Latent Effects of Exposure to Lead and the Association with Neurological Outcomes via Epigenetics and Genetics

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
2015

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Acknowledgements

I would like to begin by thanking God and family, especially my mother and father. Without that foundation, I would be nowhere.

My thesis advisors, Dr. Dana Dolinoy and Dr. Sung Kyun Park, have been absolutely amazing. Not only did Dana take me on as the first MD-PhD student in the EHS department, she also mentored me through a research project that was new ground for both of us. Dana allowed me the freedom to carve out a unique niche in the lab, and although isolating mouse neurons was painstakingly tedious, I cannot state enough how much I appreciate the support and big-picture mindset that Dana provided throughout the process. She has always had her students' best interests in mind and that realization has not escaped me either.

As for Dr. Park, I know I have probably raised his blood pressure by *at least* an amount equivalent to an IQR increase in Pb. Essentially, I am more harmful to my advisors than the Pb we study. As a teacher, Sung is a unique blend of gentle, yet intensely rigorous, and I cannot put into words how important it was for me to spend hours with him discussing how to conduct a study of environmental epidemiology. Sung has pushed me to make sure I understand the nuances of study design and analysis. The effort that Dana and Sung have put into my training is unbelievable.

As for my thesis committee members, Dr. Howard Hu was the first faculty in the School of Public Health I had contact with. His expertise in this field of using bone Pb as a marker of cumulative exposure has been extremely important.

Dr. Maureen Sartor has been extremely supportive and available whenever I have bioinformatics questions. Her expertise in analyzing genome-wide data is clearly invaluable, but she has also provided input on all parts of my dissertation.

Dr. Hank Paulson has also been a crucial member of my committee. Dr. Paulson was really the one who strongly suggested that I try genome-wide studies instead of looking for only a few candidate genes. I was fortunate enough to use the last batch of NimbleGen tiling arrays to carry this project out, and I am glad I did.

Dr. Kelly Bakulski was in the trenches with me for the most important part of my development as a scientist. For me, she has been a leader, teacher, friend, soundboard, emergency research contact, and Alps trekking partner. I learned almost every single lab skill from Kelly, as well as some coding in R. Not included in this dissertation are the plethora of other projects Kelly and I tried that did not come to full fruition: X-ray measurement of metals in human AD brain slices, effects of bisphenol A on DNA methylation in the brain, implementation of K-XRF measurements at the SPH, and many more. My only regret in interacting with Kelly is that I cannot pay her back for her invaluable guidance. I only hope I can be as unselfish, generous, and intelligent as her as I progress through my own career.

My entire lab deserves general acknowledgment, but several colleagues deserve special recognition. Dr. Chris Faulk and Amanda Barks managed the Pb-exposure mouse study from which I extracted brains. We've spent hours in the dissection room together,

not only extracting every organ from these mice, but also drafting up a zombie mouse movie, which will surely be a blockbuster. Dr. Caren Weinhouse was working on the NimbleGen tiling arrays just before I did, and her protocol and guidance was necessary for me to run the arrays myself. Tami Jones, our lab manager, cannot be thanked enough. Our whole lab would be lost without her. Everyone else in the lab has been incredibly supportive in fostering scientific discussion as well as practical lab advice.

It is also important to acknowledge the people at the Flow Cytometry Core, especially Mike Dellheim, Mike Pihalja and Aaron Robida. They ran my brain samples to isolate neurons and non-neurons. Without their expertise, this work would not have gotten done.

I am completely indebted to the Medical Scientist Training Program here at the University of Michigan. Ellen Elkin, Hilkka Ketola, and Laurie Koivupalo are amazing staff members at the MSTP and have provided me with essentials like funding guidance and healthcare. Dr. Ron Koenig has given me an opportunity of a lifetime. I was born in a rural hospital in India and subsequently grew up near the South Side of Chicago. The MSTP provides an opportunity that people from those areas can rarely dream of, much less afford financially. Ultimately, I hope to be able to utilize the skills I have learned in this training to benefit patients through both clinical and scientific means.

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Abstract

Cognitive function is measured on a spectrum that ranges from normal cognition to mild cognitive impairment to severe dementia, which may manifest itself as a neurodegenerative disease, such as Alzheimer's Disease (AD). Evidence suggests that the environment may interact with genes to influence susceptibility to cognitive decline via interaction with single nucleotide polymorphisms in genes or by modification of the epigenome. This work examines the association of a well-known environmental toxicant, lead (Pb), with cognition. The overall aim is to examine how genetics and epigenetics interact with Pb to influence susceptibility to cognitive decline.

The first specific aim of this dissertation characterizes the association between cumulative Pb exposure and longitudinal changes in scores on the mini-mental status exam (MMSE) as well as a measure of global cognition in a cohort of men in the Normative Aging Study (NAS). We use linear mixed effects models and generalized additive mixed effects models that incorporated cognitive function tests over up to 15 years. Among men 51-89 years of age at baseline, an interquartile range increase (IQR=21 μg/g) in Pb is associated with a lower baseline MMSE (β=-0.128,p=0.04) and a faster decline in the rate of MMSE (β=-0.016,p=0.04). We also report a suggestive association between patella Pb and the risk of cognitive impairment defined as MMSE score dropping below 25 using Cox proportional hazard models (hazard ratio=1.21, 95% CI:0.99-1.49).

The second aim investigates whether single nucleotide polymorphisms found to be associated with AD in genome-wide association studies modify the association between Pb and the risk of cognitive impairment in the same cohort. We analyze SNPs in genes encoding CUGBP,Elav-like family member 1 (*CELF1*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), clusterin (*CLU*), complement receptor (*CR1*), and apolipoprotein E (*APOE*). We find that an IQR increase in patella Pb confers a non-statistically significant change in risk of dropping below an MMSE score of 25 for subjects who were homozygous for the major allele at the *CR1* and *PICALM* genes. However, for carriers of the variant allele at *CR1* and *PICALM*, there was an increased risk of dropping below an MMSE score of 25 over the longitudinal observation period. The HR for *CR1* variants is 1.51 (95% confidence interval (CI): 1.03-2.21), and for *PICALM* is 1.44 (95% CI: 1.04-2.01). No significant modification of the effect of Pb on cognition by *APOE* is identified.

The third aim tests the hypothesis that DNA methylation is modified by *in utero* exposure to Pb in a mouse model. Offspring were exposed via the maternal drinking water to 0 ppm, 2.1 ppm, or 32 ppm of Pb two weeks before mating, throughout gestation, and three weeks after birth. Using NimbleGen Promoter Tiling Arrays, we probed DNA methylation levels in a neuron-specific cell population at a genome-wide level. Mice exposed *in utero* to 32 ppm Pb had 11,517 (1.7%) probes with differential methylation as compared to non-exposed mice at $p<0.005$ (lowest FDR=0.3002). Of these, 7554 (65.6%) were hypomethylated and 3963 (34.4%) were hypermethylated. We report novel exposure-dependent, differentially methylated regions associated with the following genes: histamine N-methyltransferase (*Hnmt*), selection and upkeep of intraepithelial T cells 5 (*Skint5*), Xylosyltransferase 1 (*Xylt1*), olfactory receptor 1085 (*Olfr1085*), protocadherin 19

(*Pcdh19*), and a retrotransposed gene for heterogeneous nuclear riboprotein C (*Retro-Hnrpc*). The role of *Hnmt* (histamine N-methyltransferase) is of particular interest, as it is associated with regulation of neurotransmitter levels.

Overall, this project illustrates that prior exposure to Pb can modulate cognition via interaction with genetic variants and the epigenome. This paves the way for understanding how environmental exposures exert latent effects over the lifetime of an organism vis-à-vis their interaction with the genome. Future studies need to biologically validate gene-environment interactions found in epidemiologic studies and also utilize mouse models to probe mechanisms of how the environment can modify the epigenome.

Chapter 1

Introduction

1.1 Overview of Dissertation

The current paradigm for understanding human disease involves two major features: genetic material, which is inherited and relatively unchanging; and the environment, which is constantly in flux. Inherent to the study of gene-environment interactions and disease outcomes are several challenging aspects. First, assessing the effect of exposure in a natural setting is difficult. Second, the interface between environmental toxicants and human physiology is poorly understood. Although technology to probe DNA sequences and gene expression has increased exponentially, our understanding of how genes interact to cause disease is still in its early stages. Additionally, the environment may exert effects on the epigenome, the layer "on top" of the genome that allows for its regulation. Thus, an interdisciplinary approach that utilizes both molecular and genetic epidemiology as well as molecular biology, is critical in understanding the interface between genes, the environment, and disease.

The objective for this dissertation is to characterize how chronic exposure to lead (Pb), a model neuro-toxicant, exerts effects on cognitive outcomes throughout an individual's life. We demonstrate this by utilizing epidemiologic methodology as well as mouse models to probe exploratory mechanisms of how Pb interacts with the genome. This thesis first establishes a general model delineating the association between cumulative Pb

exposure and longitudinal changes in cognition. From there, we probe the interaction of Pb with human single nucleotide polymorphisms (SNPs) associated with Alzheimer's Disease (AD) in genome-wide association studies (GWAS) and with the mouse epigenome.

1.2 Dementia and Cognitive Decline

Over the next few decades, a major challenge for medicine will be the delivery of healthcare to an aging population. In the United States (U.S.), the proportion of the population older than 65 is expected to rise from the current 13% to 19% by the year 2030 [1]. One of the devastating pathologies associated with aging is dementia, characterized by deterioration in cognition, function, and behavior. Patients with dementia are unable to perform basic activities of daily living, including dressing, feeding, and bathing. The prevalence of dementia, which is already at 35.6 million worldwide, is predicted to double every 20 years through the year 2040 [2,3]. Alzheimer's disease (AD) is the leading cause of dementia, and the risk of incident AD nearly doubles every five years after the age of 65 [2,3].

Cognitive function is thought to exist as a continuum on which dementia is the clinical endpoint. The rate of trajectory of cognition over the aging process is key in characterizing patients at risk for dementia. A normal process of aging includes an expected level of decline in memory, executive function and visuomotor ability. Patients with mild cognitive impairment (MCI), however, are a subgroup of patients that have faster declines in tests of the aforementioned functions, and are at an increased risk of developing dementia [4]. It is thus necessary to characterize factors that might precipitate or dampen the conversion from MCI to the full-blown dementia of AD or other neurodegenerative

disorders [5]. If the rate of cognitive decline can be further stratified by environmental risk factors, it may be possible to intervene in at-risk patients with neuropsychological rehabilitation [6] or pharmacologic treatments to alleviate symptoms of memory loss [7].

1.3 Alzheimer's Disease Epidemiology and Pathophysiology

AD is the most prevalent form of dementia, comprising approximately 60-80% of cases [8]. Early-onset AD (EOAD) occurs in people younger than the age of 60 and comprises about 5% of all AD cases. EOAD is associated with highly penetrant genetic mutations in the gene for amyloid precursor protein (*APP*) as well as genes in the APP processing pathway, presenilin 1 (*PSEN1*) and presenilin 2 (*PSEN2*) [9,10]. These latter two proteins, along with anterior pharynx-defective 1a (APH1a), are part of a gamma-secretase complex [9]. Along with beta-secretase 1 (BACE1), gamma-secretase cleaves APP to form the amyloidogenic cleavage products $A\beta_{40}$ and $A\beta_{42}$ that aggregate into the characteristic histopathologic plaques of AD [11]. On the other hand, the ADAM proteases (a' disintegrin and metalloproteases) such as ADAM10 and ADAM17 are alpha-secretases that act on APP to create non-amyloid-forming cleavage products [12-14].

Although genetics explains much of the susceptibility to EOAD, the etiology of late-onset AD (LOAD) is much less clear. Several studies have identified the apolipoprotein E-ε4 (*APOE-ε4*) allele as a risk factor for LOAD [15-19]. A putative function of the APOE protein is the promotion of proteolytic degradation of Aβ. The *APOE-ε*4 isozyme is thought to be less efficient in this process compared to *APOE-ε*2 [20]. Additional GWAS have identified apolipoprotein E (*APOE*), phosphatidylinositol binding clathrin assembly protein (*PICALM*), clusterin (*CLU*), complement receptor (*CR1*), and CUGBP,Elav-like family member 1

(*CELF1*) as genes that modify the risk of LOAD, among others [16,18,21,22]. *APOE*, *PICALM*, *CLU*, and *CR1* are of particular importance because they have also been shown to modify non-pathologic cognitive decline, implicating that they may modify the progression towards dementia [23-25]. However, all of the genes discovered via GWAS account for up to only 33% of the variation in liability to AD [26,27]. This suggests that either there are unknown genes that play a role in pathogenesis of disease, or that environmental exposures confer a substantial risk of developing AD dementia.

1.4 Environmental Exposure to Pb

Prior to the banning of Pb in gasoline, paint, canned goods, and other consumer products in the 1970s and 1980s in the U.S. and other countries, the general public was ubiquitously exposed to Pb [28]. Policies aimed at reducing Pb exposure such as the removal of Pb from gasoline, children's toys, and municipal drinking water have dramatically decreased adverse outcomes in children and increased economic productivity [29]. After the phaseout of leaded gasoline began in 1976 in the U.S., mean blood Pb levels (BLL) dropped from 12.8 μg/dL in 1976 to 2.8 μg/dL in 1992 [30,31].

