase in expression of this transcription factor would prevent normal B-cell differentiation. In correlation to the findings in our murine model, we expected a decrease of methylation in the promoter region of PAX5, causing increased expression of this gene.

For BLIMP-1 and XBP-1 we anticipated increased methylation, inhibiting transcription, in the promoter region of *PRDM1* and the *XPB-1* gene as proper function of

BLIMP-1 and XBP-1 is required for optimal cellular differentiation and antibody production in plasma cells. Decreased expression in these transcription factors would prevent normal B-cell differentiation and Ig secretion. In correlation to the findings in our murine model, we expected increased methylation in the promoter regions of *PRDM1* and *XBP-1*, causing decreased expression of these genes.

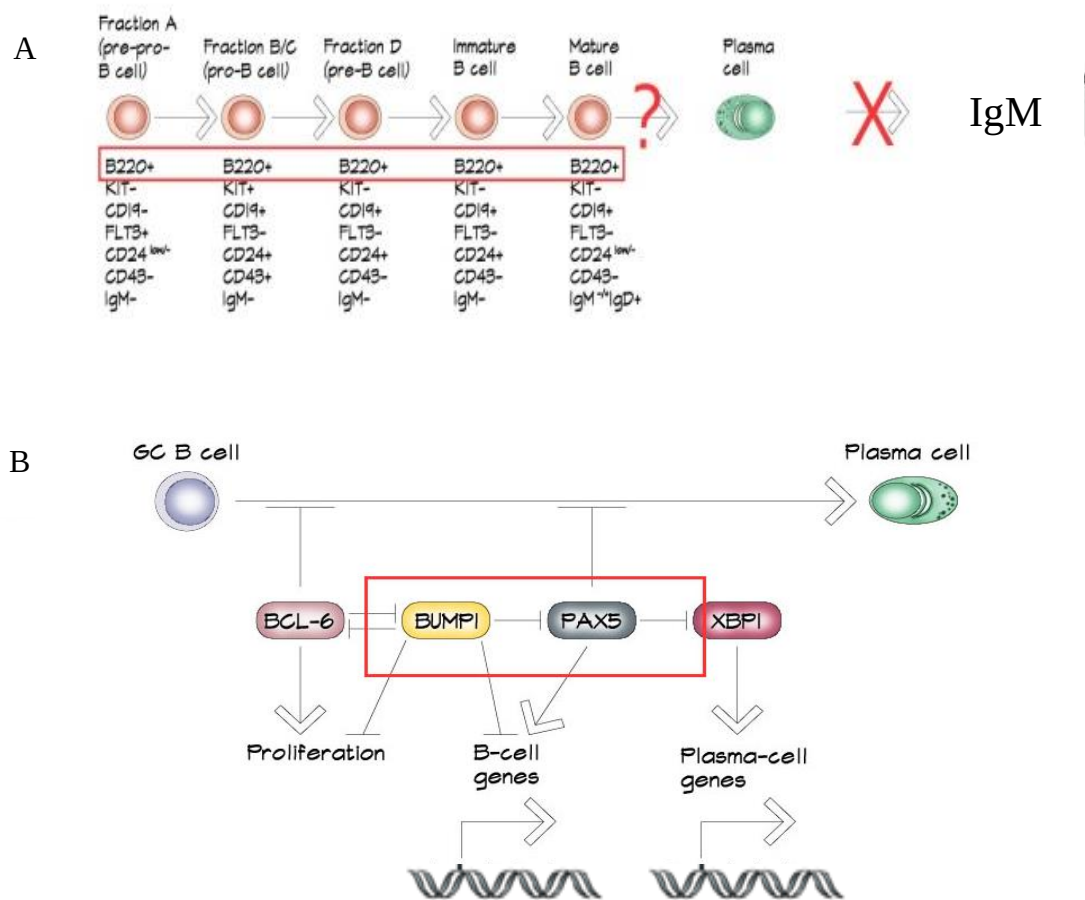


Figure 10. (A) Illustration of B220 marker presence on mouse B lymphocyte lineage adapted from (Nagasawa, 2006). (B) Illustration of the relationship of transcription factors in the B-cell to plasma cell differentiation from adapted from (Shaffer et al., 2002). Artwork by Zachary Taylor.

METHODOLOGY

Sample Collection and Preservation

B6C3F1 female mice were received at the animal facility and allowed to acclimate four to seven days before dosing. Upon arrival they were sorted such that six mice would be housed in each container. Two sets of mice, A and B, were exposed to doses of 0.01, 0.1, 1, 10, and 100 mg geological sample/kg/day, administered in a sterile phosphate-buffered saline vehicle, once a week over a four week period. Dose was administered via oropharyngeal aspiration (OA), a direct and quantifiable transmission to the lung that avoids exposure to the skin and pelt. OA does not directly compare to inhalation, however an estimate of murine inhalation can be extrapolated to human exposure (Driscoll et al., 2000). Median particle diameter given in the dose response trials was less than 5 μ m; this represents fine particles that can travel deeper into the lung and provide a greater estimation of health risk. Additional mice were exposed to comparable levels of TiO₂ allowing us to determine reactions to the dust particles independent of metal interactions. On Day 18 animals were given intraperitoneal (i.p.) injections of sheep red blood cells, T-cell-dependent antigens used to stimulate the IgM antibody response. At the end of the dosing period, peripheral blood was collected via retro-orbital bleed, and the animals were euthanized by CO₂ asphyxiation. Figure 11 depicts tissue collection for analysis. This protocol was approved by the UNLV IACUC and conducted at an AAALAC accredited facility.

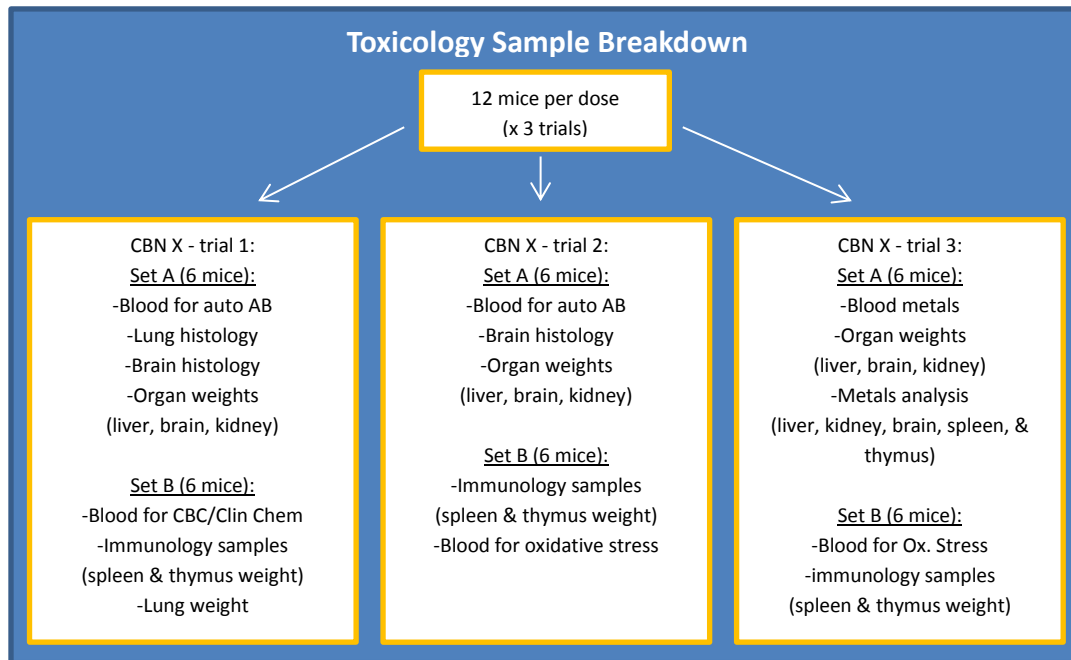


Figure 11. Toxicology sample collection breakdown. Set A each day = ANID #'s 1-6, 13-18, 25-30, 37-42, 49-54, 61-6. Set B each day = ANID #'s 7-12, 19-24, 31-36, 43-48, 55-60, 67-72. Six additional mice, ANID #'s 73-78 were also assessed for all immune endpoints as assay controls (carrier control and dex).

Spleen samples for epigenetic analysis were preserved in 1ml of RNeasy[®] and then frozen at -80 °C. When samples were shipped from UNLV to MSU they were sent overnight on dry ice, then immediately transferred to a -80 °C freezer.

DNA Extraction

Samples preserved in RNeasy[®] were brought to room temperature. Individual samples of tissue were weighed to ensure that no more than 10 mg of tissue was used for the extraction, as per the manufacturer's recommendation. Each tissue sample was teased apart with submandibular bleed lancets and placed into a labeled 1.5 ml microcentrifuge tube. Three hundred and sixty milliliters of ATL Buffer from the Qiagen[®] DNeasy Blood

& Tissue Kit (Qiagen, Valencia, CA; catalog # 69504) was added to each sample, followed by 40 µl of proteinase K. Samples were incubated at 50-56 °C until completely lysed (12-24 hours), with periodic vortexing throughout incubation. Four hundred microliters of Buffer AL was added, sample was vortexed, and incubated (50-56 °C) for 10 minutes before the addition of 400 µl ethanol (95%), and then vigorously vortexed. Six hundred microliters of each sample was added to individually labeled columns from the kit and centrifuged at 8000 rpm for 1 minute. Flow-through was discarded and the remaining 600 µl of sample was added onto the column and centrifuged. Caution was exercised when adding sample to the columns so that residual tissue and precipitate were avoided and columns were not clogged. For the first wash, 500 µl of buffer AW1 was added to each column followed by centrifugation for 1 minute at 8000 rpm and flow through was discarded. For the second wash, 500 µl of buffer AW2 was added to each column followed by centrifugation for 3 minutes at 14,000 rpm to dry the DNeasy membrane. Flow through was again discarded. Columns were transferred to a 1.5 ml labeled microcentrifuge tube and 200 µl of elution buffer added to each column. After a 1 minute incubation at room temperature, columns were centrifuged for 1 minute at 8000 rpm. For maximum yield, the eluate was re-loaded onto the column, incubated for 1 minute at room temperature and centrifuged a final time for 1 minute at 8000 rpm. Once extracted, DNA concentrations for each sample were measured with a Nanodrop spectrophotometer, and then stored at -20 °C.

Primer Design

CpG islands in the promoter regions of target genes were determined using the UCSC Genome Browser (genome.ucsc.edu) and primers for Methylation-Specific PCR (MSP) were designed using MethPrimer (Li, 2002), for the genes *PRDM1*, *XBP-1*, and *PAX5* (Table 1). For each gene, two sets of primers were created. One set recognizes and binds to the methylated sequence and the other recognizes and binds to the unmethylated sequences.

Primers were ordered from Integrated DNA Technologies (IDT), reconstituted to 100 μ M concentrations, and then diluted to 10 μ M working solutions. A touchdown gradient was run with Zymo Research Universal Methylated Mouse DNA Standard (Zymo Research, Irvine, CA; catalog #D5012) for each set of primers to optimize melting temperature to be used in RT-PCR assay. Stock primers and working solutions of primers were maintained at -20 °C when not in use.