Despite these reductions in exposure, global consumption of Pb is actually increasing today [32]. Pb is currently ranked number two on the priority list of hazardous substances by the Agency for Toxic Substances and Disease Registry [33]. At Superfund sites that are on the National Priorities List (NPL), Pb is particularly an issue due to its frequency, toxicity, and potential for human exposure [34]. $PbCl_2$ is produced at high temperatures needed for waste combustion and can be deposited into the soil and water surrounding municipal waste incineration sites [35]. Pb can also be inhaled near NPL sites

as a result of fly ash, which contains several heavy metals [35]. As of 2013, there are still over 135,000 U.S. children between ages 1-5 living with blood lead levels (BLL) greater than 5 μg/dL [36], the level beyond which the Centers for Disease Control and Prevention (CDC) recommend public health action [37]. An additional source of Pb exposure is from homes built before 1978. Leaded paint, plumbing pipes, and solder containing Pb may not have been removed from these homes, and thus serve as a persistent source of exposure via drinking water, house dust, or other kinds of ingestion [38,39]. Thus, populations that live near Superfund sites or in industrial areas with old homes are particularly vulnerable to the effects of Pb exposure [40]. Pb is a persistent environmental hazard abroad as well as in the U.S., with industrial emissions of Pb increasing across the globe [41,42]. Surprisingly, as of late 2014, areas in Afghanistan, Algeria, Iraq, Myanmar, North Korea, and Yemen have yet to ban the use of tetraethyl Pb (TEL) in gasoline [43].

The major routes of exposure to Pb are inhalation, ingestion through drinking water, and exposure to contaminated sediments and soil [44]. Of these routes, the World Health Organization estimates that 80% of the daily intake of Pb is due to ingestion of food, dirt, and dust [32,45,46]. Food-borne exposure varies depending on country, with certain populations in China receiving up to 7.7 μg/kg/day via rice consumption and drinking water, whereas the daily intake from food in the U.S. is close to 0 μg/kg [34].

1.5 Pb Pharmacodynamics

A dynamic, compartmental model can describe the body burden of Pb. The most basic version of this model states that Pb is distributed amongst three compartments: blood, soft tissue, and bone [47,48]. In adults, approximately 75-95% of the body burden of

Pb is stored in the bone compartment, 1.5-3.5% in the blood compartment, and the rest is stored in soft tissue including the brain, liver, and kidneys. In children, the distribution of Pb is skewed more towards soft tissue and away from developing bone [48]. Pb can serve to replace calcium (Ca^{2+}) in hydroxyapatite structures of bone, and thus has a longer, more stable half-life in bone as compared to other tissues. Although estimates of half-life vary depending on the population studied, blood lead is thought to decay in the body with a half-life of around 30 days [49], thereby serving as a proxy of ongoing steady-state or recently elevated exposure. On the other hand, bone Pb can be measured using K-shell X-ray Fluorescence (KXRF) in either trabecular or cortical bone, represented in this study by the patella and tibia, respectively. Trabecular bone has been reported to decline precipitously following initial measurements in men in the Normative Aging Study (NAS), followed by a relatively slower, steady state of decline [50]. This suggests that patella bone Pb represents a moving average of exogenous exposure to Pb over the last 10 years [50]. However, because of the higher turnover of trabecular bone, it is reasonable to interpret patella Pb levels as the predominant bone that provides Pb back to circulation [51]. Cortical Pb, on the other hand, has recently been shown to have a half-life ($t_{1/2}$) of 48.5 years, much longer than previously thought [50]. Thus, tibia Pb can be interpreted as the long-term cumulative exposure over much of an individual's lifetime. Interpretations of bone Pb measures vary depending on the population. Pregnant woman and the elderly, for example, have much higher rates of bone turnover, which can cause Pb mobilization into the blood compartment, where it is more toxic to tissues [52].

1.6 Pb and Effects in the Brain

1.6.1 Acute Neurotoxicity of Pb – Neurotoxicity from Pb can be classified as either acute or chronic. Acute symptoms of Pb exposure involve rapid onset of nausea, headaches, and changes in cognition. The direst effect of extreme doses of Pb is lead encephalopathy, in which the sub-acute symptoms of headache, irritability, mental dullness and tremors may worsen to paralysis, convulsions, coma, or death [53]. Acute high-dose Pb toxicity is mediated by morphological alterations of the nervous system, such as alteration of synapse formation and disruption of neuronal migration and differentiation [53,54]. Additionally, Pb can replace Ca^{2+}, thereby modulating ion channel function and neurotransmitter release [55]. Pb also acts to interfere with calcium release from the mitochondria, which promotes formation of reactive oxygen species [56,57]. Epidemiologic data at a sub-acute time-scale of toxicity (as measured by blood lead, on the order of weeks to months) in children corroborates toxicological findings. Children exposed to Pb at BLL's ranging from 10-30 μg/dL exhibit markedly decreased IQ scores relative to those with BLL's <2.4 μg/dL [58]. In a study done in Chennai, India where the mean BLL was >10 μg/dL, increases in BLLs were significantly associated with impaired visual-motor ability [59] and higher scores in anxiety and attention deficit/hyperactivity disorder (ADHD) indices [60]. In adults, increases in BLLs were significant predictors of cross-sectional tests of performance in memory and language [61]; attention, visuospatial, and visuomotor ability [62]; and mini-mental status exam (MMSE) scores [63].

1.6.2 Developmental Origins of Health and Disease and the Chronic Neurotoxicity of Pb – Mechanisms of the long-term effects of Pb are less well delineated. Indeed, it is possible that the morphological damage exacted by Pb on the brain early in life simply

carries over throughout the life course. However, there is evidence that early life Pb exposure may act in accordance with the Developmental Origins of Health and Disease (DOHaD) hypothesis, which states that environmental insults ranging from nutrient starvation to active exposure to toxicants cause damages to the developing body that manifest once the organism is older [64,65]. Indeed, early life exposure to Pb has been shown to result in AD-like pathology in monkeys. In monkeys exposed to Pb in the first year of life, histopathological lesions of Aβ plaques were found 20 years later, in conjunction with upregulation of genes important in the APP pathway [66].

Increasing evidence points to epigenetics as a potential mechanism by which Pb exerts latent, long-term effects [31]. The epigenome is the set of factors other than the DNA sequence that exerts control over genetic regulation. Although every cell in an individual carries the same complement of DNA, its epigenome varies across different cell types [67]. Thus, it is critical to analyze epigenetic changes in a cell type-specific context. One of the major covalent epigenetic regulators of the genome that is relatively stable over time, yet susceptible to environmental perturbations early in life, is DNA methylation. Prior work from our lab exposing viable yellow Agouti mice (A^{vy}) *in utero* to Pb has revealed dose-specific changes in DNA methylation, indicating that early Pb exposure influences epigenetic reprogramming [68]. The epigenome is vulnerable to change at multiple points in life, but due to waves of reprogramming during embryogenesis, effects of *in utero* exposure are especially cogent [69]. Additionally, as shown in a study of human LOAD cases vs controls, there appears to be a modest discordance in DNA methylation in LOAD cases [70]. Rats [71] and monkeys [72] administered physiologically relevant levels of Pb (blood levels~19-26 μg/dL) early in life had an increase in 8-oxo-dG, a marker for oxidative stress

found on DNA, in the brain when measured later in life. A decrease in DNA methyltransferase activity (DNMT) was also observed in the monkey brains. Together, these findings indicate that early exposure to Pb may modulate oxidative stress and DNA methylation pathways, and ultimately, gene expression relevant to neurological outcomes that may manifest later in life.

To our knowledge, there are no epidemiologic studies that correlate very early-life Pb exposure to cognitive outcomes. However, long-term Pb exposure can be measured in tibia and patella bone, as mentioned previously. In the NAS population, a community dwelling cohort, patella Pb was significantly associated with a change in MMSE score from the first visit to second visit [72]. Tibia and patella Pb were associated with visual-spatial tests [73]. However, both of these studies looked at only the difference in scores between two consecutive tests. A longitudinal study which looks at several repeat measures would be more useful in characterizing the association between environmental risk factors, genetics, and cognitive decline over time.

1.7 Specific Aims of this Thesis

1.7.1 Analysis of Cumulative Pb Exposure and Longitudinal Change in Cognition –

The rate of cognitive decline is accelerated in patients with MCI who are at risk for dementia. Understanding the effect of environmental exposures can help identify how patients respond to environmental insults. The first aim of this thesis was to establish a model of the association of cumulative Pb exposure with the rate of change in scores on the MMSE. We also tested the association of cumulative Pb exposure with individual tests of

cognition spanning memory, language, and visuospatial domains; as well as a summary score of a battery of cognitive tests that we termed "global cognition".

1.7.2 Gene-Environment Interactions and their Association with MMSE Scores –

APOE, *PICALM*, *CLU*, and *CR1* all modify the susceptibility to AD [16] and cognitive decline [23-25]. *CELF1* is another risk susceptibility gene for AD, and is also associated with plasma homocysteine levels [74]. Since Pb is associated with plasma homocysteine as well [75], *CELF1* is a particularly interesting candidate for study of gene-environment interaction. The second aim of this thesis uses the model developed in the first aim to understand how cognition-related genes modify susceptibility to Pb exposure-related cognitive decline.

1.7.3 In Utero Pb exposure and Neuron-specific Epigenetic Changes in Mice –

The third objective of this thesis is to explore modification of DNA methylation as a possible mechanism by which Pb may exert a chronic, latent effect. Studies by Zawia *et al* suggest that early life exposure to Pb may modulate DNA methylation at a genome-wide level [76]. Additionally, Alzheimer's cases show modest differences in DNA methylation as compared to control [70]. It is possible that larger changes occur in one cell type, but are masked when evaluated in bulk tissue. No prior studies have conducted genome-wide DNA methylation analyses in neuron-specific cells extracted from mice that were subject to any kind of environmental exposure. The third aim of this thesis is to characterize genome-wide differential methylation in neurons of 10-month old mice that were exposed to varying levels of Pb *in utero* through maternal drinking water.

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CHAPTER 2

The association of Pb exposure and longitudinal changes in Mini-Mental Status Exam scores, global cognition and domains of cognition over time

2.1 Abstract

Lead exposure is associated with declines in cognitive function, including screening measures such as the Mini-Mental Status Exam (MMSE) as well as measures of specific cognitive domains. However, little is known about the associations between bone Pb concentrations and longitudinal changes in cognition over the course of 10-15 years. Our aim was to examine the longitudinal associations of cumulative lead exposure with baseline level and changes in scores on the MMSE, individual tests of cognition, and global cognition, which is a summary score of a battery of cognitive tests. In a subcohort of the VA Normative Aging Study (n=741 for MMSE, n=715 for global cognition between 1993 and 2007), we used linear mixed effects models to estimate the associations between patella or tibia bone Pb concentrations, estimates of cumulative exposure, and repeated measures of cognition (MMSE scores, individual tests of cognition, and global cognition). Among men 51-89 years of age at baseline, patella Pb was associated with MMSE, both at baseline and longitudinally over a period of 15 years. Patella Pb was not associated with summary scores of global cognition at baseline or longitudinally. Tibia Pb was not associated with baseline measures or changes in MMSE or global cognition. Associations between Pb and

baseline level or change in individual tests of cognition varied by domain. We found an association between Pb and changes in cognition over time in several measures of cognition, including MMSE and individual tests.

2.2 Introduction

Approximately 13 percent of the US population is aged 65 and older, and that demographic is expected to reach 19 percent by 2030 [1], leading to increased numbers of older adults susceptible to cognitive impairment and neurodegenerative diseases [2,3]. Worldwide, an estimated 35.6 million people were living with dementia in 2010, and this figure is expected to nearly double by 2030 [4]. Cognitive impairment is part of a spectrum that deviates from normal aging, eventually leading to dementia. Dementia and mild cognitive impairment are associated with reductions in memory, visuospatial, orientation, and language domains.

Exposure to lead (Pb) adversely affects cognition, independent of age-related cognitive decline. In adults, Pb exposure from occupational or environmental sources has been inversely associated with scores on the Mini Mental Status Exam (MMSE) [5-8]. Patella Pb was associated with declines in scores on the MMSE, [9] as well as measures of visuospatial and visuomotor ability [10], indicating that a mobilized, accumulated Pb burden may impact cognition [11]. Combined, these studies indicate that multiple markers of cognition are needed to evaluate the effects of Pb on global as well as specific measures of cognition.

Prior research has only measured changes in MMSE or other cognitive tests over two visits. In most cognitive aging studies, mean MMSE score increases between first and second repeat tests, attributed in part to a learning effect, where subjects recognize questions from previous tests [12,13].

This study evaluated the associations between long term Pb exposure and longitudinal changes in cognition repeatedly measured for over 10 years. As such, the learning effect can be accounted for over more tests per subject. Multiple repeat measures over time analyze change in score, allowing for less susceptibility to confounder bias and can also strengthen the case for lead being part of pathophysiology in causing cognitive decline. Our hypothesis is that bone Pb is associated with decline over 10-15 years, and we tested this by evaluating the association between Pb and the following measures: MMSE, a created summary score called global cognition, and domain-specific individual tests.