Table 1. MSP Primer Sequences

Gene	Direction	Sequence	bp	Position	CpG Count
Methylated					
<i>PRDM1</i>	Forward	5'- TTTGTCGGGTTGAGTAGTATTGAC-3'	228	chr10:44458551-4445889	33
	Reverse	5'-TCAATATTAATAAAAAAATCTACGAC-3'			
<i>PAX5</i>	Forward	5'-TTATTCGGTTAGTTGTGGTTTGTC-3'	194	chr4:44712906-44713335	37
	Reverse	5'-ACGCTTTCTAATCCTTAACCTCGAT-3'			
<i>XBP-1</i>	Forward	5'-ATGTGAGATTATGTTTTGTTTTTCGT-3'	167	chr11:5520641-5525993	88
	Reverse	5'-CTCTAAACCTCTTACACACCTTCG-3'			
Unmethylated					
<i>PRDM1</i>	Forward	5'- TTTGTTGGGTTGAGTAGTATTGATG-3'	228	chr10:44458551-4445889	33
	Reverse	5'- TCAATATTAATAAAAAAATCTACAAC-3'			
<i>PAX5</i>	Forward	5'-TTTATTTGGTTAGTTGTGGTTTGTT-3'	197	chr4:44712906-44713335	37
	Reverse	5'-CCACACTTTCTAATCCTTAACCTCAAT-3'			
<i>XBP-1</i>	Forward	5'-ATGTGAGATTATGTTTTGTTTTTGT-3'	168	chr11:5520641-5525993	88
	Reverse	5'-TCTCTAAACCTCTTACACACCTTCAA-3'			

Bisulfite Conversion

The bisulfite conversion was performed with either the Zymo EZ DNA Methylation™ or Zymo EZ DNA Methylation-Gold™ kits (Zymo Research, Irvine, CA; catalog # D5001, D5005). Extracted DNA samples were brought to room temperature, gently vortexed, and briefly centrifuged to bring the entire sample to the bottom of the tube. The sample volume added to each tube was dependent on the DNA yield from the extraction. All samples volumes were calculated such that 500 ng would be used for each conversion.

For the Zymo EZ DNA Methylation™ kit, 5 µl of dilution buffer was added to 500 ng of DNA from sample and the total volume adjusted with molecular grade water for a final volume of 50 µl. Samples were incubated at 37 °C for 15 minutes followed by the addition of 100 µl of conversion reagent (sodium metabisulfite) and mixed. Samples were then incubated in the dark at 50 °C for 12-16 hours. After incubation, samples were set in an ice bath for a minimum of 10 minutes. Four hundred microliters of binding buffer (guanidine hydrochloride) was added to the polypropylene microcentrifuge column with a silica-based matrix. One hundred and fifty microliters of the converted sample was then added to the column and mixed with the buffer. Columns were spun at 15,200 x g for 30 seconds. Sample was washed with 100 µl of buffer then centrifuged at 15,200 x g for 30 seconds. After the wash, 200 µl of desulphonation buffer (sodium hydroxide and water) was added to the column and left at room temperature for 15 minutes followed by centrifugation at 15,200 x g for 30 seconds. Samples were washed twice more with 200 µl of buffer followed by elution of the converted DNA with 10 µl of

elution buffer (Tris/EDTA). Samples were either immediately used or stored at -80 °C for future use.

Samples converted using the Zymo EZ DNA Methylation-Gold™ kit combined 130 µl of conversion reagent (sodium metabisulfite) with 500 ng DNA sample and adjusted the total volume to 150 µl with molecular grade water. Samples were then placed in a thermocycler where they incubated at 98 °C for 10 minutes followed by 64 °C for 2.5 hours. Six hundred microliters of binding buffer (guanidine hydrochloride) was added to the polypropylene, silica-based matrix microcentrifuge column with 150 µl of the sample, and then mixed. Columns were centrifuged at 15,200 x g for 30 seconds. Sample was washed with 100µl of buffer then centrifuged at 15,200 x g for 30 seconds. After the wash, 200 µl of desulphonation buffer (sodium hydroxide and water) was added to the column and left at room temperature for 15 minutes followed by centrifugation at 15,200 x g for 30 seconds. Samples were washed twice more with 200 µl of buffer followed by elution of the converted DNA with 10 µl of elution buffer (Tris/EDTA). Samples were either immediately used or stored at -80 °C.

The process behind bisulfite conversion denatures double stranded DNA (dsDNA) using heat (50 °C) to expose the nucleotides. Next, sodium bisulfite is incubated with the sample. During this time the sulfate will bind to the 6' carbon of the unmethylated cytosine, but the presence of a methyl group on the 5'carbon precludes sulfonation from occurring. Sodium hydroxide and water are added to promote the hydrolytic deamination of the cytosines that were originally unmethylated, followed by an alkali desulfonation, converting the original nitrogenous base into a uracil (Figure 12).

The sample is then washed in preparation for polymerase chain reaction (PCR). The resulting differences in the DNA sequences provide methylation specific binding for the methylated and unmethylated primers.

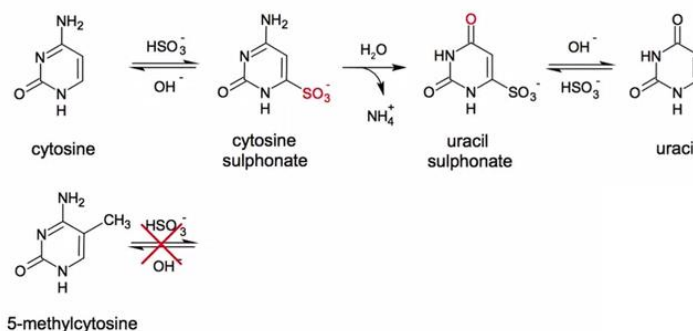


Figure 12. Illustration of the bisulfite conversion process

Real-Time Polymerase Chain Reaction

Bisulfite converted DNA samples from the 0 mg/kg and 100 mg/kg dose groups were amplified using the final concentration of 200 pM of each primer in the presence of 1x BioRad iTaq™ Universal SYBR® Green Supermix (BioRad, Hercules, CA; catalog # 172-5120). Amplifications were performed on a MyIQ thermocycler (BioRad) with initial incubation at 95 °C for 30 seconds, 40 cycles of 95 °C for 15 seconds, 55.6 °C for 30 seconds followed by a melt curve analysis. Universal methylated mouse DNA standards from EpigenDx (Hopkinton, MA; catalog # 80-8063- MGHM5 and 80-8064- MGUM5) were bisulfite converted and amplified alongside the NDRA samples to ensure adequate bisulfite conversion and binding of MSP primers. The methylated and unmethylated controls also served as reference points when determining the methylation status for each sample.

Agarose Gel Electrophoresis

A Thermo Scientific Horizontal Electrophoresis System (Model D2) and its components were used to cast and run 2% agarose gels. Two microliters of 6X blue/orange loading dye from Promega (Madison, WI; catalog # G118) was added to each sample, and mixed. The electrophoresis system was filled with 1X TBE buffer. A DNA molecular weight marker (100 pb ladder) from Amresco[®] (Solon, OH; catalog # K880-250UL) was run as a reference. Samples were run for 50-60 minutes at a current of 100mAmps then analyzed with an UltraThinLED Illuminator (The Gel Company, San Francisco, CA; catalog # TLB-01). Images were taken using the Imager smart phone gel doc (The Gel Company, San Francisco, CA; catalog # GDR-500), uploaded to a computer, and annotated.

Methylation Scoring

The methylation status of each sample was dichotomized into methylated and unmethylated groups using both sets of primers. Exponential amplification occurs early with the methylated primers when the DNA is methylated resulting in low C_t values. This same sample will not readily amplify with the unmethylated primer set, resulting in a late exponential amplification and corresponding high C_t value. Conversely, unmethylated samples will amplify readily with the unmethylated primers but not with the methylated primer set. The control DNA with high or low methylation status is used to visualize and correct differences in hybridization efficiency between primer sets.

5-mC % Enzyme-Linked Immunosorbant Assay (ELISA)

DNA extracts of 0 mg/kg and 100 mg/kg dose groups for CBN1, CBN5, CBN6, and CBN7, trials one and two, were thawed and then vortex to ensure adequate mixing of sample. DNA concentrations were calculated such that 100 ng of DNA from each sample was added to a PCR tube containing the respective amount of 5-mC coating buffer. A Standard curve was created using a mixture of methylated and unmethylated control DNA with 0, 5, 10, 25, 50, 75, and 100% methylation concentration points. Standard curve controls and samples were each run in duplicate. All DNA was run in a thermocycler at 98 °C for 5 minutes to denature the double stranded DNA. Immediately following the denaturation, all samples were transferred to ice for 10 minutes. The entire volume of denatured DNA and buffer (100 µl) was transferred to the ELISA plate, covered with foil, and incubated at 37 °C for one hour to allow the DNA to bind to the plate. Following the incubation, the buffer was discarded from the wells and the plate washed three times with 200 µl of 5-mC ELISA buffer. After the washing, 200 µl of 5-mC ELISA buffer was added to each well the plate covered with foil and set in the 37 °C incubator for 30 minutes. After the incubation buffer was discarded from the wells. One hundred microliters of an antibody mixture consisting of Anti-5-Methylcytosine (1:2000) and Secondary Antibody (1:1000) in 5-mC ELISA buffer was added to each well. The plate was covered with foil and incubated at 37 °C for an hour. Following the final incubation the antibody mixture was discarded from the wells and the plate washed three times with 200 µl of 5-mC ELISA buffer. One hundred microliters of horseradish peroxidase (HRP) developer was then added to each well. Color was allowed to develop

for 20-30 minutes at room temperature. Plate absorbance was measured at 405 nm in 1 second intervals on a Perkin Elmer Victor³ 1420 Multilabel Counter microplate reader with Wallac 1420 Manager software. The concentration of each standard curve point was plotted as a function of absorbance and the curve fitted with a log curve. The percentage of 5-mC in each DNA samples was calculated using the line equation (Equation 1.1) from the standard curve and the mean absorbance taken from the samples run in duplicate.

$$\%5\text{-mC} = e\{(\text{absorbance} - \text{y-intercept})/\text{slope}\} \quad (1.1)$$

A t-test was run to determine if there was a statistically significant change in 5-methylcytosine percentage between 0 mg/kg and 100 mg/kg dosed mice.

Clinical Chemistry & Hematology

For hematology and clinical chemistry endpoints, blood from anesthetized animals was collected into a microtainer tube containing EDTA, to prevent the sample from coagulating. Once collected, samples were sent overnight to the MVDL in Bozeman, MT, for hematology and clinical chemistry analysis. Hematology parameters included: white blood cells (WBC; $10^9/\text{L}$), red blood cells (RBC; $10^9/\text{L}$), hemoglobin (HGB; g/dl), hematocrit (HCT; %), mean corpuscular volume (MCV; fl), mean corpuscular hemoglobin (MCH; pg), mean corpuscular hemoglobin concentration (MCHC; %), red cell distribution (RDW; %), platelet count ($10^9/\text{L}$), neutrophils (%), neutrophils ($10^9/\text{L}$), lymphocytes (%), lymphocytes ($10^9/\text{L}$), monocytes (%), monocytes ($10^9/\text{L}$), eosinophils (%), eosinophils ($10^9/\text{L}$). Clinical chemistry parameters included:

creatine phosphokinase (CPK; IU/I), aspartate aminotransferase/serum glutamic oxaloacetic transaminase (AST/SGOT; IU/I), alanine aminotransferase/serum glutamic pyruvate transaminase (ALT/SGPT; IU/I), alkaline phosphatase (ALKP; IU/I), glucose (mg/dl), cholesterol (mg/dl), total protein (g/dl), albumin (g/dl), globulin (g/dl), phosphate (mg/dl), blood urea nitrogen (BUN; mg/dl), creatinine (mg/dl), and total bilirubin (mg/dl). Hematologies were run on all samples; however clinical chemistries performed were dependent on the volume of sample provided and were only run when sufficient volume was available. Hematology and clinical chemistry analyses were not performed in duplicate or triplicate as were other assays.

For determination of blood metal and metalloid concentrations, blood from anesthetized animals was collected into a microtainer tube containing heparin, to prevent the sample from coagulating. All blood tubes were weighed before and after collection of blood to determine the weight of each specimen. Once collected, samples were frozen at -80°C and then shipped to the Laboratory Services Bureau of the Montana Department of Public Health and Human Services for analysis of metal and metalloid concentrations. Metals/metalloids included: arsenic, cadmium, chromium, lead, magnesium, manganese, molybdenum, nickel, strontium, vanadium, and zinc. Blood metal/metalloid concentrations were not performed in duplicate or triplicate as were other assays.

RESULTS AND DISCUSSION

Dust CharacterizationCBN1

The median diameter of 4.39 μm from CBN1 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 2. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 3). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations, 55,100 $\mu\text{g/g}$ and 21,600 $\mu\text{g/g}$ respectively (Table 2). CBN1 measured the lowest concentration of Cr relative to the other CBN units, and the highest amount of Cu.

Table 2. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from CBN 1.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.39	55090	70	33	511	21595	9.4	69	79	62	618	<0.47	<3.0	13	<8.3	25	4.7

* Median diameter (μm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 3. Arsenic speciation results of dusts collected from CBN 1.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 1/PM4/100812	ND (<0.27)	21.4	ND (<0.20)	ND (<0.18)	$\mu\text{g/g}$

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

CBN2

The median diameter of 4.49 μm from CBN2 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 4. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 5). Of the metals in this sample, aluminum and iron were the found to have the highest concentrations at 59,400 $\mu\text{g/g}$ and 26,700 $\mu\text{g/g}$ respectively (Table 4). When comparing the different concentrations of metals in $\sim\text{PM}_{4.7}$ samples between CBN1 and CBN2, As and U concentrations in CBN2 were approximately two-fold higher than CBN1. Mn and Sr were approximately 30% less in CBN2 when compared to CBN1. Of all the combination units, CBN2 represents the highest measured amount of Cs and U, and the lowest amounts of Co, Cu and Pb. CBN2 also has the second highest concentration of arsenic at 140 $\mu\text{g/g}$.

Table 4. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from CBN 2

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.49	59377	74	39	387	26658	8.2	25	106	137	424	<0.47	<3.0	26	<8.3	23	8.6

* Median diameter (μm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 5. Arsenic speciation results of dusts collected from CBN 2.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 2/PM4/061112	ND (<0.27)	26.7	ND (<0.20)	ND (<0.18)	$\mu\text{g/g}$

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

CBN3

The median diameter of 4.13 μm from CBN3 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 6. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 7). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations at 59,993 $\mu\text{g/g}$ and 28,564 $\mu\text{g/g}$ respectively (Table 6). When comparing the different concentrations of metals among combination units, CBN3 reflects the lowest amounts of As (17 $\mu\text{g/g}$), Cs (9.3 $\mu\text{g/g}$), Pb (23 $\mu\text{g/g}$), and U (2.2 $\mu\text{g/g}$).

Table 6. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from CBN 3.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.13	59993	62	44	552	28564	11	32	94	17	328	<0.47	<3.0	9.3	<8.3	23	2.2

* Median diameter (μm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table7. Arsenic speciation results of dusts collected from CBN 3.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 3/PM4/061112	ND (<0.26)	4.04	ND (<0.19)	ND (<0.17)	$\mu\text{g/g}$

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

CBN4

The median diameter of 4.05 μm from CBN4 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 8. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 9). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest

concentrations 79,700 µg/g and 33,300 µg/g respectively (Table8). When comparing the different concentrations of metals with other combination units, CBN4 reflects the highest amounts in Al, V, Cr, Zn, Sr and Pb.

Table 8. Total elemental concentration (µg/g in dry sample) of dusts collected from CBN 4.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.05	79651	100	54	752	33266	14	37	135	71	666	<0.47	<3.0	15	<8.3	34	4.9

* Median diameter (µm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 9. Arsenic speciation results of dusts collected from CBN 4.

Sample ID	AS(III)	AS(V)	MMAAs	DMAAs	Units
CBN 4/PM4/061212	ND (<0.29)	15.7	ND (<0.22)	ND (<0.19)	µg/g

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAAs = Monomethylarsonic acid

DMAAs = Dimethylarsinic acid

CBN5

The median diameter of 4.6 µm from CBN5 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 10. All arsenic in the sample was determined to be pentavalent (As⁵⁺) (Table 11). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations, 74,500 µg/g and 33,300 µg/g respectively (Table 10). CBN5 measured the highest concentration of As at 227 µg/g.

Table 10. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from CBN 5.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.6	74530	99	43	357	33313	9.7	33	124	227	321	<0.47	<3.0	22	<8.3	44	13

* Median diameter (μm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 11. Arsenic speciation results of dusts collected from CBN 5.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 5/PM4/061112	ND (<0.30)	73.1	ND (<0.22)	ND (<0.20)	$\mu\text{g/g}$

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

CBN6

The median diameter of 3.14 μm from CBN6 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 12. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 13). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations, 47,700 $\mu\text{g/g}$ and 21,200 $\mu\text{g/g}$ respectively (Table 14). CBN6 measured the lowest relative concentrations of Al, V, Fe, Zn, and Pb relative to the other CBN units.

Table 12. Total elemental concentration ($\mu\text{g/g}$ in dry sample) of dusts collected from CBN 6.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
3.14	47692	56	35	376	21208	8.3	28	71	24	445	<0.47	<3.0	9.4	<8.3	23	2.3

* Median diameter (μm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 13. Arsenic speciation results of dusts collected from CBN 6.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 6/PM4/100812	ND (<0.23)	6.93	ND (<0.17)	ND (<0.15)	µg/g

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

CBN7

The median diameter of 4.37 µm from CBN7 and a total digestion chemical composition, determined by ICP-MS, of the dust are shown in Table 14. All arsenic in the sample was determined to be pentavalent (As^{5+}) (Table 15). Of the metals in this sample, aluminum and iron concentrations were the found to have the highest concentrations, 73,200 µg/g and 36,300 µg/g respectively (Table 14). CBN7 measured the lowest relative concentrations of Mn and Sr, and the highest amounts of V, Fe, and Co relative to the other CBN units.

Table 14. Total elemental concentration (µg/g in dry sample) of dusts collected from CBN 7.

Median*	Al	V	Cr	Mn	Fe	Co	Cu	Zn	As	Sr	Cd	Sb	Cs	Tl	Pb	U
4.37	73239	103	50	294	36340	10	30	97	24	285	<0.47	<3.0	24	<8.3	26	5.1

* Median diameter (µm)

< indicates value is below method quantitation limit (MQL). Value presented is MQL.

Table 15. Arsenic speciation results of dusts collected from CBN 7.

Sample ID	AS(III)	AS(V)	MMAs	DMAs	Units
CBN 7/PM4/061112	ND (<0.27)	6.16	ND (<0.20)	ND (<0.18)	µg/g

All results reflect the applied dilution

All results reported as received (wet weight)

ND = Not detected at the applied dilution

MMAs = Monomethylarsonic acid

DMAs = Dimethylarsinic acid

PRDM1

Methylation scores were determined for all 0 mg/kg and 100 mg/kg dosed mice from CBN1 trial one and two, CBN5 trial one and two, CBN6 trial one and two, and CBN7 trial one and two. All animals were determined to have no methylation in the promoter region of the *PRDM1* transcription factor gene (Table 16).

Table 16. *PRDM1* Methylation

CBN	Dosage (mg/kg)	n	Methylated	Unmethylated
1	0	10	0%	100%
	100	12	0%	100%
5	0	11	0%	100%
	100	12	0%	100%
6	0	11	0%	100%
	100	12	0%	100%
7	0	12	0%	100%
	100	11	0%	100%

PAX5

Methylation scores were determined for all 0 mg/kg and 100 mg/kg dosed mice from CBN1 trial one and two, CBN5 trial one and two, CBN6 trial one and two, and CBN7 trial one and two. All animals were determined to have no methylation in the promoter region of the *PAX5* transcription factor gene (Table 17).

Table 17. *PAX5* Methylation

CBN	Dosage (mg/kg)	n	Methylated	Unmethylated
1	0	10	0%	100%
	100	12	0%	100%
5	0	11	0%	100%
	100	11	0%	100%
6	0	11	0%	100%
	100	12	0%	100%
7	0	12	0%	100%
	100	11	0%	100%

XBP-1

Methylation of the *XBP-1* gene was not determined due to improper primer amplification of the control DNA. It is expected that a redesign of the primer pairs and adjustments to the PCR protocol would provide reliable results.

5-mC % Global Methylation

5-mC% was determined for all 0 mg/kg and 100 mg/kg dosed mice from CBN1 trial one and two, CBN5 trial one and two, CBN6 trial one and two, and CBN7 trial one and two (Figure 13).

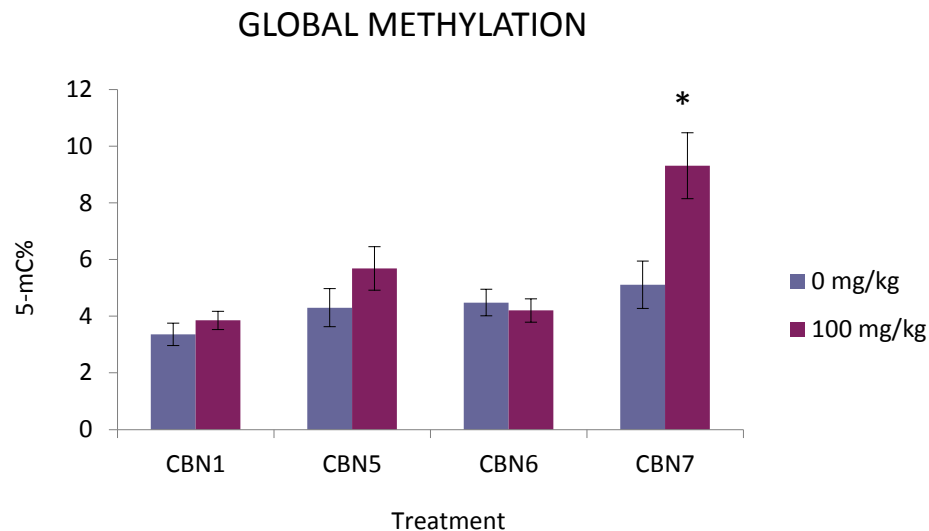


Figure 13. 5-mC% of 0 mg/kg dose group and 100 mg/kg dosed mice exposed to dust from CBN1, CBN5, CBN6, and CBN7. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 9-10 animals. Data presented are representative of two trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$) within treatment.