2.3 Methods

2.3.1 Study Population – This research was conducted on a subgroup of the VA Normative Aging Study, which is a longitudinal cohort established in Massachusetts in 1963.[14] Healthy men (n=2,280), between the ages of 21-80, were recruited and participated in clinical examination and complete health and lifestyle questionnaires every 3 to 5 years. At enrollment, men were excluded from the study if they had a past or present history of heart disease, cancer, diabetes, gout, asthma, sinusitis, bronchitis, peptic ulcer, or blood pressure greater than 140/90 mm Hg. Starting in 1993, 1131 men underwent a battery of tests to assess cognitive function (described below).

From 1991 until 1999, 876 participants had bone Pb measurements. The overlap of subjects with both patella and tibia bone lead measurement and cognitive measurement included 795 subjects. We further excluded subjects who had a patella Pb or tibia Pb measure with uncertainty greater than 15 μg/g (n=2) or 10 μg/g (n=6), respectively, and this yielded 788 subjects (n=2397 observations). Measurements with high uncertainty in bone Pb usually indicate excessive subject movement during measurement.[15] For the MMSE analysis, we excluded subjects who did not have an MMSE score (n=43), yielding 776 subjects at baseline (n=2245 observations). Finally, we excluded subjects with missing data on covariates: education level (n=30 subjects), and alcohol intake (n=8 subjects)). This yielded a final number of 741 subjects with at least one MMSE assessment and full covariate data for the MMSE analysis. The sixth (n=20) and seventh assessments (n=1) for MMSE were dropped to account for influential outliers, resulting in 2132 total observations for the 741 subjects.

In order to validate the longitudinal regression, we also ran a logistic regression model using the MMSE score outcome. The model tested the association between bone Pb and the OR of a subject registering an MMSE score less than 25 at any time point after baseline. In this logistic model, we excluded subjects with baseline MMSE scores less than 25. Additionally, in order to pickup on any possible point in time where the subjects score dropped below our specified cutoff of MMSE <25, we had to assess change in score, and thus it was necessary to exclude any subjects who did not have at least two tests. Additionally, we did not drop the sixth and seventh assessment, although one patient with seven assessments had a baseline score less than 25, and was therefore dropped. This resulted in 521 total subjects at baseline.

A similar exclusion based on missing data for covariates of interest yielded 715 subjects with at least one measure of global cognition (n=1410 observations). Here, the fourth assessment was dropped to account for influential outliers, resulting in 1365 total observations for the 715 subjects analyzed for changes in global cognition.

2.3.2 Exposure Assessment – Bone lead was measured using K-shell X-ray fluorescence (KXRF) spectroscopy as previously described.[10] Briefly, ^{109}Cd gamma rays excite the K-shell electrons of Pb embedded in bone, which emit an X-ray photon that can then be detected. An ABIOMED KXRF Instrument was used to measure lead at the tibia and the patella, corresponding to cortical and trabecular bone, respectively. The KXRF beam collimator was directed perpendicular to the tibial midshaft and at a 30^{o} angle from the horizontal for the patella for 30-minute measures.[10,16-18]

2.3.3 Cognitive assessments – Several cognitive screening tools were used for this analysis: the MMSE, NES2 (Neurobehavioral Evaluation System 2),[19] CERAD (Consortium to Establish a Registry for Alzheimer's Disease),[20] and WAIS-R (Wechsler Adult Intelligence Scale-Revised).[21] The MMSE assesses overall cognition by testing several domains including memory, visuospatial ability, attention, language, and orientation. Validated in multiple populations, the MMSE is widely used as a screening test for dementia,[22] and to assess cognitive decline in non-demented populations.[23] Declines of the MMSE over time may indicate underlying pathologies such as Alzheimer's disease, where the MMSE declines by an average of 1.8-4.2 points per year,[24-26] or normal

cognitive declines associated with aging. The MMSE includes 30 questions, but the present analysis did not include "Which County are we in?" because counties in Massachusetts do not have political meaning. Thus, the maximum score was 29. These measures were taken repeatedly over 20 years, generally every 3 years.

We also assessed scores from 7 individual tests used in the NES2, CERAD, or WAIS-R, which have been described elsewhere [6,27,28]. Two of the tests, word list total recall and verbal fluency (both CERAD), are generally associated with language. The digit span backward sum test (WAIS-R), total number recalled for digit span test (WAIS-R), and word list delayed recall test (CERAD) are proxies for memory. The pattern recognition test (NES2) and visual drawings summary score (CERAD) serve as proxies for visuospatial ability. These tests do overlap in their associations with memory, language and visuospatial ability, but are mentioned only in order to group tests into categories for easier interpretation. In addition to assessing each of these tests individually, we calculated a summary score of these tests, referred to as "global cognition", which we tested as a separate proxy for worsening neurological impairment. To standardize comparisons in creating the summary score, we calculated z-scores for each test by subtracting the observed value for any subject at any time from the mean baseline cognitive score, and divided that value by the standard deviation of the baseline cognitive score. Overall global cognition was assessed by average z-scores of 6 of the tests (total number recalled for digit span backward was excluded due to similarity with digit span backward sum).

2.3.4 Other Covariates – We included age at first cognitive test, highest education level and baseline smoking status, and alcohol intake as covariates in our regression model. Smoking status was assessed by questionnaire and subjects were re-categorized as "current smokers", "had smoked in the past and quit", or "had never smoked". Alcohol intake was assessed by whether a person had more than two alcoholic beverages per day. Education level was categorized from years of education separately for the MMSE and the NES analyses. For the MMSE analysis, subjects were categorized as not having finished high school (<12 years), those who were high school graduates or completed some college (12-15 years), and college graduates (>16 years).

2.3.5 Data Analysis – The datasets used in this study were managed using SAS software (version 9.4 for Windows) and all analyses were performed using R Software version 3.1.0. We used linear mixed effects models to assess the association between baseline Pb exposure and longitudinal changes, separately in each of the measures of cognition described above. Our baseline model included baseline patella Pb, time from first visit, the interaction of Pb and time, and baseline covariates (age, alcohol intake, smoking, and education). Linear mixed effects modeling allows for differences in the number of repeated measures across a subject's visits. Our linear mixed effects model included random intercepts for individual as well as random slopes for time in order to account for correlations among the repeated measurements.

To account for the learning effects with MMSE and global cognition, we ran a second model, referred to as the First-test indicator model, that was the same as the basic model,

with the addition of an indicator variable to adjust for whether or not a test was the subject's first assessment.

Model 1 (Basic Model):

$$Cognitive\ test \approx B_0 + B_1 \times Pb + B_2 \times Time_{fromVisit1} + B_3 \times (Time_{fromVisit1} \times Pb) + B_4 \times Age_{FirstCognitiveTest} + B_5 \times Education + B_6 \times SmokingStatus + B_7 \times Alcohol$$

Model 2 (First-test Indicator model):

$$Cognitive\ test \approx B_0 + B_1 \times Pb + B_2 \times Time_{fromVisit1} + B_3 \times (Time_{fromVisit1} \times Pb) + B_4 \times Age_{FirstCognitiveTest} + B_5 \times Education + B_6 \times SmokingStatus + B_7 \times Alcohol + B_8 \times FirstTestIndicator$$

This model, similar to one employed in prior studies [29] can partially account for the learning effect by separating the change from visit 1 to visit 2, and regressing based on longitudinal changes from subsequent visits. We found no difference among the results for these models. However, because MMSE is susceptible to a learning effect, as we observed in our study (Table 2), we chose to report analyses based on the first-test indicator model. Additionally, MMSE has a ceiling effect and may not be best modeled linearly. Thus, we also ran a cox proportional hazards model where the dichotomous outcome was whether or not a patient dropped below a cutoff score of 25 on the MMSE assessment at any point during enrollment. The longitudinal analyses of individual tests and summary measure of global cognition were reported from the basic model only.

We performed several sensitivity analyses to test the robustness of our findings. First, there is a possibility for a selection bias related to health factors driving cohort participation in general and also the exclusion criteria at initial NAS recruitment. In order to minimize this, we excluded subjects who were greater than 45 years of age at the time of NAS study enrollment because people over 45 would be more likely to exhibit health symptoms that would keep them out of the NAS and thus be more susceptible to these selection pressures. As another sensitivity analyses, we repeated our analyses with additional covariates including the history of a diagnosis of either hypertension, stroke, chronic heart disease or myocardial infarction, as those might be potential mechanisms by which cognitive decline is occurring and have been previously associated with Pb exposure.

2.4 Results

2.4.1 Descriptive Statistics – Among the 741 men with MMSE scores, mean patella Pb concentrations were 30.64 μg/g (SD=19.44) and tibia Pb was 21.62 μg/g (SD=13.33) (Table 1). The average participant age was 67.77 (SD: 6.82) at the time of the first cognitive assessment (51.4-98.0 years). The mean time between cognitive assessments was 4.8 years. The mean MMSE score for all subjects increases by 0.4 from visit 1 to visit 2, suggestive of a learning effect (Table 2). However, we see a subsequent decline of 0.6 points in mean scores from visit 2 to visit 5. There was a negligible increase in the Z-score measure of global cognition between visits 1 and 2, and a decrease in the mean z-score by visit 3.

2.4.2 Associations between Lead Concentrations and MMSE – Table 3 presents longitudinal associations of bone lead with changes in MMSE score from the linear mixed effects models. At time=0 (baseline), each IQR difference in patella Pb (21 ug/g) was associated with a 0.15 point decrease in MMSE score ($p = 0.04$). IQR differences in tibia Pb (15 ug/g) were not significantly associated with baseline MMSE scores ($p = 0.15$). As expected, MMSE scores display an overall decline with time. There was a 0.096 point decrease in MMSE score per year when patella Pb was modeled ($p < 0.0001$), and a 0.095 point decrease per year when tibia Pb is modeled ($p < 0.0001$). We observed a significant association between the interaction term for time and patella Pb and MMSE, indicating faster declines in MMSE scores as patella Pb levels increase (Table 3). On average, MMSE scores were 0.016 points lower per IQR increase in baseline patella Pb per year of follow-up (p-value = 0.04) (Figure 1). This suggests that for every 10 years of follow-up the MMSE scores are expected to drop by 0.16 points per IQR increase in baseline patella Pb. We found a less significant interaction between tibia Pb and time ($p = 0.19$).

We also assessed whether or not each subject dropped below a cutoff MMSE score of 25 (Table 4). An IQR increase in patella Pb was associated with a 1.21 times greater risk of having an MMSE score dip below the cutoff of 25 (95% confidence interval (CI) of HR: 0.99-1.49; $p = 0.07$). The same analysis done with tibia Pb yields an insignificant association with MMSE score (HR=1.31; 95% CI: 0.89-1.94; $p = 0.18$).

2.4.3 Associations between Lead Concentrations and Global Cognition – As expected, time was significantly associated with global cognition z-scores, with a 0.15 point decrease in z-score associated with 1 year of follow-up time for either analysis including tibia or

patella Pb ($p < 0.0001$ for both). Although the association of tibia Pb and patella Pb with baseline global cognition z-scores did not reach the alpha-level of 0.05 for significance, there was a slight suggestion of association ($p=0.16$ for tibia Pb and $p=0.069$ for patella Pb). Additionally the interaction between Pb and time was not significantly associated with Z-scores of global cognition in either measure of bone Pb.

2.4.4 Associations between Lead Concentrations and Cognitive Domains – In order to organize results from individual tests, each test was assigned loosely to a domain, and is reported under that domain in Table 6. The trajectories of each cognitive test score varied by domain (language, memory, visuospatial). In the language domain, there was a significant main association between time and patella and tibia Pb, respectively. There was a 0.014 point decrease in the word list total recall z-score for every interquartile increase in patella Pb per year of follow-up ($p = 0.04$). A similar result was seen for the word list delayed recall test, which is a similar test, although shown here as different domain. An IQR increase in patella Pb was associated with 0.014 point decrease in word-list delayed recall per year of follow-up ($p = 0.03$). There were no significant associations between changes in language or memory domain tests and the interaction between tibia Pb and time of follow-up.

In the visuospatial domain, the baseline mean visual drawings summary score was associated with a 0.108 point decrease for every IQR increase in patella Pb. Patella Pb concentration was not associated with pattern recognition scores over time ($p_{interaction\ PbxTime} = 0.35$) or visual drawings summary score over time ($p_{interaction\ PbxTime} = 0.57$) We also saw an overall decrease with time in both test scores included in the visuospatial domain.

Pattern recognition scores decreased by 0.017 points per year of follow-up (p = 0.026), and visual drawing summary score declined by 0.043 points per year of follow-up (p < 0.0001). Tibia Pb parameter estimates were within 5% of the values we reported with patella Pb. However, there was an additional significant interaction between time and tibia Pb not found with patella Pb in the visuospatial domain. Every IQR increase in tibia Pb was associated with a 0.016 point *increase* in the visual drawing summary z-score per year change in time ($p_{interaction\ PbxTime}$ = 0.03).

2.4.5 Sensitivity Analyses – Our sensitivity analyses yielded similar results for the parameters of interest ($\beta_{Time\ Of\ Follow\ Up}$, β_{Pb}, $\beta_{Pb*Time\ Of\ Follow\ Up}$) within 10% of magnitude of the association found in the primary analysis. This was true for the analysis in which we excluded subjects who were >45 years of age at enrollment as well as the analysis in which we accounted for history of hypertension, stroke, chronic heart disease or myocardial infarction. There was one exception in the model in which subjects older than 45 years at entry into the NAS were excluded. The magnitude of the association between MMSE and the interaction of tibia Pb and time was 31% of that found in the primary analysis ($\beta_{Pb*Time\ Of\ Follow\ Up} = 0.0034$, $p = 0.72$).

2.5 Discussion

We have further characterized the association between cognition and the interaction of bone Pb with time of follow-up. Our data suggest that the longitudinal trajectory of the MMSE score is associated with bone Pb levels and does change with time,

which is consistent with previous literature. As shown in table 2, MMSE scores slightly rose from visit 1 to 2 (on average), but then declined. The decline in MMSE scores from visits 2 to 5 was consistent with trends observed previously in the NAS [9].

In order to contextualize the effect size of parameter estimates, we reported interquartile ranges of Pb for tibia and patella. For example, the magnitude of association between each IQR increase in patella lead and MMSE score is 1.2 times that of the average yearly cognitive decline. In other words, an individual with an amount of patella Pb that is 21 ug/g higher is predicted to have a cognitive score that is 83% of that in an individual who aged an additional year without the additional IQR increase in Pb. Thus, our findings suggest that patella Pb has an important effect compared to age, a factor known to influence cognition [13]. The effect that patella Pb has over time is 16% of the average yearly decline in MMSE, as seen by the interaction term of $\beta_{Pb*Time\ Of\ Follow\ Up}$. In other words, an individual in our study would have to age 0.16 years in order to predict the same decline associated with an IQR increase in patella Pb.

Each individual cognitive test can be roughly assigned to a domain of cognition, although there is overlap between domains. In our analysis, the language domain included word list total recall and verbal fluency; the memory domain included digit span backward sum, total number recalled for digit span, and word list delayed recall; and the visuospatial domain included pattern recognition and visual drawings summary score. Age has varying effects on different domains and tests of cognition. For example, the individual tests of cognition that decline with age, when measured separately, have parameter estimates for $\beta_{Time\ Of\ Follow\ Up}$ that are 13-40% smaller in magnitude relative to the same parameter

estimates found using global cognition measures, suggesting that aging may have a cumulative effect on overall cognition rather than each individual test.

Our findings are consistent with other studies. It has been established that cross-sectional cognitive test scores vary with bone Pb [6,7,9,30]. Additionally, in the same population, the change in cognition was measured by the difference in MMSE scores between the first two visits only [9]. Although the previous study did not incorporate up to five visits, it still observed a significant association between increasing Pb and decreasing MMSE (one-IQR higher patella Pb concentration associated with -0.24 point change in MMSE), which was about 1.5 times the magnitude of what was found in our present study. Our data add to these findings by assessing longitudinal repeat measures from 15 years rather than 3-4 years. The decline of scores over this longer period of time is larger than the practice effect, allowing for effective modeling. Other studies of longitudinal change in cognition have noted similar trends as well [11,29,31].

Additionally, we noted that several individual cognitive test scores (word list recall, both delayed and total) decreased faster with higher exposure to Pb. Although we did notice a positive interaction between time and tibia Pb in our visual summary score, the main effect of time and the main effect of tibia Pb were still inversely associated with MMSE. Additionally, our study finds a significant inverse association between the visual drawings sum test and both tibia and patella Pb, respectively. Overall, the visuospatial and language domain had significant associations with either tibia or patella Pb and time, or the interaction of Pb and time.

MMSE scores are not easily converted to Z-scores because they are more prone to non-linear change. In other words, a decrease from an MMSE score of 28 to 27 is much less severe than a decrease from 25 to 24. Thus, we also performed a separate logistic regression analysis of MMSE scores that takes into account the non-continuous nature of the variable. Our results from that analysis supported the findings of the linear mixed effects analysis. The results of the longitudinal model suggest that the trajectory of MMSE scores over time will vary depending on Pb exposure (Table 3). The logistic analysis supplements this finding, suggesting that patella Pb exposure may be associated with a clinically important shift in cognition.

A strength of the present study is the use of Pb measured in both cortical and trabecular bone, corresponding to the tibia and patella. These sites were chosen because of their differences in half-life. Reports on the half-life of Pb in bone vary by site as well as with factors such as age, prior exposure, and other conditions that modify bone turnover. Trabecular bone is reported to have an elimination half-life ($t_{1/2}$) of 8-20 years [16,32]. Cortical bone has a much longer half-life, with estimates ranging from 10-48.6 years [16,18,32]. Tibia bone Pb measures may be interpreted as the cumulative lead exposure in our population, whereas patella bone Pb serves as the predominant bone that provides Pb from bone resorption back to circulation [32]. This may explain why, in our analyses, patella Pb had a more statistically significant association with MMSE scores relative to tibia Pb.

Our study also has the possibility for selection bias due to loss to follow up. However, in data not shown, neither patella Pb nor tibia Pb was associated with number of follow-up visits. Our sensitivity analysis excluding people with less than three visits did not

affect parameter estimates. Additionally, MMSE and global cognition models were analyzed adjusting for follow-up time, and it was not significantly associated with the outcome.

The major strength of our study is that it employs longitudinal data from up to five visits, allowing us to observe changes in cognition over time. Additionally, bone Pb is a well-established biomarker of long-term Pb exposure. Our data employ both tibia and patella bone Pb measurements, allowing us to assess the effect of varying measures of long-term Pb exposure. Although a number of subjects have migrated over the 50 years of the study, the NAS is a cohort of men who have lived in a relatively homogeneous geographic area, allowing us to adjust for known confounders and mitigating the effect of unknown confounders. We have used longitudinal models to measure the association of an important toxicant with changes in cognition over time. Such models can be used for future purposes to assess the role of interaction amongst exposures or between gene and environment over time.

Table 2.1: Baseline Study Characteristics – Normative Aging Study. Boston, Massachussets

	MMSE	Global Cognition
Number of Subjects	741	715
Mean Age in years (SD)	67.77 ± 6.82	68.43 ± 7.11
Mean Patella Pb in µg/g (SD)	30.64 ± 19.44	30.47 ± 19.65
Mean Tibia Pb in µg/g (SD)	21.62 ± 13.33	21.39 ± 13.31
Education in number of years (%)		
<12	33 (4.4)	33 (4.6)
12-15 yrs	555 (74.9)	532 (74.4)
16+	153 (20.6)	150 (21.0)
Smoking Status (%)		
Never	221 (29.8)	209 (29.2)
Current Smoker	47 (6.3)	44 (6.2)
Former Smoker	473 (63.8)	462 (64.6)
More than 2 alcoholic beverages per day? (%)		
No	585 (78.9)	560 (78.3)
Yes	156 (21.1)	152 (21.3)

A total of 741 subjects who met restriction criteria and had both Pb and MMSE measures were included in the study. A total of 715 subjects had both Pb and individual test scores (which were used for creation of global cognition measure).

Table 2.2: Mean Cognition Scores (MMSE and "Global" Cognition) over time

Visit	N subjects	Mean MMSE (±SD)	N subjects	Mean Global Cognition Score (±SD)
1	707	26.9 ± 1.4	715	1.45E-06 ± 3.6
2	556	27.1 ± 1.6	452	0.0096 ± 3.8
3	404	26.7 ± 1.8	198	-0.12 ± 3.8
4	265	26.7 ± 1.8	n/a	n/a
5	125	26.5 ± 2.0	n/a	n/a

No subjects who had data available for bone Pb, cognitive tests and covariates had more than three visits, and thus visits 4 and 5 are designated as "n/a" in the column for mean global cognition score.

Table 2.3 - Association between Pb and MMSE over time

Patella Pb			
Parameter	**β-Estimate**	**95% CI**	**p-value**
IQR change in Pb	*-0.150*	(-0.275, -0.025)	*0.02*
Time	*-0.096*	(-0.118, -0.074)	*<0.0001*
IQR change in Pb*time	*-0.016*	(-0.031, -0.0009)	*0.04*
Tibia Pb			
Parameter	**β-Estimate**	**95% CI**	**p-value**
IQR change in Pb	-0.098	(-0.229, 0.034)	0.15
Time	*-0.095*	(-0.117, -0.073)	*<0.0001*
IQR change in Pb*time	-0.011	(-0.026, 0.005)	0.18

Each exposure (patella or tibia) was included as a predictor in a separate model. Covariates included baseline age, smoking status, education level, and alcohol consumption. Interpretation of parameters: Pb – baseline association of IQR change in patella Pb and MMSE; Time – change in MMSE for every IQR increase in Pb per year change in time; Pb*Time – change in MMSE for every IQR increase in Pb per year change in time. The IQR for patella Pb was 21 ug/g and for tibia Pb was 15 ug/g.

Table 2.4: Hazards ratios of MMSE decline in association with Bone Pb Levels

	Point Estimate	95% CI	p-value
HR associated with an IQR increase in Patella Pb	1.21	(0.99, 1.49)	0.07
HR associated with an IQR increase in Tibia Pb	1.05	(0.82,1.35)	0.70

Cox proportional hazards regression describing hazard ratio (HR) of having MMSE score drop below 25 (≤24) and patella or tibia Pb exposure. All models were adjusted for education, smoking status, and alcohol intake.

Table 2.5 – Association of Pb and Global Cognition over time

Patella Pb			
Parameter	**β-Estimate**	**Confidence Interval**	**p-value**
IQR change in Pb	-0.250	(-0.518, 0.019)	0.07
Time	*-0.147*	(-0.187, -0.107)	*<0.0001*
IQR change in Pb*time	-0.027	(-0.069, 0.014)	0.20
Tibia Pb			
Parameter	**β-Estimate**	**Confidence Interval**	**p-value**
IQR change in Pb	-0.206	(-0.453, 0.089)	0.16
Time	*-0.148*	(-0.184, -0.106)	*<0.0001*
IQR change in Pb*time	0.002	(-0.040, 0.038)	0.93

This was modeled similarly to the regression shown in Table 2.3. Global cognition was created as the average of the sum of z-scores of 6 of the individual test. All models were adjusted for education, smoking status, and alcohol intake.

Table 2.6: Association of IQR increase in Pb and individual cognitive tests over time

Patella Pb	*Effect of Baseline Pb*		*Effect of Time*		*Effect of Time*Pb Interaction*	
	Parameter	P-value	Parameter	P-value	Parameter	P-value
Language Domain						
Word List Total Recall	0.008	0.83	**-0.024**	**<0.001***	**-0.014**	**0.04***
Verbal Fluency	-0.04	0.29	**-0.018**	**0.008***	-0.007	0.29
Memory Domain						
Digit span backward sum	-0.041	0.28	0.001	0.85	-0.005	0.52
DSBT (total #recalled for digit span)	-0.045	0.24	-0.004	0.55	-0.008	0.26
Word List delayed Recall	0.013	0.74	**-0.03**	**<0.0001***	**-0.014**	**0.03***
Visuospatial Domain						
Pattern Recognition, #correct	-0.053	0.15	**-0.017**	**0.03***	0.004	0.57
Visual Drawings, sum	**-0.108**	**<0.01***	**-0.043**	**<0.0001***	0.007	0.35
Tibia Pb	*Effect of Baseline Pb*		*Effect of Time*		*Effect of Time*Pb Interaction*	
	Parameter	P-value	Parameter	P-value	Parameter	P-value
Language Domain						
Word List Total Recall	0.04	0.33	**-0.024**	**0**	-0.006	0.42
Verbal Fluency	-0.05	0.24	**-0.018**	**0.01**	-0.005	0.47
Memory Domain						
Digit span backward sum	-0.039	0.36	0.001	0.87	-0.001	0.92
DSBT (total #recalled for digit span)	-0.046	0.29	-0.004	0.53	-0.004	0.58
Word List delayed Recall	0.076	0.07	**-0.031**	**<0.0001***	-0.008	0.26
Visuospatial Domain						
Pattern Recognition, #correct	-0.072	0.08	**-0.017**	**0.03***	0.006	0.47
Visual Drawings, sum	**-0.142**	**<0.01***	**-0.043**	**<0.0001***	**0.016**	**0.03**

Linear mixed effects regression of the association between individual cognition tests and patella or tibia Pb exposure, with adjustment education, smoking status, and alcohol intake.