Global methylation was found to be significantly increased in mice exposed to 100 mg/kg dust from CBN7 when compared to control mice. The control and high dust exposure comparison for CBN1, CBN5, and CBN6 did not reach statistical significance.

Following these findings, dose groups 0.01, 0.1, 1, and 10 were assayed with the 5-mC% ELISA to investigate a global methylation dose response; however, there was no statistically significant change from control in the additional dose groups (Figure 14).

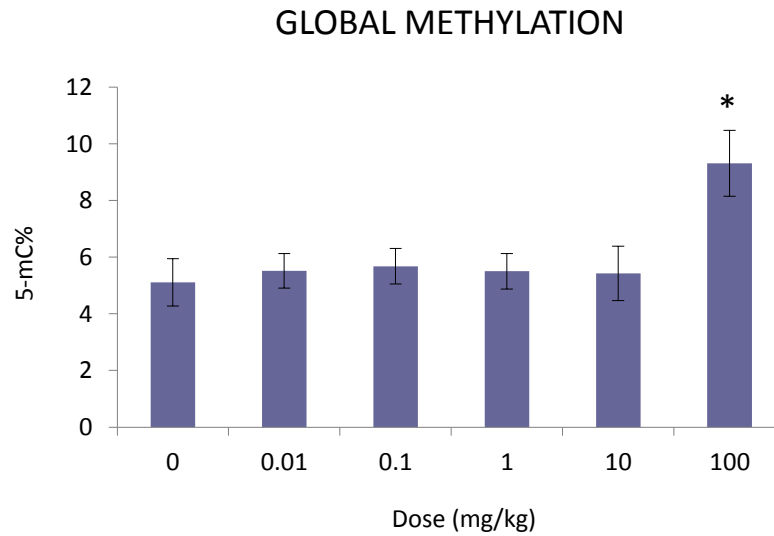


Figure 14. 5-mC% of all treatments of dosed mice exposed to dust from CBN7. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 8-10 animals. Data presented are representative of two trial days. The (*) indicates a response statistically different from the control group ($p < 0.05$) within treatment.

Clinical Chemistry & Hematology

CBN1

None of the hematological endpoints measured in mice dosed with dust samples from CBN1 were statistically significant relative to responses in the 0 mg/kg group nor were any changes dose-responsive in nature. Similarly, clinical chemistry endpoints did not vary by dose; however, plasma creatinine was dose-responsively increased in mice exposed to 0.01 mg/kg to 100 mg/kg (Figure 15).

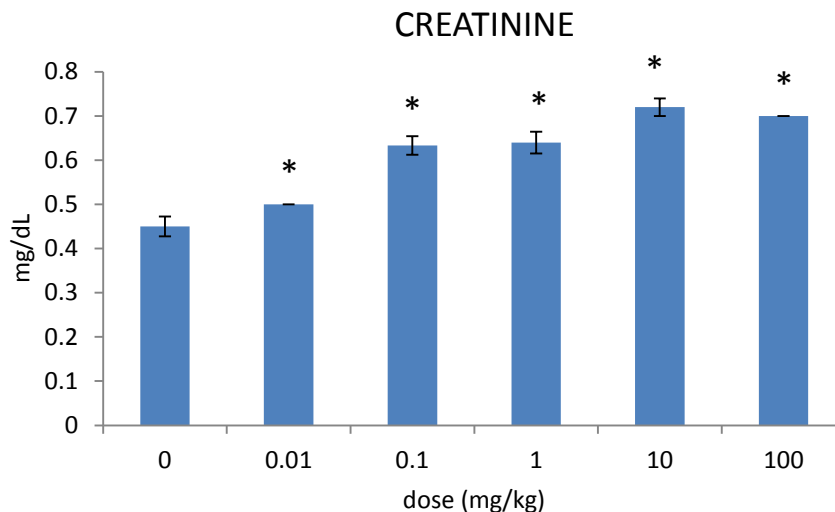


Figure 15. Serum creatinine levels measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit CBN1 (vegetated and non-vegetated sand dunes). Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the control group ($p < 0.05$).

This increase in serum creatinine was one of the most sensitive parameters affected to dust samples from CBN1. The significant difference from control mice at the 0.01 mg/kg dose and the dose responsively decreased IgM antibody production established the LOAEL for this combination unit to be 0.01 mg/kg. A no observed adverse effect level could not be determined.

CBN2

Of the hematological endpoints measured in mice dosed with dust samples from CBN2, only the percentage of eosinophils was statistically significantly increased relative to the 0 mg/kg group, though changes were not dose-responsive. Of the clinical chemistry endpoints, BUN was significantly decreased at 10 and 100 mg/kg exposures, and plasma

creatinine showed a dose-responsive increase from 1 mg/kg to 100 mg/kg (Figures 16-17).

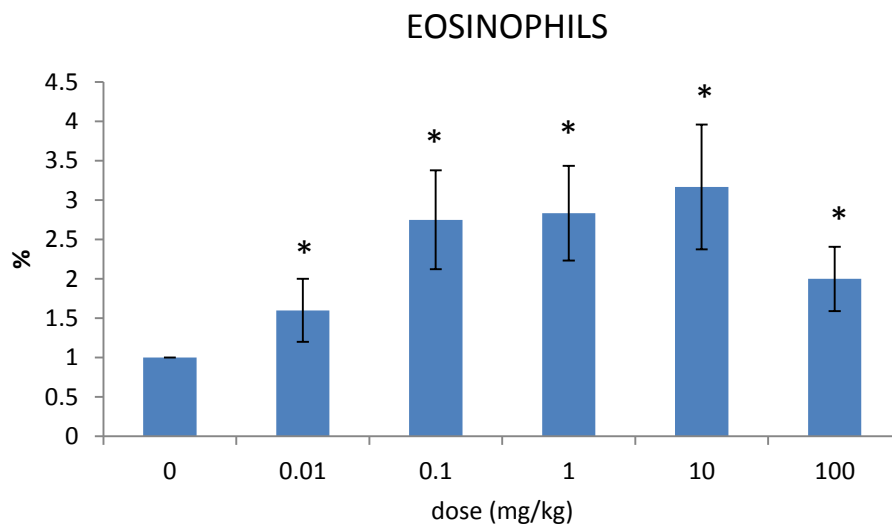


Figure 16. Of the hematology endpoints assessed, the percent of eosinophils in the white blood cell differential was increased in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN2. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

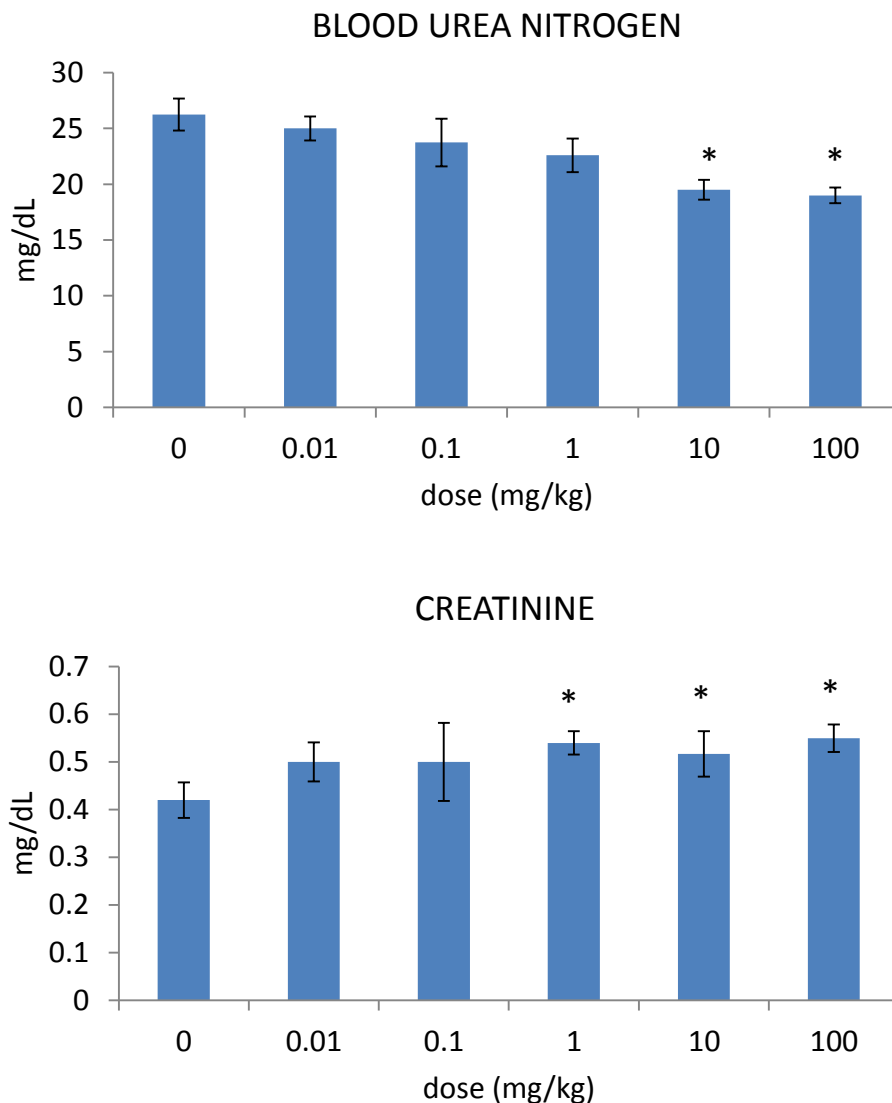


Figure 17. Of the clinical chemistry endpoints assessed the serum blood urea nitrogen and serum creatinine were altered in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN2. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

Eosinophils were significantly increased from 1 to 1.5% at 0.01 mg/kg exposure level. This change, albeit statistically significant, may not be biologically meaningful in

terms of confirming toxicity or an impairment to health without further investigation. Similarly, an increase to 3% eosinophils in the white blood cell differential at 1 and 10 mg/kg exposure levels does not necessarily demonstrate a clear link to toxicity. These changes, however, may provide weight of evidence towards investigating possible allergic or related changes in immune function.

CBN3

No statistically significant changes in hematological endpoints were observed at any dose. Of the clinical chemistry endpoints measured in mice dosed with dust samples from CBN3, only albumin levels collected from animals exposed to 10 or 100 mg/kg were statistically significantly increased (by about 10%) relative to the 0 mg/kg group. (Figure 18).