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CHAPTER 3

Effect Modification by Cognition-related Polymorphisms of the Association between Cumulative Lead Exposure and Cognitive Decline

3.1 Abstract

Bone lead is a significant predictor of declines in scores on the Mini Mental Status Exam (MMSE) in older adults, indicating that accumulated lead burden may impact cognitive function. It is unknown if genes associated with Alzheimer's disease and cognitive impairment modify the association between lead and cognitive function. We aimed to evaluate whether *APOE* (rs429358 and rs7412), *PICALM* (rs3851179), *CLU* (rs11136000) *CR1* (rs6656401), and *CELF1* (rs7933019) polymorphisms found to be associated with Alzheimer's disease in genome-wide association studies modify the association between cumulative lead exposure and incident cognitive impairment defined by having an MMSE score<25. A subset of men from the VA Normative Aging Study (n=574, mean age=67.2) had genotypes, bone lead, and MMSE scores assessed. Mixed effects regression as well as Cox proportional hazards modeling was used to assess the interaction of genotype and Pb to modify the hazard ratio (HR) of cognitive impairment over 12-15 years of follow-up. After adjusting for age, smoking status, alcohol intake, and education, the HR associated with an interquartile range (21 μg/g) increase in patella lead was 2.15 (95% confidence interval (CI), 1.36-3.41) for subjects heterozygote at the *CR1* locus, but it was 1.11 (95% CI,

0.85-1.44) for subjects with no *CR1* minor allele ($p_{interaction}$=0.01). For subjects who were homozygous for minor allele at *PICALM*, the HR associated with and IQR increase in patella lead was 3.54 (95% CI, 0.95-13.25), whereas a null association was found for men with no minor allele ($p_{interaction}$=0.08). We found no significant effect modification by ApoE. This study suggests that genetic variants associated with Alzheimer's disease and cognitive impairment may influence vulnerability to lead's effect on cognitive decline.

3.2 Introduction

As the proportion of the population that is over the age of 65 increases [1], the burden of neurological diseases is expected to increase along with it [2-5]. Although full-blown dementia is the most damaging clinical endpoint, it is increasingly becoming evident that neurological impairment occurs on a spectrum of worsening cognitive function. Dementia as a result of neurodegenerative disease such as Alzheimer's disease (AD) is a major concern, but mild cognitive impairment may be a transition stage from normal cognition into dementia [6,7]. There is clinical utility in the prediction of factors that push people along the spectrum of neurological function towards dementia in general, AD-related or otherwise [8].

Both genetic and environmental factors are thought to be associated with cognition. A meta-analysis of 74,046 individuals identified 11 new loci for susceptibility to AD [9], in addition to those that were previously well characterized [10-14]. Several reports suggest that approximately 24-33% of the variation in liability to AD disease can be explained by common single nucleotide polymorphisms (SNPs) [15,16]. Some AD-associated loci of note

are at ApoE-associated alleles rs429358 and rs7412, and near the phosphatidylinositol binding clathrin assembly protein (*PICALM*), clusterin (*CLU*), complement receptor-1 (*CR1*), and CUGBP, Elav-like family member 1 (*CELF1*) genes. In addition to their association with AD, many of these genes are also associated with several indicators along the spectrum of cognitive decline, including declines in mini-mental status exam (MMSE) scores [17], principle components of fluid-type cognitive tests [14], and trajectories of global cognition and attention tests [18].

Although genetic associations account for some of the variation in cognitive impairment, a major portion of the variance in cognitive liability is associated with either as-of-yet unknown genetic and epigenetic factors, or with environmental perturbations. Lead (Pb), well known to be associated with cognitive decline, is defined by several different measures. Bone Pb, an indicator of cumulative exposure to Pb, has been found to be associated with cross-sectional cognitive measures of language, memory and visuospatial ability [19,20] as well as with MMSE [21]. Of note, bone Pb levels also shown associations with longitudinal changes in a limited number of cognitive tests[22], also including the MMSE [23]. The first aim of this thesis expands on these studies by including data from at least 5 visits in a longitudinal analysis. We also found a significant association of patella Pb with longitudinal MMSE scores.

Individuals with the ApoE-ε4 are more likely to have lower cross-sectional scores on individual tests of cognition [24]. However, the interplay of genetics and environmental exposure in the association with longitudinal changes in global cognition as measured by the MMSE is of key interest in the current study. Pb has been shown to be associated with *APOE* expression levels [25], and *APOE* variants at rs7412 and rs429358 are CpG switches

[14]. A CpG switch occurs when a variant allele creates or deletes a new CpG site that may be methylated. This suggests that methylation may play a role in how Pb modifies ApoE. Additionally, there is a precedent for a gene-environment interaction between Pb and SNP's: genes in the iron metabolism [26] and oxidative stress [27] pathways have been shown to modify the association between Pb and cognition. Given prior association of Pb with expression of genes associated with neuro-degeneration, the current study aims to determine whether *APOE*-associated alleles rs429358 and rs7412, as well as *PICALM*, *CLU*, *CR1*, and *CELF1* modify the effect of Pb on longitudinal MMSE scores.

3.3 Methods

3.3.1 Study Population – This research was conducted on a subgroup of the VA Normative Aging Study, which is a longitudinal cohort established in Massachusetts in 1963 [28]. Healthy men (n=2,280), between the ages of 21-80, were recruited and participated in clinical examination and complete health and lifestyle questionnaires every 3 to 5 years. At enrollment, men were excluded from the study if they had a past or present history of heart disease, cancer, diabetes, gout, asthma, sinusitis, bronchitis, peptic ulcer, or blood pressure greater than 140/90 mm Hg. Starting in 1993, 1131 men underwent a battery of tests to assess cognitive function (described below).

From 1991 until 1999, 876 participants had bone Pb measurements. The overlap of subjects with both patella bone lead measurement and a mini-mental status exam (MMSE) assessment included 795 subjects. Our exclusion method followed that described in the study population section of Aim 1. Briefly, from the subjects who had an MMSE assessment

and bone lead measurements, we further excluded subjects who had a patella Pb or tibia Pb measure with uncertainty greater than 15 μg/g (n=2) or 10 μg/g (n=6), respectively, and this yielded 788 subjects (n=2397 observations). Measurements with high uncertainty in bone Pb usually indicate excessive subject movement during measurement [29]. For the MMSE analysis, we excluded subjects who did not have an MMSE score (n=43), yielding 776 subjects at baseline (n=2245 observations). Finally, we excluded subjects with missing data on covariates: education level (n=30 subjects), and alcohol intake (n=8 subjects)). This yielded a final number of 741 subjects with at least one MMSE assessment and full covariate data for the MMSE analysis. The sixth (n=20) and seventh assessments (n=1) for MMSE were dropped to account for influential outliers, resulting in 2132 total observations for the 741 subjects. 644 of these subjects had genotype information for *PICALM* and *CR1*, 645 for *CLU*, 632 for *CELF1*, and 600 for the ApoE alleles rs7412 and rs429358.

In order to validate the longitudinal regression, we also ran a Cox proportional hazards model using the MMSE score outcome. The model tested the association between bone Pb and the hazard ratio of a subject registering an MMSE score less than 25 at any time point after baseline. In this hazards model, it was necessary to exclude subjects that did not have follow-up MMSE test results (n=167). This resulted in 574 total subjects at baseline. 516 of these subjects had genotype information for *PICALM*, 514 for *CR1*, 515 for *CLU*, 504 for *CELF1*, and 494 for the APOE alleles rs7412 and rs429358.

3.3.2 Genotyping – Genetic polymorphism measurements for the APOE allele included rs429358 and rs7412. For the APOE SNPs, as well as for *PICALM* (rs3851179), *CR1*

(rs6656401), and *CLU* (rs11136000), genotyping was done as previously described [17]. Briefly, multiplex PCR assays were designed using Sequenom SpectroDESIGNER software by inputting sequences containing the single nucleotide polymorphism (SNP) site and 100 bp flanking sequence on either side of the SNP. Most assays were genotyped using the Sequenom MassArray MALDI-TOF mass spectrometer (SpectroDESIGNER, Sequenom). Assays that failed to multiplex were genotyped using TaqMan 5′ exonuclease (Applied Biosystems, Foster City, CA) and ABI PRISM 7900 Sequence Detector System.

The SNP associated with *CELF1*, rs7933019, was extracted from a study of folate network genes [30]. Briefly, genotyping was performed in the region 2kb on either side of the gene via an Illumina GoldenGate custom genotyping panel. SNP selection encompassed 2 kb on either side of the gene to include promoter and/or regulatory region variants.

3.3.3 Pb Exposure Assessment – Bone lead was measured using K-shell X-ray fluorescence (KXRF) spectroscopy as previously described [31]. Briefly, ^{109}Cd gamma rays excite the K-shell electrons of Pb embedded in bone, which emit an X-ray photon that can then be detected. An ABIOMED KXRF Instrument was used to measure lead at the tibia and the patella, corresponding to cortical and trabecular bone, respectively. The KXRF beam collimator was directed perpendicular to the tibial midshaft and at a 30^{o} angle from the horizontal for the patella for 30-minute measures [31-34]. Our previous study found more significant associations with patella Pb, so to simplify results, we present only patella Pb analyses.

3.3.4 Cognitive assessments – The cognitive screening tool used for this analysis was the mini-mental status exam, or MMSE. The MMSE assesses overall cognition by testing several domains including memory, visuospatial ability, attention, language, and orientation. Validated in multiple populations, the MMSE is widely used as a screening test for dementia [35], and to assess cognitive decline in non-demented populations [36]. Declines of the MMSE over time may indicate underlying pathologies such as Alzheimer's disease, where the MMSE declines by an average of 1.8-4.2 points per year [37-39], or normal cognitive declines associated with aging. The MMSE includes 30 questions, but the present analysis did not include "Which County are we in?" because counties in Massachusetts do not have political meaning. Thus, the maximum score was 29. These measures were taken repeatedly over 20 years, generally every 3 years.

3.3.5 Data Analysis – The datasets used in this study were managed using SAS software (version 9.4 for Windows) and all analyses were performed using R Software version 3.1.0. We used linear mixed effects models to first assess the main association between the genotype of each subject and longitudinal changes. Linear mixed effects modeling allows for differences in the number of repeated measures across a subject's visits. Our model included random intercepts for individual as well as random slopes for time in order to account for correlations among the repeated measurements. Model 1 (shown below) included genotype, time from first visit, the interaction of genotype and time, baseline covariates (age, alcohol intake, smoking, and education), and an indicator variable termed "FirstTestIndicator", which adjusted for whether an MMSE assessment was the subject's first or not. This variable, similar to one employed in Aim 1 as well as prior studies [22],

can partially account for the learning effect by separating the change from visit 1 to visit 2, and regressing based on longitudinal changes from subsequent visits.

Model 1 (Longitudinal model):

$$MMSE \approx B_0 + B_1 \times SNP + B_2 \times Time_{fromVisit1} + B_3 \times (Time_{fromVisit1} \times SNP) + B_4 \times Age_{FirstCognitiveTest} + B_5 \times Education + B_6 \times SmokingStatus + B_7 \times Alcohol + B_8 \times FirstTestIndicator$$

Model 2 (Cox Proportional Hazards Model):

$$\log\left(\frac{\lambda_1(t \mid X_k)}{\lambda_0(t)}\right) \approx B_0 + B_1 \times Pb + B_2 \times SNP + B_3 \times Age_{FirstCognitiveTest} + B_4 \times Education + B_5 \times SmokingStatus + B_6 \times Alcohol$$

Additionally, MMSE has a ceiling effect and may not be best modeled linearly. Thus, we also ran a cox proportional hazards model (Model 2 above), where the hazard ratio $\frac{\lambda_1(t \mid X_k)}{\lambda_0(t)}$ is the relative risk of the event at time *t* that any subject's MMSE score drops below 25, adjusted for variables defined by X_k.

3.3.6 Gene-Environment Interaction Analysis – We tested the association of the interaction of Pb and SNP status on change in MMSE by incorporating a three-way interaction term between Pb, SNP, and Time into Model 1.

Model 3 (Gene-Environment Longitudinal Model):

$$MMSE \approx B_0 + B_1 \times Pb + B_2 \times SNP + B_3 \times Time_{fromVisit1} + B_4 \times (Time_{fromVisit1} \times SNP) + B_5 \times (Time_{fromVisit1} \times Pb) + B_6 \times (Pb \times SNP) + B_7 \times (Time_{fromVisit1} \times Pb \times SNP) + B_8 \times Age_{FirstCognitiveTest} + B_9 \times Education + B_{10} \times SmokingStatus + B_{11} \times Alcohol + B_{12} \times FirstTestIndicator$$

As shown in Model 4, the association of the interaction of Pb and genotype on the MMSE hazards ratio was assessed by adding an interaction term between Pb and SNP to Model 3.

Model 4 (Gene-Environment Cox Proportional Hazards Model):

$$\log\left(\frac{\lambda_1(t \mid X_k)}{\lambda_0(t)}\right) \approx B_0 + B_1 \times Pb + B_2 \times SNP + B_3 \times Pb \times SNP + B_4 \times Age_{FirstCognitiveTest} + B_5 \times Education + B_6 \times SmokingStatus + B_7 \times Alcohol$$

3.3.7 Genotype modeling – Based on gene distributions shown in supplementary information (Figure S1), we used two methods to model the association of each SNP with MMSE. The two-degree-of-freedom (two-DF) approach compares the heterozygous (Aa) to the wild-type (AA) reference as well as the homozygous variant (aa) to the wild-type. The dichotomous model combines subjects who are carriers of the variant group [homozygous variant (aa) + heterozygous (Aa)] and compares them to the reference wild-type (AA). These methods were chosen over a method in which the homozygous variant (aa) is the reference because some of our SNPs of interest had very low minor allele frequency (Supplementary Figure S1).

3.4 Results

3.4.1 Descriptive statistics – Among the total 741 men with MMSE scores, mean patella Pb concentrations were 30.64 μg/g (SD=19.44) and tibia Pb was 21.62 μg/g (SD=13.33) (Table 1). The average participant age was 67.77 (SD: 6.82) at the time of the first cognitive assessment (51.4-98.0 years). The number of subjects used in the Cox proportional hazards analysis was 574. The sample size varied based on genotype, as summarized in Supplementary Figure 3.S1. The frequency of homozygous minor allele carriers was below 5% for the following SNPs: ApoE rs7412 (0.6%), ApoE rs4293583 (1.8%), and CR1 (3.7%). Given the low frequency of the minor allele of these SNPs, we also report dichotomous analyses in which carriers of at least one allele are combined into one group. All genes were in Hardy-Weinberg equilibrium (Table 3.2, $p_{HWE}>0.05$)

3.4.2 Main Association between SNPs and MMSE – We first examined the main association between SNPs and MMSE. The overall results are reported in Table 3, and three SNPs that approached statistically significant levels of association are described here. The homozygous minor group at the *PICALM* SNP was associated with a 0.408 point lower baseline MMSE score (p=0.053). The other associations were not significant, but were consistent across the dichotomous model and two-DF model. The variant carrier group, as defined by the dichotomous model, is associated with a 0.205 point lower baseline MMSE score (p=0.08), and a decline that is less steep, relative to the wild-type group, by 0.022 (p=0.18). Although the values for the *PICALM* SNP do not reach an α-level <0.05, the minor allele is generally associated with a negative baseline MMSE score, but an increase in MMSE

score over time relative to wild-type. These longitudinal results conflict with those found in the Cox proportional hazards (Cox PH) model in which the variant *PICALM* allele has a non-statistically significant protective association ($HR_{\text{any carrier}}$=0.679, p=0.109).

The minor allele for CR1 had a significant association with baseline MMSE. Using the two-DF model, the heterozygous group had 0.25 (p=0.045) lower baseline MMSE score relative to wild-type group, and the homozygous variant group had a 1.11 point (p<0.001) lower MMSE score at baseline. The association of allelic status and *change* in MMSE over time is given by the interaction terms (SNP x Time). The heterozygous group had a 0.048 point-per-year higher slope (p=0.008), and the homozygous variants had a 0.100 point-per-year higher slope (p=0.002), relative to wild-type, respectively. The dichotomous model was consistent with the longitudinal model, with the variant carrier group being associated with a 0.35 point lower baseline MMSE score (p=0.004), but a 0.054 point-per-year lower slope, or a less steep of a decline (p=0.002). No associations between MMSE and CR1 analyzed in the Cox proportional hazards model were close to an α-level of 0.05.

The group that was heterozygous at APOε allele rs429358 had a significant positive association with baseline MMSE, with the baseline score being 0.39 points higher (p=0.001). Although the variant group had a lower baseline score, this association was not significant. The heterozygous group had a steeper decline over time in MMSE (-0.05 points-per-year, p=0.007). The results of the dichotomous model supported this effect. The variant carrier group was associated with a 0.39 point increase in baseline MMSE score (p=0.005) relative to the homozygous wild-type group. The variant carriers were associated with a

0.055 point-per-year steeper decline (p=0.004). Results from the Cox PH model where rs429358 was modeled using the two-DF method suggest that the homozygous minor group had a 4.84 times higher hazards ratio (HR) of having an MMSE<25 compared to the wild-type group (p=0.003). However, the HR for the heterozygous carriers was not significant (HR=0.770, p=0.44), nor was it significant in the dichotomous model ($HR_{any\ carrier}$=0.99, p=0.98).

3.4.3 Interaction between Pb and SNPs on change in MMSE – We next examined the association of the interaction between Pb and genotype with change in MMSE. Overall results are noted in Table 4, and we focus here on PICALM, CLU, CR1 and rs429358 because they had near-significant associations between the interaction term [*Pb x Gene*] with MMSE in the Cox PH models.

As shown in Figure 2, the HR associated with an IQR (21 μg/g) change in Pb in the subjects homozygous for the major PICALM allele was 1.04 (95% CI: 0.77-1.40), but the HR associated with Pb in the homozygous minor group was 3.54 (95% CI: 0.95-13.25). The p-value for interaction of Pb and homozygous minor allele status was p=0.08. In the dichotomous model, the HR for subjects with at least one minor allele was 1.44 (95%CI: 1.04-2.01). Although the effect of Pb in this group is significant, the p-value of estimate of the interaction effect of Pb and genotype is p=0.13. In the longitudinal model, the beta term for interaction of Pb with the homozygous minor group (two-DF model) is -0.023 (p=0.011), suggesting that in this group, there was a 0.483 point *decrease* in baseline MMSE for every IQR increase in patella Pb (21 ug/dL). There was no significant interaction with time in the longitudinal model.

For the *CR1* SNP, the Cox PH model (two-DF model) also suggested a significant interaction between Pb and genotype. The HR associated with Pb in the homozygous major group was 1.11 (95% CI: 0.85-1.44), compared to that in the heterozygous group (HR=2.15, 95% CI: 1.36-3.41), with an interaction p-value of p=0.01. The effect of Pb in the homozygous minor group was not significant, nor was the interaction term. The longitudinal two-DF model does not suggest significant interactions between Pb and *CR1* genotype (p>0.10). However, the longitudinal dichotomous model suggests that for every IQR increase in patella Pb, there was a 0.231 point decrease (p=0.086) in baseline MMSE in the subjects who had either one or two minor alleles (A/G + A/A) compared to wild-type *CR1* (G/G). We note here that the significance level for this interaction did not satisfy α-level<0.05.

The β-estimate for the three-way interaction of [*Time* x *Pb* x *CLU heterozygotes*] was 0.056 (p=0.008) in the two-DF model and 0.051 (p=0.013) for minor allele carriers (C/T + T/T vs C/C) in the dichotomous model. The β-estimate for interaction of Pb and time was -0.042 (p=0.004, data not shown). This suggests that in the wild-type group, every IQR increase in Pb is associated with an additional decrease in MMSE score of 0.042 points. However, in the heterozygous group (C/T vs C/C) the magnitude of the association of Pb with time is dampened. Thus, for C/T group, every IQR increase in Pb is associated with 0.021 *increase* change in MMSE score. These results were consistent with those found in the Cox PH model, in which results did not reach statistical significance. The hazard ratio for Pb in subjects who were homozygous major was 1.49 (95% CI: 0.98-2.28). The HR for Pb in any carriers of the minor allele was 1.05 (95% CI: 0.79-1.41).

ApoE locus rs429358 also had significant three-way interaction β-estimates in both the dichotomous and two-DF models. In the reference homozygous major group (T/T), the interaction of an IQR change in Pb and time was associated with a 0.013-point decrease in MMSE over time (p=0.19, data not shown). The β-estimate associated with the parameter [*Time* x *IQR change in Pb* x *rs429358 (T/C)*] was -0.046 (p=0.06) in the two-DF model and -0.041 (p=0.09) for minor allele carriers (T/C + C/C) in the dichotomous model. Thus for the T/C group, every IQR increase in Pb was associated with a (0.013+0.042)=0.055 point decrease in MMSE per year of time (or per year of age increase). Cox PH modeling using dichotomous genotype modeling supported the direction of association found in longitudinal models, but results did not reach statistical significance. The HR association for Pb in subjects who were homozygous major was 1.19 (95% CI: 0.95-1.50), whereas that found in subjects who were carriers of at least one minor allele was 1.71 (95% CI: 0.96-3.05). The association of interaction between Pb and genotype on the HR was not significant using either dichotomous or two-DF models of rs429358.

3.5 Discussion

In our study of older men in the NAS, genes known to be associated with neurodegeneration modified the association between cumulative lead exposure and declining cognitive function. Cross-sectional MMSE scores were lower for subjects with the minor allele of *PICALM*, but over time, the decline in MMSE scores is less. Cox proportional hazards modeling suggested that subjects who were *PICALM* heterozygotes had a lower HR of having their MMSE drop below 25, but that result was not statistically significant (Figure 1). In prior work, the minor allele of *PICALM* has been associated with a protective effect on

cognitive decline as measured by the 3MS, an expanded measure of the MMSE [18]. The *PICALM* minor allele also had an OR of 0.84 in a meta-analysis of genome-wide association studies (GWAS) of AD.[9] Despite this protective main association, we found that carriers of the minor allele are more susceptible to the deleterious association between Pb and MMSE decline although the parameter for the interaction did not reach α-level<0.05 (p=0.08 in two-DF model; p=0.13 in dichotomous model). *PICALM* is not known to interact with Pb pathways, so this result was surprising. If the biological interaction was additive, one would expect an effect of the minor allele that protected against Pb-associated cognitive decline. The *PICALM* tagging SNP in this study is intronic and is thought to be associated with expression [40]. It is possible that Pb modifies expression of genes via altering DNA methylation [41-43]. Thus, the protective effect the minor allele may be dampened by the presence of Pb.

Similar to *PICALM*, we noted that the minor allele for *CR1* was associated with lower baseline MMSE scores. However, the decline of MMSE was smaller for both heterozygous and homozygous minor groups relative to the group that was homozygous major. This may be an artifact of the ceiling effect of MMSE scores because no significant association was found between *CR1* and the hazard ratio of MMSE score dropping below 25 over the study period. Prior work suggests that *CR1* rs6656401 is also associated with increased odds of Alzheimer's disease [9]. The CR1 variant does seem to exacerbate the deleterious effect of Pb on MMSE scores, as seen by Cox proportional hazards models (Table 5, Figures 1 &2). Like *PICALM*, the *CR1* SNP we are observing is intronic, and likely associated with expression.

The ApoE allele rs429358 is of special interest because of its association with cognition in various studies. It is one of two loci that define ApoE-ε4 status, which confers a greater risk of developing Alzheimer's [9]. In a GWAS of cognitive decline as defined by a principal component-derived fluid-type general intelligence score, the presence of the major "T" allele at rs429358 was associated with less cognitive decline [14]. We observed the same main association in the heterozygous group, which had a significantly higher HR for MMSE<25 as compared to the homozygous T/T group. Results from our longitudinal analysis suggest that the minor allele mitigates the deleterious effect of Pb on cognition at baseline, whereas ApoE- ε4 status is associated with lower cross-sectional cognitive scores in a prior study[24]. However, our results also show that with time, the effect of the variant rs429358 is actually to exacerbate the deleterious association between Pb and cognition, which is consistent with the aforementioned paper[24] and others [17].

CLU showed some statistically significant effects in the longitudinal model, in which we observed a dampened association between Pb and change in MMSE scores such that the slope of *CLU* minor allele carriers was less steep than that of subjects homozygous for the *CLU* SNP. This was in concordance with the association of rs11136000 with increased odds of Alzheimer's disease [9]. However, this is the opposite of its association with cognitive decline [18].

Our data suggest an association between *CELF1* and baseline MMSE scores. The minor allele homozygous group had lower baseline scores, but their slope was less steep than subjects who were homozygous major for the *CELF1* SNP. However, this was not reproduced in the dichotomous model or the Cox proportional hazards model, suggesting

that the result may be an artifact of the ceiling effect of MMSE. We did notice an increasing trend of magnitude of HR's associated with Pb (Figure 3.2), but this was not significant (p_{trend}=0.35).

This analysis was subject to the same limitations that are part of many gene-environment studies. Many of our associations were near-significant, and this may simply be due to the lack of power to detect associations in interactions. As stated elsewhere, when analyzing gene-environment interactions to detect true associations, it will be necessary to pool studies in meta-analyses [44]. Although we have reproduced some of the results of similar studies [14,17,18], our analysis is unique in that it looks at the effect of gene-environment on a longitudinal outcome. It will be necessary to reproduce these results in different populations using similar longitudinal outcomes. Additionally, the modifying effects we did see may be caused by proximal genes that are in linkage disequilibrium with our SNPs of interest. However, we contend that this is unlikely because our SNP selection was based on well-characterized prior associations with cognition and AD.

Despite these limitations, this is the first analysis of AD-associated SNPs and their interaction with an environmental exposure to modify cognitive trajectories. This work demonstrates that variation in genes associated with AD may serve to modify the risk of cognitive decline that is associated with cumulative Pb exposure. This subset of variant allele carriers may experience a decline in cognition that is more substantial than previously considered. Our findings point to additional research avenues to elucidate mechanistic pathways that incorporate the effects of environment. This may facilitate the development of preventive and treatment measures to abate cognitive decline. As chronic

Pb exposure continues to be a public health concern, our findings stress the importance of policy to reduce the use of Pb as well as preventive measures to help avoid exposure.