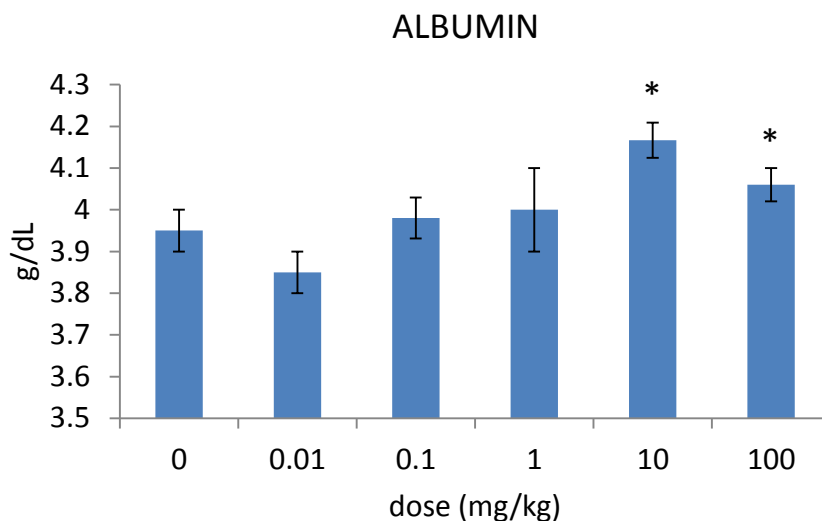


Figure 18. Albumin levels measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface units 3.1-3.5 combined CBN3. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

CBN4

Of the hematological endpoints measured in mice dosed with dust samples from CBN4, HGB and MCV were endpoints with statistical differences between exposed and unexposed animals. HGB concentration was elevated by about 5%, on average, in mice following exposure to 1, 10, and 100 mg/kg relative to mice from the 0 mg/kg group (Figure 19). In these same dose groups, MCV was decreased by about 20% in each group relative to concentrations in the 0 mg/kg group (Figure 19). Of the clinical chemistry endpoints, ALT and plasma creatinine were altered. Following exposure to 0.1-100 mg/kg, ALT was decreased by 48.8% to 62.6% relative to the 0mg/kg group (Figure 20). Creatinine levels were increased by 10% in both the 10 and 100 mg/kg dose groups relative to the 0 mg/kg dose group (Figure 20).

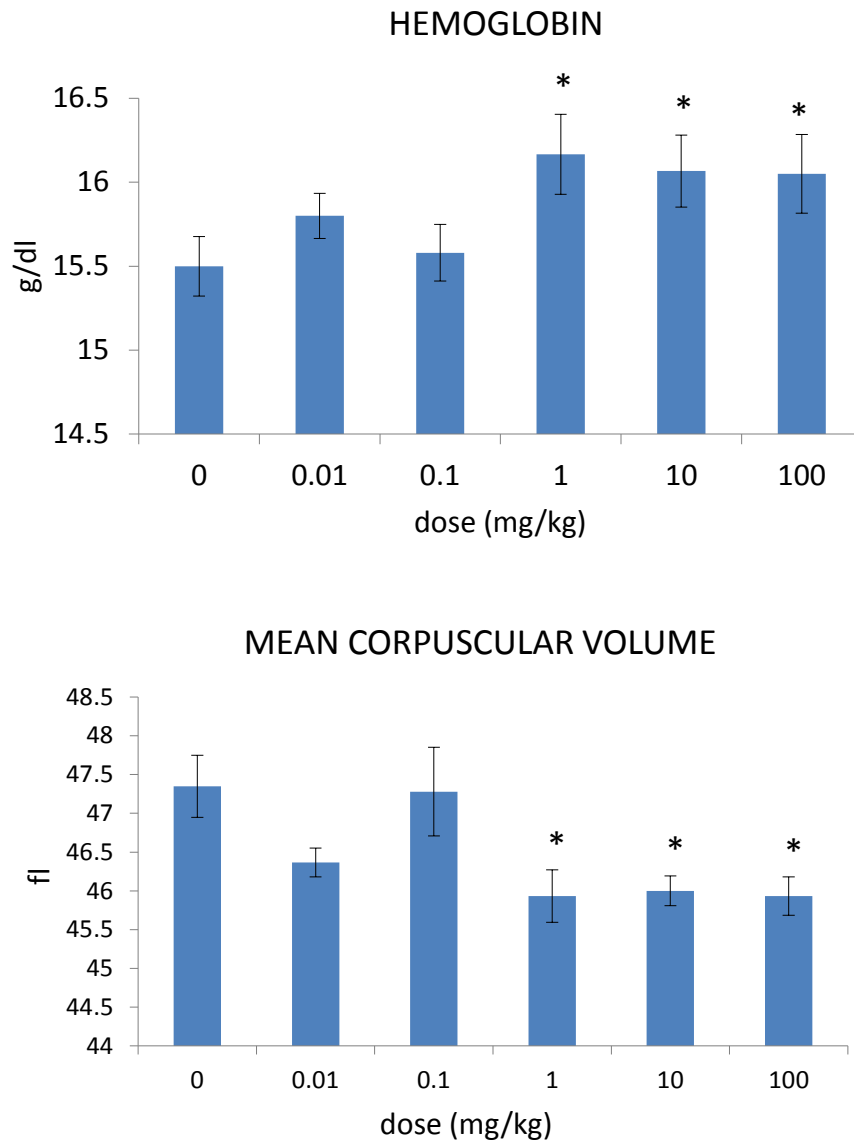


Figure 19. Hematological endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface units 4.1-4.3 and 2.1 combined CBN4. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

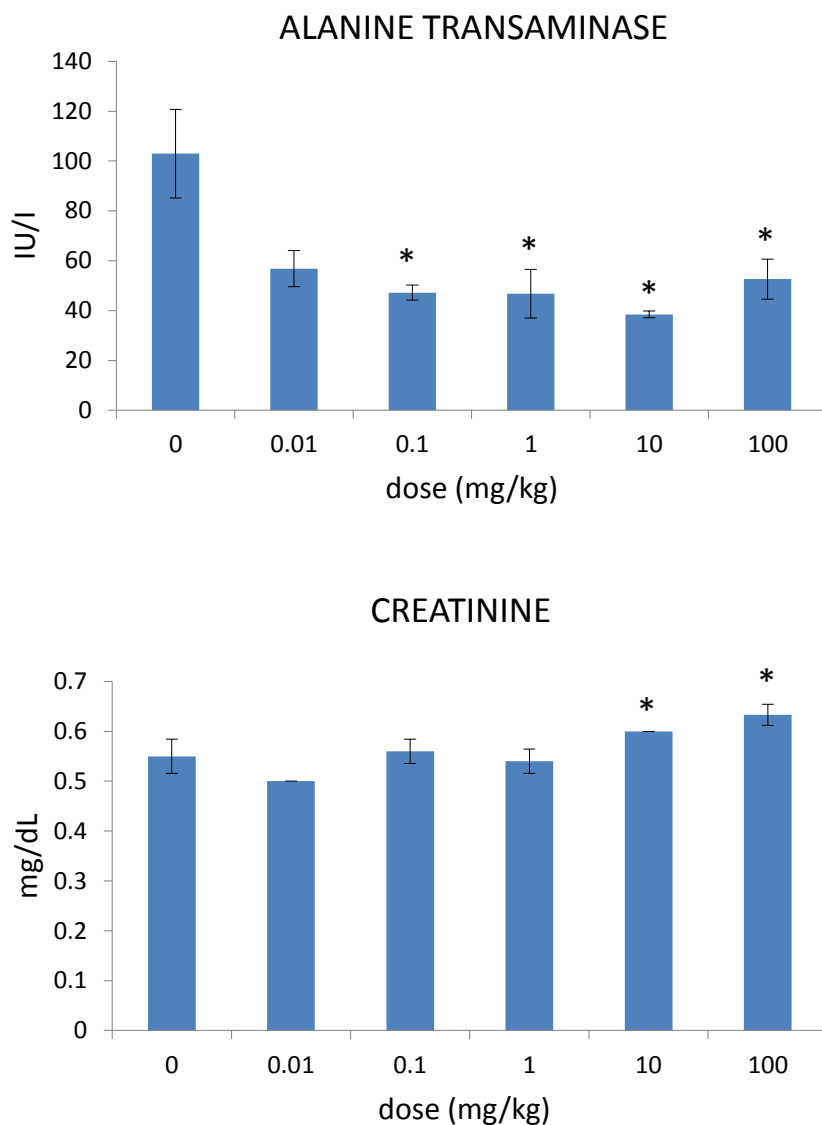


Figure 20. Clinical chemistry endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface units 4.1-4.3 and 2.1 combined CBN4. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

ALT is a serum biomarker of organ health contributed to the NOAEL and LOAEL for CBN4. Hemoglobin was elevated and MCV was reduced relative to the

0mg/kg group after exposure to 1, 10, and 100 mg/kg. Additionally, ALT was reduced relative to the 0 mg/kg group after exposure to 0.1 to 100 mg/kg. Hemoglobin is a protein associated with red blood cells that binds oxygen. In general, elevated serum hemoglobin is suggestive of anemia, or a condition where red blood cells break down or are destroyed (Mehta, 2012). MCV is a measure of the size of red blood cells; a reduction in MCV indicates that the red blood cells are small and also can be used as an indicator of anemia (Mehta, 2012). The elevated serum hemoglobin and decreased MCV associated with exposure to higher concentrations of dust from CBN4 indicates that its combination of dust and heavy metals can be toxic to red blood cells at the high exposure concentrations and for the duration of this study. Additional studies are warranted to determine the potential mechanisms by which dust from CBN4 affects red blood cells.

ALT is a liver enzyme that may increase in detectable concentrations in the serum when the liver is damaged or diseased (Dufour, 2010); however, reductions in ALT are more challenging to interpret as it is not generally a marker of a specific disease. Increases in serum creatinine were observed after exposure to 10 and 100 mg/kg. Serum creatinine is a marker of kidney function and increasing levels suggest nephrotoxicity. Glomerular filtration rate (GFR) is typically used to assess renal function and is often determined by creatinine clearance. Creatinine is a byproduct of muscle metabolism and is transported in plasma before excretion in the urine. This production happens at a fairly constant rate and is relative to muscle mass. The reliability of creatinine production makes it a reliable analytes for assessing GFR in humans. However, estimating GFR based on creatinine clearance in the kidneys requires knowledge of gender, age, and

weight as these parameters typically relate to muscle mass. We could not directly translate the calculation for human estimated GFR to our murine model; however, increased serum creatinine concentrations are relative to decreased GFR (Kaplan, 2010).

CBN5

Of the hematological endpoints measured in mice dosed with dust samples from CBN5, MCV levels were statistically and significantly decreased in 0.1 mg/kg to 100 mg/kg, while MCHC levels were increased in 0.1 mg/kg through 100 mg/kg exposed groups (Figure 22). Of the clinical chemistry endpoints plasma creatinine was significantly increased at 10 mg/kg and 100 mg/kg (Figure 21).