Table 3.1: Descriptive Statistics by Model Type

	Longitudinal Model	Cox PH Model
N Subjects	741	574
Mean Age (SD)	68.1 (7.0)	67.2 (6.6)
MMSE Mean (SD)	26.6 (1.8)	26.8 (1.7)
Patella Mean (SD)	30.6 (19.4)	29.6 (20.0)
Tibia Mean (SD)	21.6 (13.3)	20.9 (13.5)
Education (Freq %)		
Less than 12 years	74 (10.0)	61 (10.6)
High school graduate	263 (35.5)	206 (35.9)
Some college	193 (26.0)	143 (24.9)
College graduate	211 (28.5)	164 (28.6)
Smoke (Freq %)		
Never	221 (29.8)	177 (30.8)
Current smoker	47 (6.3)	30 (5.2)
Former smoker	473 (63.8)	367 (63.9)
TwoDrink (Freq %)		
< 2 drinks / day	585 (78.9)	452 (78.7)
≥ 2 drinks / day	156 (21.1)	122 (21.3)

Table 3.2: Allele Frequencies in the Normative Aging Study by Model Type

Longitudinal Model	Gene Frequencies (%)			
	Major	Heterozygous	Minor	HWE p-value
CELF1	300 (47.5)	276 (43.7)	56 (8.9)	0.51
rs7412	514 (85.7)	82 (13.7)	4 (0.6)	0.71
rs429358	452 (75.3)	137 (22.8)	11 (1.8)	0.87
PICALM	280 (43.5)	302 (46.9)	62 (9.6)	0.13
CLU	226 (35.0)	322 (49.9)	97 (15.0)	0.31
CR1	435 (67.5)	185 (28.7)	24 (3.7)	0.44
Cox PH Model				
CELF1	236 (46.8)	223 (44.2)	45 (8.9)	0.45
rs7412	423 (85.6)	68 (13.8)	3 (0.6)	0.88
rs429358	372 (75.3)	112 (22.7)	10 (2.0)	0.65
PICALM	222 (43.0)	247 (47.9)	47 (9.1)	0.06
CLU	344 (66.9)	148 (28.8)	22 (4.3)	0.24
CR1	180 (35.0)	259 (50.3)	76 (14.8)	0.27

HWE: Hardy-Weinberg Equilibrium, HWE p-value < 0.05 indicates disequilibrium in the gene pool.

Table 3.3: Main Association of SNPs on MMSE

Longitudinal Model	**CELF-1**		**PICALM**		**CLU**		**CR1**		**rs7412**		**rs429358**	
2 DF	β	p-val	β	p-val	β	p-val	β	p-val	B	p-val	β	p-val
Heterozygous	0.185	0.127	-0.160	0.190	0.124	0.331	***-0.255***	***0.045***	0.100	0.550	0.441	***0.002***
Hmz Minor	0.030	0.886	***-0.408***	***0.053***	0.159	0.374	***-1.105***	***<0.001***	-0.005	0.990	-0.230	0.597
Time*Heterozygous	-0.003	0.872	0.025	0.147	-0.022	0.208	***0.048***	***0.008***	0.009	0.690	-0.053	***0.007***
Time*Hmz Minor	-0.012	0.678	-0.006	0.861	-0.014	0.603	***0.101***	***0.009***	0.080	0.380	-0.076	0.234
Dichotomize												
Any carrier	0.159	0.169	***-0.205***	***0.080***	0.132	0.275	***-0.354***	***0.004***	0.096	0.571	0.388	***0.005***
Time*any carrier	-0.005	0.785	0.022	0.182	-0.021	0.222	***0.054***	***0.002***	0.013	0.565	-0.055	***0.004***
Cox Prop Haz Model (OR reported)	**CELF-1**		**PICALM**		**CLU**		**CR1**		**rs7412**		**rs429358**	
2 DF	HR	p-val	HR	p-val	HR	p-val	HR	p-val	HR	p-val	HR	p-val
Hetero	1.009	0.970	***0.672***	***0.108***	1.115	0.685	1.013	0.962	0.696	0.340	0.770	0.439
Minor	0.994	0.989	0.758	0.648	1.049	0.901	0.502	0.347	0.000	0.995	4.843	***0.003***
Dichotomize												
Any carrier	1.007	0.977	***0.679***	***0.109***	1.099	0.710	0.939	0.807	0.675	0.301	0.992	0.978

Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Table 3.4A: Interaction between Pb and Genes to Modify MMSE - Longitudinal Model

Longitudinal	**CELF-1**		**PICALM**		**CLU**		**CR1**		**rs7412**		**rs429358**	
2 DF	β	p-val	β	p-val	β	p-val	β	p-val	β	p-val	β	p-val
IQR Pb	-0.090	0.298	-0.046	0.647	-0.163	0.144	-0.067	0.416	-0.116	0.119	***-0.164***	***0.034***
Htz	0.176	0.145	-0.153	0.205	0.130	0.306	***-0.261***	***0.040***	0.103	0.551	***0.448***	***0.001***
Minor	0.040	0.849	-0.345	0.101	0.114	0.537	***-0.986***	***0.002***	-0.634	0.598	-0.224	0.614
Time*Htz	-0.005	0.763	0.026	0.122	-0.011	0.527	***0.049***	***0.007***	0.004	0.882	***-0.060***	***0.002***
Time*Minor	-0.012	0.686	-0.005	0.875	-0.012	0.654	***0.099***	***0.013***	0.324	0.108	-0.052	0.489
IQR Pb * Htz	-0.029	0.831	-0.041	0.761	0.044	0.749	-0.169	0.233	0.150	0.474	***0.335***	***0.037***
IQR Pb * Minor	***-0.446***	***0.072***	***-0.474***	***0.011***	0.025	0.920	-0.256	0.270	-1.032	0.549	0.166	0.767
Time*IQR Pb*Htz	0.000	0.999	0.006	0.761	***0.056***	***0.008***	0.029	0.168	-0.032	0.314	***-0.046***	***0.063***
Time*IQR Pb*Minor	***0.069***	***0.024***	0.032	0.265	0.010	0.790	0.025	0.453	0.346	0.153	0.077	0.530
Dichotomize												
IQR Pb	-0.092	0.290	-0.047	0.641	-0.164	0.142	-0.068	0.408	-0.115	0.120	***-0.166***	***0.032***
Any minor	0.150	0.195	***-0.193***	***0.099***	0.129	0.286	***-0.348***	***0.005***	0.101	0.551	***0.396***	***0.004***
Time*any minor	-0.005	0.763	0.023	0.156	-0.009	0.589	***0.054***	***0.001***	0.006	0.794	***-0.061***	***0.001***
IQR Pb*Any minor	-0.106	0.406	-0.163	0.201	0.040	0.765	***-0.222***	***0.086***	0.136	0.510	***0.338***	***0.031***
Time*IQR Pb*any minor	0.016	0.387	0.012	0.508	***0.051***	***0.013***	***0.030***	***0.109***	-0.029	0.347	***-0.041***	***0.093***

Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Table 3.4B: Interaction between Pb and Genes to Modify MMSE - Cox PH Model

Cox Prop Haz Model (HR reported)	**CELF-1**		**PICALM**		**CLU**		**CR1**		**rs7412**		**rs429358**	
2 DF	HR	p-val	HR	p-val	HR	p-val	HR	p-val	HR	p-val	HR	p-val
Pb	1.006	0.320	1.002	0.801	***1.019***	***0.065***	1.005	0.442			1.008	0.158
Htz	1.005	0.984	***0.633***	***0.074***	1.166	0.582	0.913	0.746			0.731	0.375
Minor	1.034	0.942	0.446	0.342	1.178	0.671	0.560	0.430			***4.783***	***0.004***
Pb*Htz	1.009	0.437	1.013	0.237	0.982	0.144	***1.032***	***0.010***			1.020	0.235
Pb*Minor	1.020	0.568	***1.060***	***0.075***	0.998	0.923	0.969	0.554			1.010	0.744
Dichotomize												
Pb	1.006	0.325	1.002	0.767	***1.019***	***0.065***	1.005	0.475	1.008	0.129	1.008	0.159
Any minor	1.006	0.982	***0.629***	***0.065***	1.150	0.596	0.884	0.647	0.688	0.346	0.951	0.873
Pb*Any minor	1.010	0.380	1.016	0.131	0.984	0.162	1.015	0.156	1.015	0.486	1.017	0.236

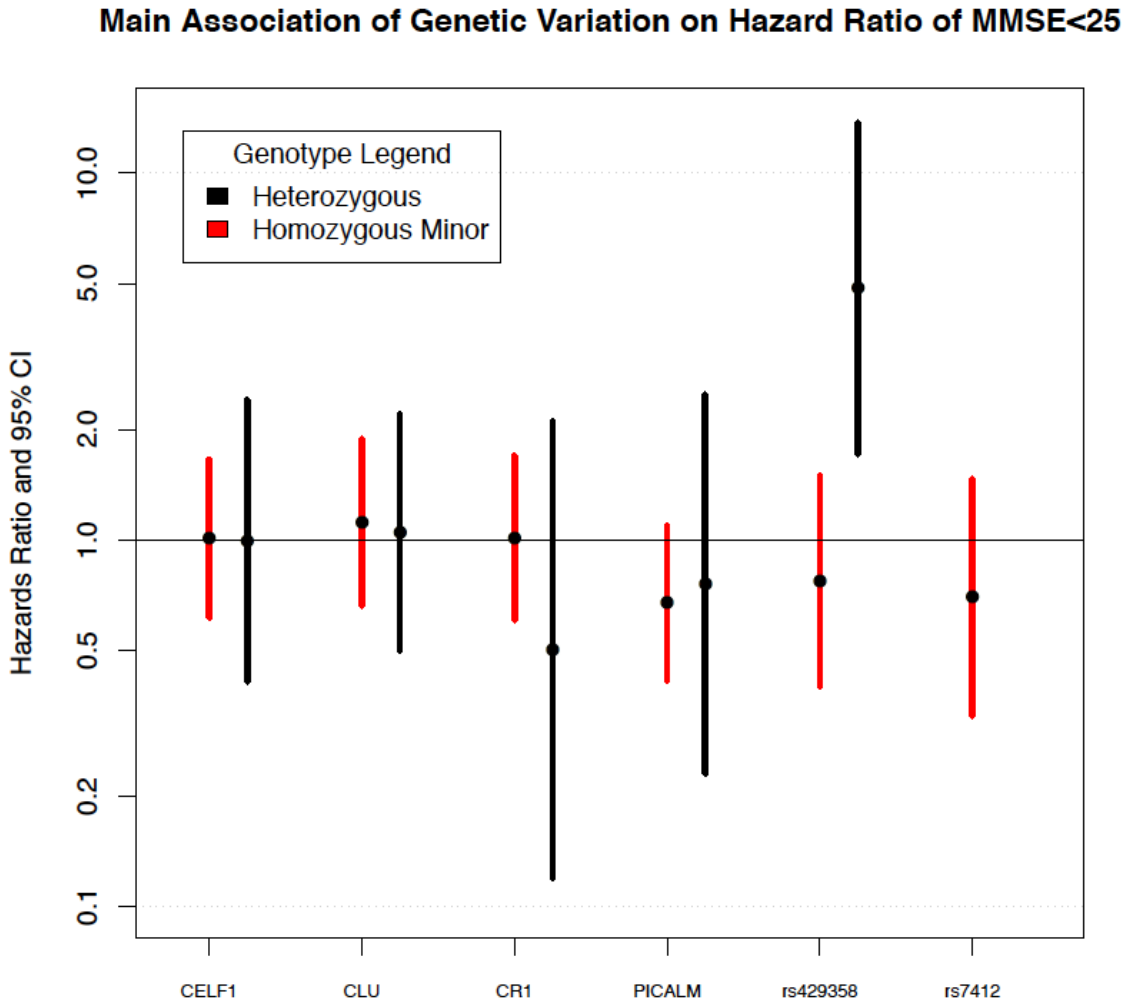
Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Table 3.5: Association of IQR change in Pb by Genotype to modify HR of MMSE<25

Two DF Model				Dichotomous Model			
Gene	**Genotype**	**HR**	**95% CI**	**Genotype**	**HR**	**95% CI**	**p-trend**
CELF1	Homozygous C/C	1.15	0.88-1.50	Homozygous C/C	1.14	0.88-1.50	0.35
	Variant C/G	1.38	0.91-2.10	"G" Carrier	1.4	0.94-2.10	
	Variant G/G	1.73	0.42-7.12				
PICALM	Homozygous G/G	1.04	0.77-1.40	Homozygous G/G	1.05	0.78-1.41	0.06
	Variant A/G	1.35	0.95-1.93	"A" Carrier	1.44	1.04-2.01	
	Variant A/A	3.5	0.95-13.25				
CR1	Homozygous G/G	1.11	0.85-1.44	Homozygous G/G	1.1	0.84-1.43	0.55
	Variant A/G	2.15	1.36-3.41	"A" Carrier	1.51	1.03-2.21	
	Variant A/A	0.57	0.06-5.12				
CLU	Homozygous C/C	1.49	0.98-2.28	Homozygous C/C	1.49	0.98-2.28	0.33
	Variant C/T	1.03	0.75-1.41	"T" Carrier	1.05	0.79-1.41	
	Variant T/T	1.42	0.55-3.65				
rs429358	Homozygous T/T	1.19	0.94-1.51	Homozygous T/T	1.19	0.94-1.50	0.27
	Variant T/C	1.78	0.93-3.43	"C" Carrier	1.71	0.96-3.05	
	Variant C/C	1.47	0.41-5.27				
rs7412	Homozygous C/C	1.19	0.95-1.50	Homozygous C/C	1.19	0.95-1.50	0.47
	Variant C/T	1.57	0.67-3.67	"T" Carrier	1.62	0.70-3.74	
	Variant T/T	1	0-0				