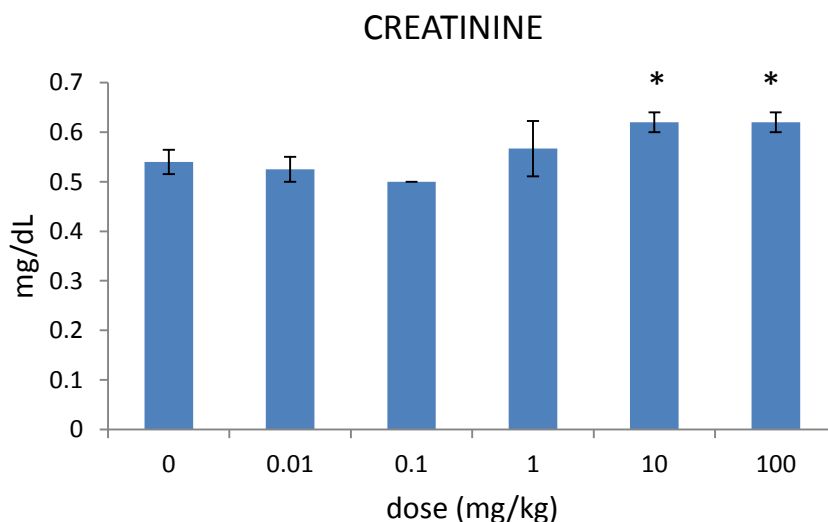


Figure 21. Of the clinical chemistry endpoints assessed, significant changes were demonstrated in serum creatinine in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN5. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. This symbol (*) indicates a response statistically different from the control group ($p < 0.05$).

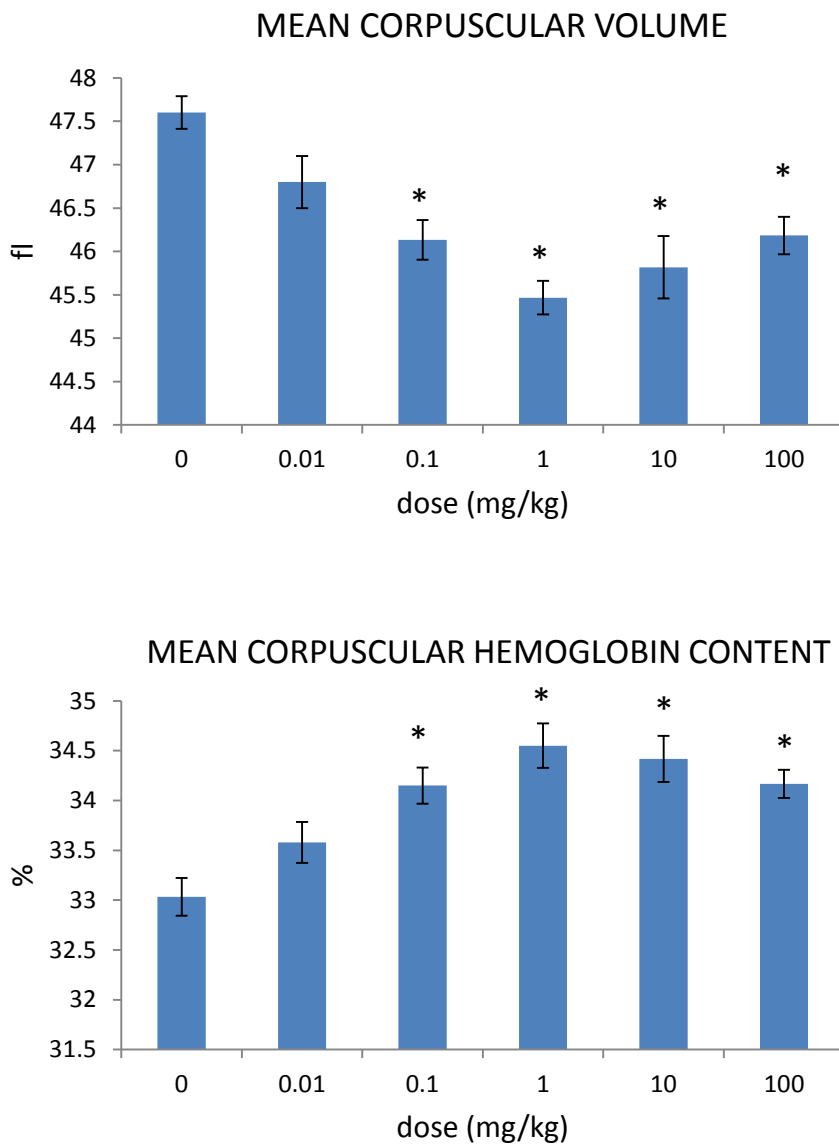


Figure 22. Of the hematology endpoints assessed, significant changes were demonstrated in red blood cell indices in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN5. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. This symbol (*) indicates a response statistically different from the control group ($p < 0.05$).

CBN6

Of the hematological endpoints measured in mice dosed with dust samples from CBN6, increased neutrophil (PMN) percentage (shown) was corroborated by increased absolute numbers of PMNs in peripheral blood as both were statistically and significantly increased at 100 mg/kg using raw data (Figure 24). Of the clinical chemistry endpoints, plasma creatinine was dose responsively increased from 0.01 mg/kg to 100 mg/kg using ranked transformed data (Figure 23).

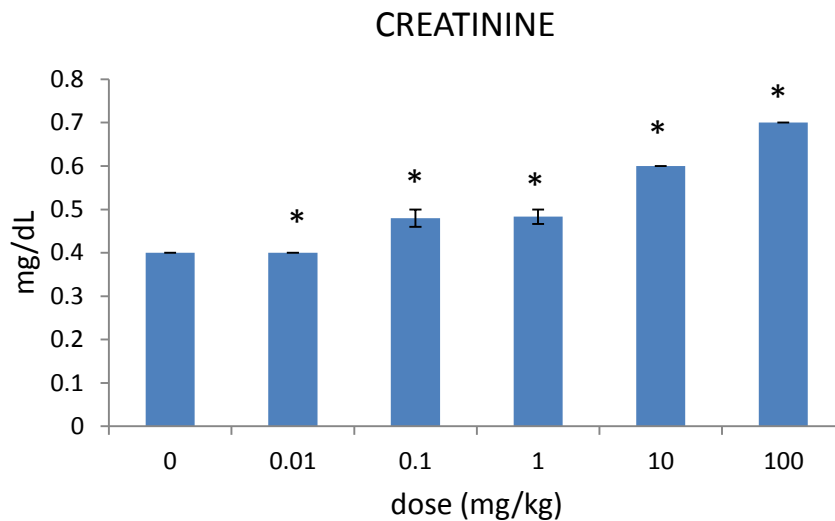


Figure 23. Of the clinical chemistry endpoints assessed, significant changes were demonstrated in serum creatinine in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN6. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the control group ($p < 0.05$).

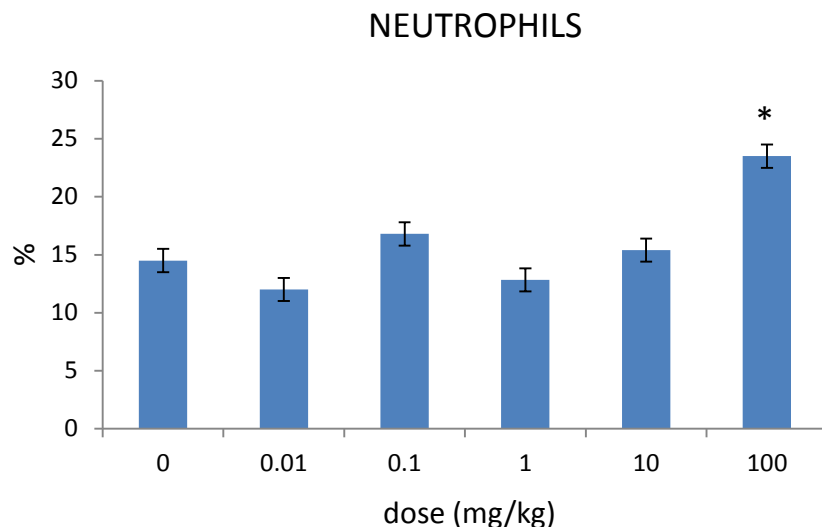


Figure 24. Of the hematology endpoints assessed, significant changes were demonstrated in blood neutrophil percent in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA CBN6. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. Data presented are representative of one trial day. The (*) indicates a response statistically different from the control group ($p < 0.05$).

We also saw an increase of relative and absolute neutrophil numbers in mice exposed to 100 mg/kg dust from CBN6. Though this increase was statistically significant, we were unable to find any biologically relevant relationship between increased PMNs and metal exposure. Typically, increased levels of neutrophils are associated with inflammation, bacterial infection, or leukemia (Murphy, 2012).

CBN7

Several of the hematological and clinical chemistry endpoints measured in mice dosed with dust samples from CBN7 were altered. In the 10 and 100 mg/kg groups, WBC counts were increased by 93.8% and 109.4%, respectively, and lymphocyte concentrations were increased by 92.3% and 111.6%, respectively. Monocyte

concentrations were increased by 247% in the 100 mg/kg group relative to the 0 mg/kg group (Figures 25-26). Of the clinical chemistry endpoints (Figures 27-28), blood urea nitrogen was decreased by 21.1% to 30.9% after exposure to 1 mg/kg to 100 mg/kg and plasma creatinine was significantly increased by 4.3% in the 100 mg/kg group, relative to the 0 mg/kg group, giving a BUN:creatinine ratio of 50:1. A dose responsive increase (from 60% to 120%) in total bilirubin levels from animals dosed with 0.1 mg/kg through 100 mg/kg also was observed.

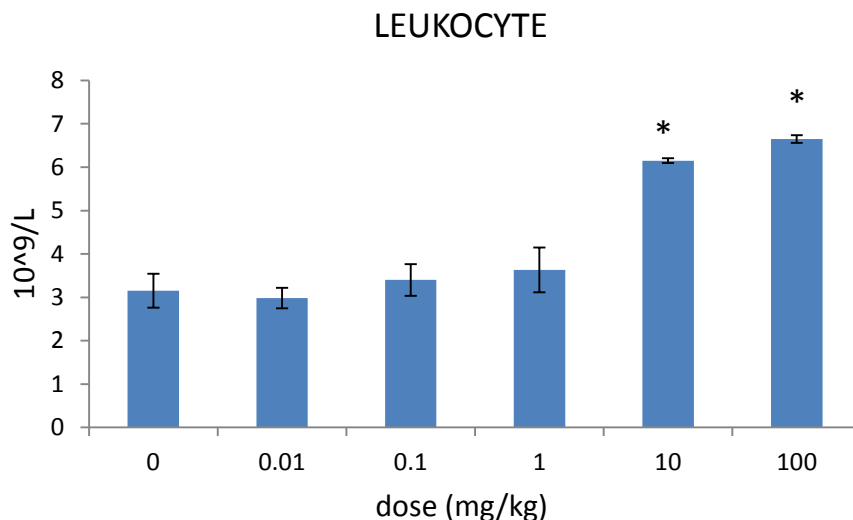


Figure 25. Of hematological endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit 2.3 CBN7 leukocytosis was observed. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0mg/kg group ($p < 0.05$).

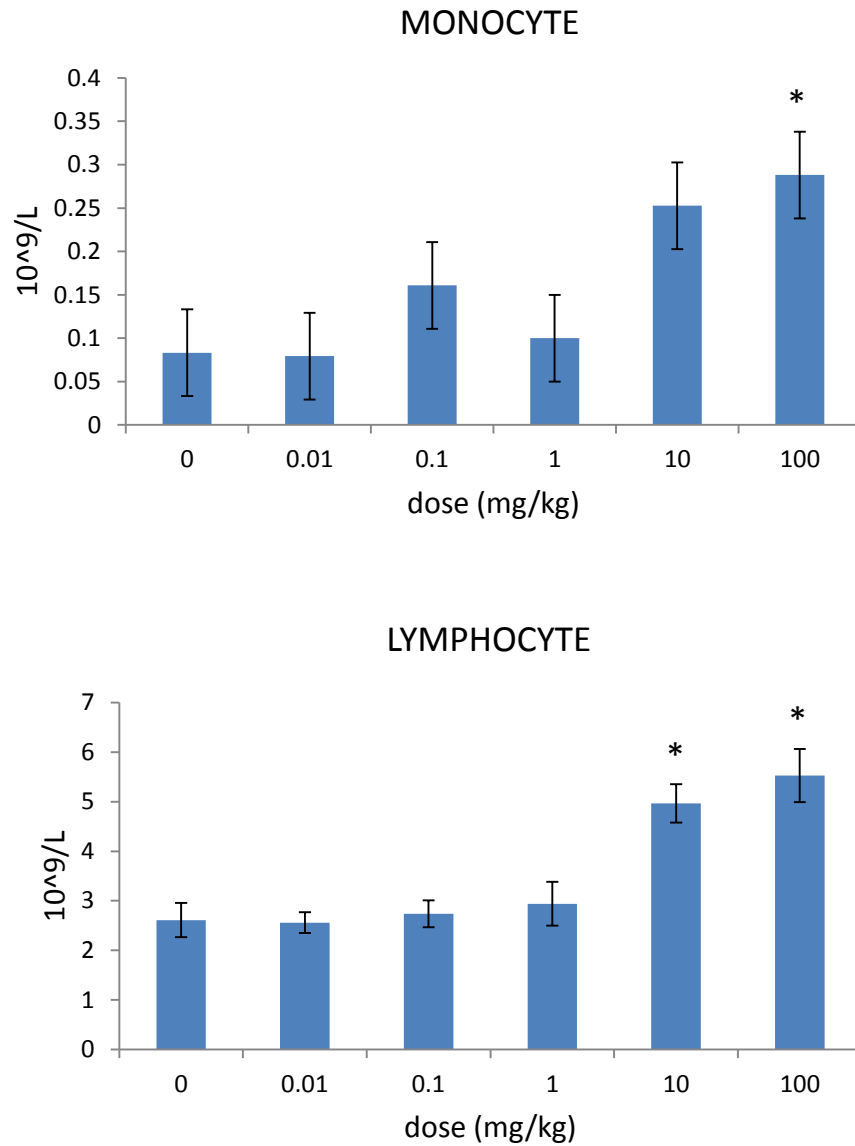


Figure 26. Of hematological endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit 2.3 CBN7 monocytes and lymphocytes were observed to be increased in the high dose groups. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0mg/kg group ($p < 0.05$).

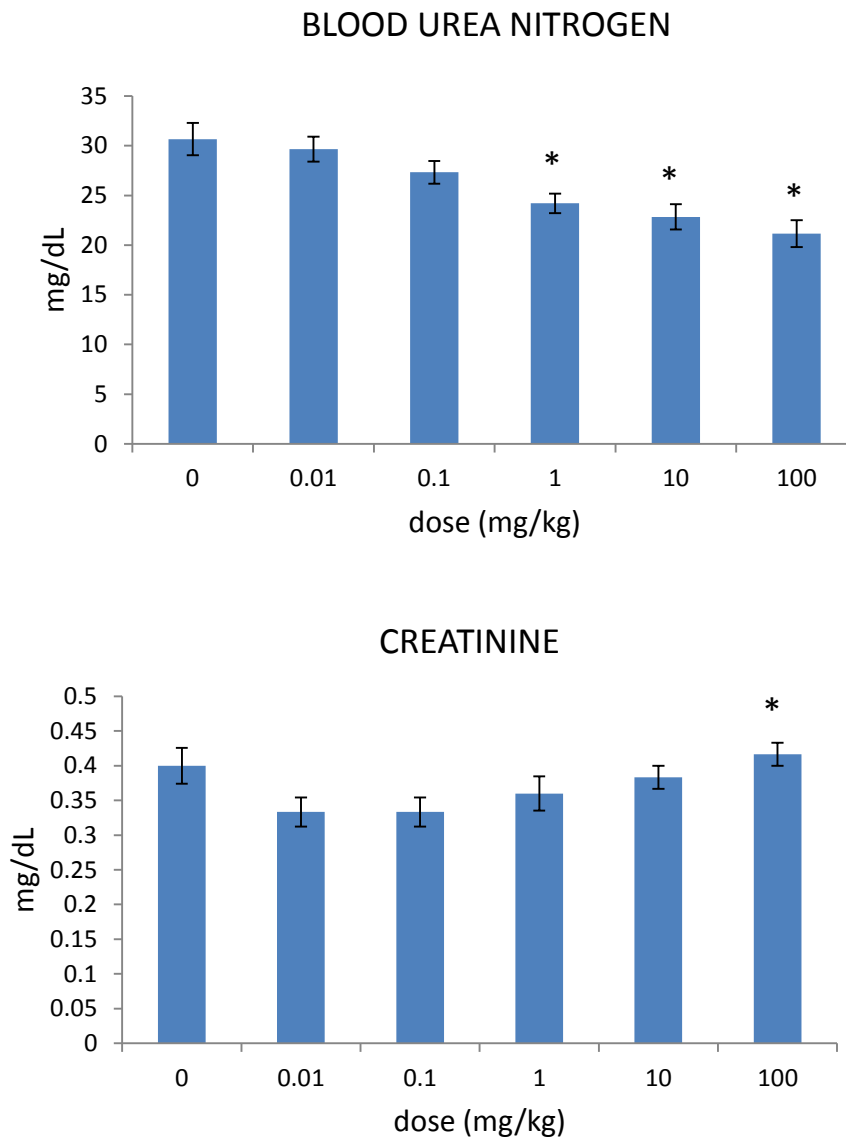


Figure 27. Of clinical chemistry endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit 2.3 CBN7 serum blood urea nitrogen and serum creatinine were altered. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

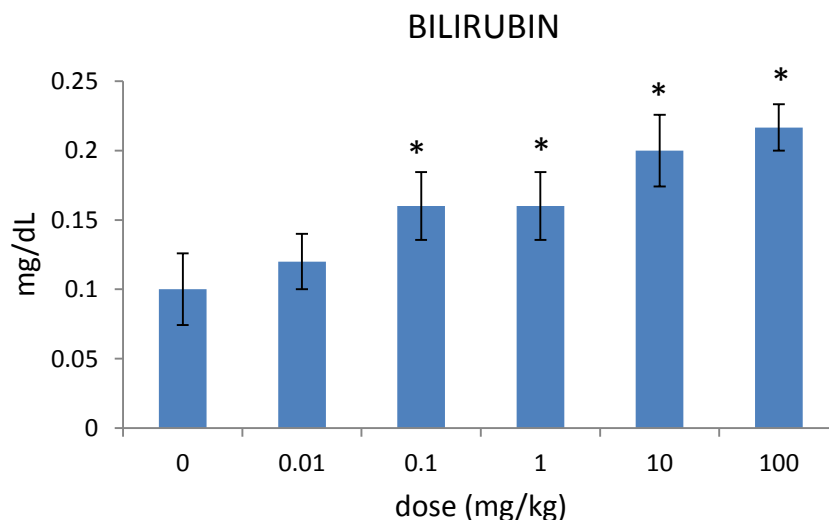


Figure 28. Of clinical chemistry endpoints measured in adult female B6C3F1 mice following oropharyngeal aspiration exposure to dust samples from NDRA surface unit 2.3 CBN7 total bilirubin was increased. Data are presented as mean $\pm$ standard error of the mean. Sample size for each group was 5-6 animals. The (*) indicates a response statistically different from the 0 mg/kg group ($p < 0.05$).

The combination of elevated WBCs, lymphocyte concentration, and monocyte concentration observed in the two highest dose groups, are typically indicative of inflammation, bacterial infection, leukemia, stress, or anemia (Murphy, 2012). In humans, other factors, such as cigarette smoking or certain drugs, also may increase hematological endpoints, but these do not pertain to the present study. Often, splenomegaly (an increase in the size of the spleen) accompanies increases in these endpoints, but this was not observed in animals from the afflicted dose groups. Without additional studies, it is not possible to determine exactly why these values were increased in animals exposed to 10 or 100 mg/kg of dust from CBN7; the most likely explanation is that they were experiencing systematic stress or inflammation as a result of the exposure.

Additionally, changes were observed in BUN, plasma creatinine, and total bilirubin levels. These can be indicative of both liver and kidney damage and possibly indicate liver and kidney toxicity associated with exposure to the higher concentrations of dust samples from CBN7.

Discussion

This study was included to explore a possible mode of action to understanding the biological process affecting decreased IgM secretion in our murine model. Previous studies demonstrating effects that metals and particulate matter have on epigenetic mechanisms provided sound reasoning for investigating epigenetic markers in the mice exposed to dust from NDRA. Though histone modification changes have been associated with arsenic and chromium exposure (Ren et al., 2011; Sutherland and Costa, 2003; Zhou et al., 2008, 2009) we chose to evaluate CpG island methylation as it is heavily studied and a well understood epigenetic marker with fairly streamlined and economic assays available for laboratory detection. We picked transcription factors known to highly influence the cellular differentiation of B-cells to plasma cells in anticipation they would provide an explanation for decreased immunoglobulin secretion if alterations were found. Spleen tissue was chosen as B-cell differentiation occurs in the germinal center of this organ.

Though we looked into elements that could rationally explain the observed effects in our mice, other factors remain to be evaluated. Additional transcription factors that were considered but not incorporated into our study were Bcl-6 and IRF4, which are also

integral to the B-cell to plasma cell differentiation pathway. An interference with the presentation of antigen to T-cells, the T-cell ability to present antigen to B-cells, or interferences with cytokines involved in the promotion of plasma cell differentiation could also be responsible for the decreased antibody production. It is also important to consider the fact that the transcription factor genes we explored could have been suppressed by other epigenetic means such as histone modifications, chromatin remodeling, or microRNAs.

Identifying epigenetic changes due to exposure of NDRA dust would indicate an underlying biological mechanism of the immune profile changes and further characterize the toxicological profile. Particulate matter and a number of the individual metals we evaluated are known to alter the epigenome. The dust from NDRA is a complicated mixture of geogenic minerals with variable chemical compositions and particulate matter sizes. These inherent properties add an extra dimension of difficulty when evaluating toxicity. Chemicals within mixtures may interact in vivo having additive or synergistic effects that contribute to toxicity. In future studies the individual components of the NDRA dust can be explored in isolation and combinations to uncover the trigger of the immunologic shift. Whole genome sequencing of bisulfite treated DNA will provide target genes associated with this exposure.

The LOAEL established in this study was 0.01 mg/kg, while a NOAEL could not be determined. The LOAEL was based on an immunological and a kidney measure. That is, a dose-responsive suppression of IgM antibody production and increasing levels of serum creatinine were the most sensitive parameters altered by exposure to dust samples

from CBN1. Immunotoxicity occurred at dust concentrations where no overt toxicity was indicated by a change in body weight from animals exposed to 0 mg/kg, which is often an indicator of overt or systemic toxicity. The immunological parameters affected in this study are known to be predictive of increased disease susceptibility (Luster et al., 1992, 1993) and, therefore, are regarded as a reliable marker for immunotoxicity.

Nain et al., (2012) reported that serum biochemical changes are detectable in blood urea nitrogen, potassium, chloride, and ALT from subchronic arsenic exposure. However, this was not observed in our study. Dose-responsive increases in serum creatinine were observed following exposure to dust samples from CBN1, which contained 62 µg/g (ppm) arsenic, 25 µg/g lead, 511 µg/g manganese, and 4.7 µg/g uranium (Table 2). Serum creatinine was increased in mice dosed with 10 mg/kg and 100 mg/kg from CBN5 which contained 227 µg/g arsenic, 44 µg/g lead, 357 µg/g manganese, and 13 µg/g uranium (Table 10). Serum creatinine is a marker of kidney function and increasing levels suggest nephrotoxicity. GFR is typically used to assess renal function and is often determined by creatinine clearance. Creatinine is a byproduct of muscle metabolism and is transported in plasma before excretion in the urine. This production happens at a fairly constant rate and is relative to muscle mass. The reliability of creatinine production makes it a reliable analyte for assessing GFR in humans. However, estimating GFR based on creatinine clearance in the kidneys requires knowledge of gender, age, and weight as these parameters typically relate to muscle mass. We could not directly translate the calculation for human estimated GFR to our murine model; however, increased serum creatinine concentrations correlate with decreased GFR

(ATSDR, 2007b; Kaplan, 2010). Changes to serum creatinine can be linked to altered kidney function that is associated with exposure to uranium, manganese, lead, and arsenic.

We observed dose-responsive increases in serum creatinine and decreases in BUN following exposure to CBN2 dust exposure. This is somewhat consistent with Nain et al., (2012) who report serum biochemical changes reflective of nephrotoxicity following subchronic arsenic exposure. CBN2 contains approximately a two-fold increase in As and U over CBN1. Exposure to CBN1 affected creatinine levels only, while CBN2 affected both BUN and creatinine levels. Typically, serum BUN and creatinine will track together when kidney function is impaired. It is rational that increased concentrations of As and U might contribute to the expression of these serum kidney biomarkers in CBN2.

Red blood cell size and hemoglobin capacity were affected following exposure to CBN5. The MCHC was increased beginning at 0.1 mg/kg through all higher exposure levels. This is a measure of the concentration of hemoglobin in a given volume of packed RBC. Conversely, the MCV was decreased. It is likely that the MCHC was increased due to the size of the red blood cells (MCV) decreasing without a concurrent reduction of hemoglobin content inside the cell. Lead is associated with microcytic, hypochromic anemia, which would account for the decreased MCV; however, a decrease in intracellular hemoglobin would have also been anticipated as Pb is understood to have direct effects on hemoglobin synthesis. CBN4 and CBN5 are the only two combination units to show any effect on red blood cell physiology and it should be noted that they contained the highest concentrations of both lead and aluminum compared to other

combination units, both metals known to interfere with the ALAD enzyme in heme biosynthesis.

It is intriguing that mice dosed with dust from CBN7 were affected to a greater extent compared to any other CBN unit. This is peculiar because there was nothing significant about the area or its dust characterization. It had an average particle size and none of the metal concentrations stand out compared to the other treatments. It did have slightly elevated concentrations of Fe and V, but these two metals have very little correlation to pathology. It is also interesting that the only CBN unit we were able to detect epigenetic changes in was CBN7. These results show that the complex mixture of dust from the NDRA can induce epigenetic changes in high concentrations; however it does not explain why we saw IgM depression or provide insight as to why CBN7 seems to have the greatest impact in the mice.

Though the flow cytometric and PFC assays are good measures of immunity, they do not directly measure immunocompetence of immune system function. Host resistance challenge models are often used to corroborate other evaluations of immunity by inoculating dosed animals with either *Listeria monocytogenes* or *Streptococcus pneumoniae* and observing the ability to fight infection. This would be a good follow-up study to more definitively describe resistance to infection in our murine model; however, within the scope of our study we did not have the opportunity to perform this analysis.

Another sound parameter to assess immune function is measurement of cytokines known to be instrumental to development, maturation, differentiation, and response of the immune system. Cytokines can be measured by the ELISPOT, an ELISA type assay,

quantitation of mRNA concentration for given cytokines. IL-1 α , IL-10, IL-36, and IFN- γ are some important cytokines to phagocytic cell function and their role in recruiting and presenting antigen to T-cells. IL-1, IL-2, IL-4, IL-6, IL-7, IL-8, IL-9, and IL-10 are critical for the maturation and function of T-cells. IL-4 and IL-6 are secreted by T-cells as part of their role of aiding B-cells in maturation and proliferation in preparation for the process of differentiating into plasma cells and synthesizing specific antibodies. These cytokines are integral to the maturation and proliferation of B-cells in order for them to adequately differentiate into antibody secreting plasma cells (Klaassen, 2013; Murphy, 2012).

This study identified toxicological effects in a murine model that corresponded to relevant human exposure. What remains unknown is whether those that ride at the Nellis Dunes report higher than usual increases in respiratory diseases. In the state of Nevada, public health tracking of disease did not begin until the late 1990's and limited data regarding disease patterns are available. Additionally, the Las Vegas area is comprised of a transient population further complicating epidemiology studies. To learn more about how these murine data might correlate with disease, I would propose to examine hospital admissions related to lung pathology such as asthma, pneumonia, bronchitis, and COPD, as well as incidence of cardiovascular disease or stroke during and after the windy season to look for disease trends that might associate with dust exposure.

Though epigenetics has been understood since the early twentieth century, its implications to disease and medicine did not emerge until the 1980's when loss of DNA methylation was observed to be associated with colorectal cancers (Feinberg, 2008). This

understanding has led to the inquiry of clinical applications through epigenetic biomarkers not only in cancer but for neurodegenerative diseases and potentially even diabetes, or even epigenetic therapies for the treatment of disease (García-Giménez. 2012). A recent study by Suárez-Álvarez et al. (2013) is investigating the use of CpG methylation and histone modifications as biomarkers after solid organ transplant (SOT) to identify early signs of graft rejection and monitoring immunosuppressive therapy.

As we have made progress in identifying relationships between epigenetics and cancer, or other epigenetic pathological correlations, there is much still to be learned about epigenetics and its potential roles in affecting immunity. Assessing immune integrity is not only important in the clinical laboratory, but also necessary for development of safe new therapies, and epidemiological and public health studies. A number of drugs and environmental chemicals are known to modulate immune function, including: immunosuppression, hypersensitivity, asthma, inflammation, and autoimmunity (Calderón-Garcidueñas, 2009; Looker, 2014; Nadeau, 2010; Pollard, 2010). Establishing biomarkers to assess immunological competency can be difficult due to the dynamic nature of the immune system. Furthermore, immunological testing methods are incomplete. Current methods of assessing immune competency include enumerating white blood cell populations and type, functional natural killer cell activity in blood, autoantibody profiles, inflammatory markers in serum, and for population studies monitoring vaccine responses (Diamandis, 2010). However, the problem with these studies is that they only offer limited insight to the overall view of a complex system. The immune system is vulnerable to toxicants, and alterations are typically

observed in this system prior to damage in other systems such as the liver, kidney and heart. Therefore, expanding and refining tools for assessing immunological competency is critical to public health biomonitoring, and early detection of disease. Immune competency can be defined a number of ways and involves consideration of the innate, adaptive, humoral, and cellular components of the immune system. New “omics” technology is promising for mechanistic and screening applications that could provide insight about immune function.

Utilizing methylation marks could be more reliable than current methods of biomonitoring as these profiles are relatively stable and fairly impermeable to fluctuation regardless of physiological state and/or sample collection and storage conditions (How Kit, 2012; Ladics, 2007). For these reasons, epigenetic markers that can be used in place of physiological biomarkers as predictors of alterations in the fluctuating immune system should be considered. The use of epigenetics in cancer diagnosis and treatment has paved the way for DNA methylation as a diagnostic and therapeutic tool. From this, equipment and high throughput protocols already exist in a number of clinical laboratories. This existing epigenetics set up in clinical laboratories, and the rate with which the relationship between epigenetics and cancer has moved from the research lab into the clinical setting shows promise for utilizing epigenetic markers in other aspects of health care.

Data sets such as the International Human Epigenome Consortium (IHEC) (www.encodeproject.org/ENCODE), the NIH Roadmap Epigenomics program (www.roadmapepigenomics.org), and BLUEPRINT (www.blueprint-epigenome.eu)

and DEEP (www.deutsches-epigenom-programm.de) are developing reference data for the epigenome and some more specific regarding hematopoietic cells and tissues. These reference epigenome databases provide important baseline data of epigenetic marks that occur in normal and specific populations such as children, adults, and seniors. Therefore, epigenetic marks can be used as a biomarker and compared to reference genomes to determine if aberrant marks are indicative of pathology in an individual.

In this study, immunosuppression to geogenic dust from NDRA was detected for all sites surveyed. Furthermore, we identified other changes in health parameters that mechanistically linked to the exposure, such as Pb and Al concentrations and red blood cell results, and many that did not. Screening the DNA for global methylation changes allowed us to examine potential modes of action that had not manifest with more specific methods of detection. Because significantly different levels of 5-mC% were only detected in CBN7 and the response was not found to be dose responsive, it could be determined that CpG methylation was not the cause for decreased IgM production and that other avenues should be explored rather than further investigation of CpG methylation of additional transcription factor genes.

From this study we can conclude that the complex mixture of metals occurring in geogenic dust from the NDRA affects immunity, liver and kidney function, and the hematopoietic system. We can also show that epigenetic markers have the potential for alterations at high concentrations. Our investigation of methylation markers in the B-cell to plasma cell differentiating pathway did not produce a mode of action responsible for IgM suppression in mice, however it did direct us towards looking into other epigenetic

marks to further elucidate what is happening in the murine model. We will continue to search for an answer by next evaluating histone modifications as a potential mode of action for immunoglobulin suppression in the B6C3F1 mice exposed to dust from the NDRA.

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