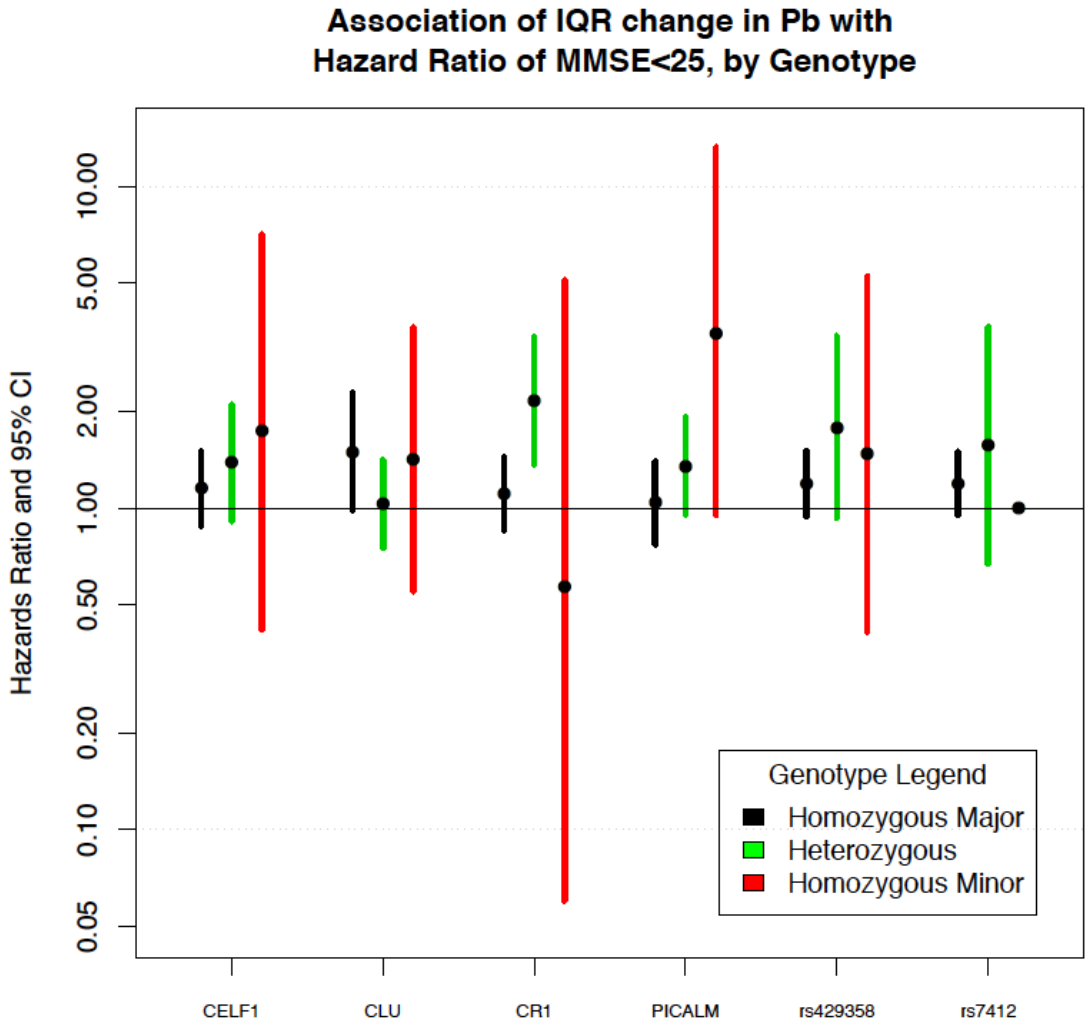
Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Figure 3.1: Genetic Variation association with hazard ratios of MMSE<25



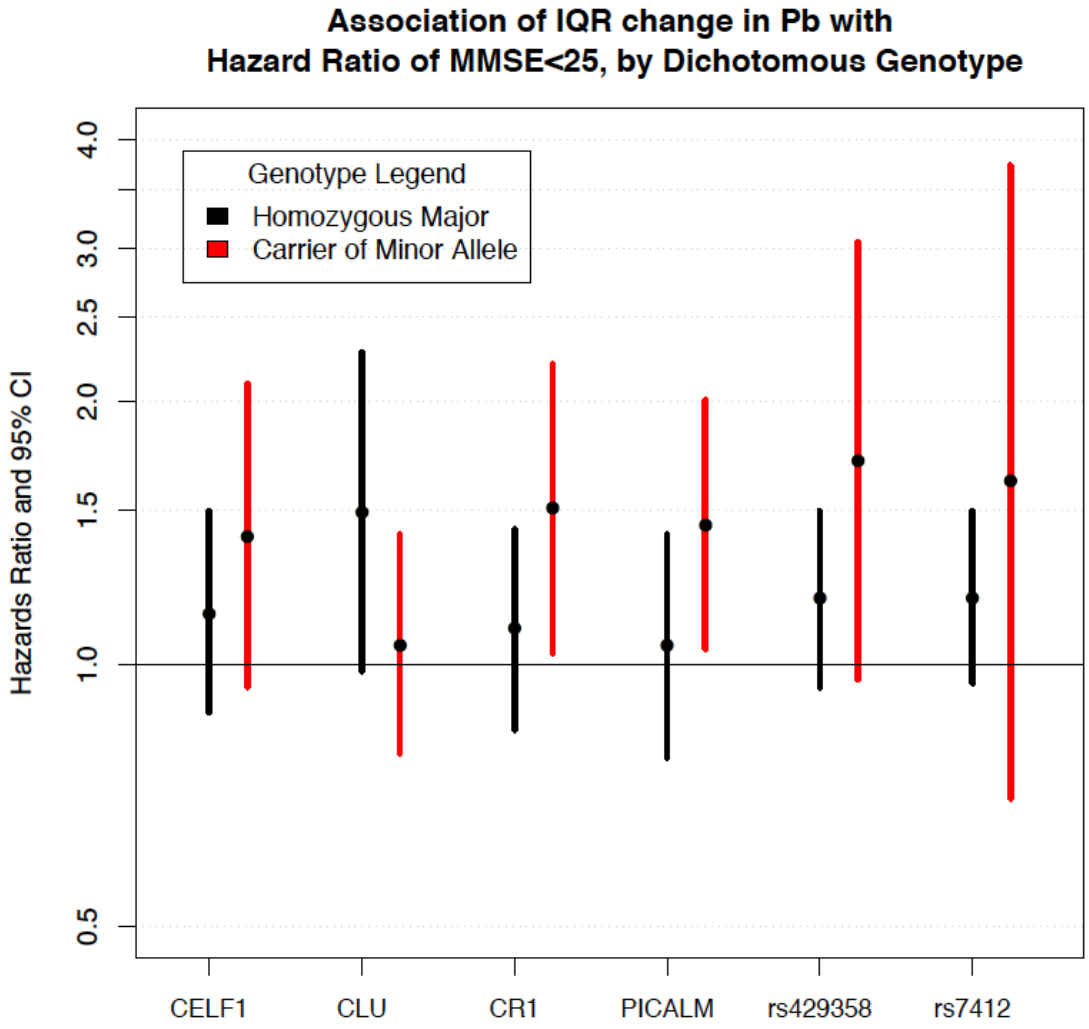
Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Figure 3.2: Association of IQR change in Pb with hazard ratio of MMSE<25, by genotype



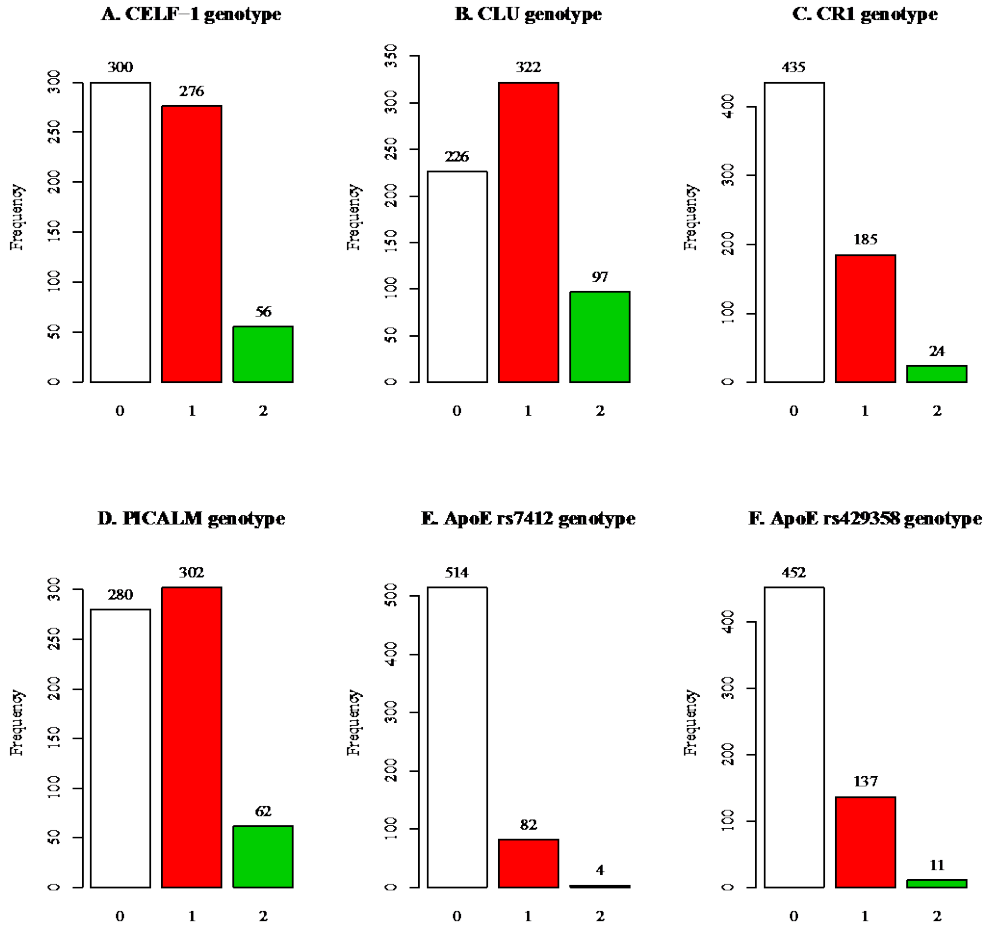
Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Figure 3.3: Association of IQR change in Pb with hazard ratio of MMSE<25, by dichotomous genotype



Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

Figure 3.S1: Genotype frequencies in subjects with Pb measurements



X-axis indicates genotype – 0=Homozygous major allele, 1=Heterozygous, 2=Homozygous minor allele. Abbreviations – *CELF1*: CUGBP, Elav-like family member 1; *PICALM*: phosphatidylinositol binding clathrin assembly protein; *CLU*: clusterin; *CR1*: complement receptor-1; rs7412 and rs429358 are two functional SNPs associated with *APOE*

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CHAPTER 4

In Utero Lead Exposure and Neuron-specific Epigenetic Changes in Mice

4.1 Abstract

Frontal cortex neuronal cells have distinct DNA methylation signatures compared to non-neurons. In addition, the methylation landscape of neuronal cells varies depending on neuronal activity, based on *in vivo* research in adult mice. The methylome of neuronal cells may be plastic throughout the lifespan, and it may be particularly sensitive to environmental conditions such as lead (Pb) exposure. Studies in rodents and monkeys exposed to Pb in the first few weeks and first year of life suggest that epigenetic sensitivity is associated with neuropathology in late life. Epigenetic epidemiology research in the brain has studied mixed cell types in whole tissue samples, but it is now clear that epigenetic analyses that target specific cell types may be more informative, particularly since neurons are known to represent approximately only 10% of cells in CNS tissue. The *in utero* exposure groups consisted of offspring exposed via the maternal drinking water to 0 ppm, 2.1 ppm, or 32 ppm of Pb acetate two weeks before mating, throughout gestation, and three weeks after birth. Using NimbleGen Promoter Tiling Arrays, we probed DNA methylation levels in the neuron-specific cell population at a genome-wide level. After applying the bioinformatics bumphunting method and a family-wise error rate cutoff of 0.3, we report 6 novel exposure-dependent differentially methylated regions associated with

the following genes: *retro-Hnrnpc*, *Skint5*, Xylt, *Olfr1085*, *Pcdh19*, and *Hnmt*. The roles of *Hnmt* (histamine N-methyltransferase) and *Pcdh19* (protocadherin 19) are of particular interest for future studies. *Hnmt* is involved in neurotransmitter metabolism and *Pcdh19* is implicated in neurodevelopment.

4.2 Introduction

4.2.1 Alzheimer's Disease, Pb, & the Developmental Origins of Health and Disease

Hypothesis – Extensive epidemiologic studies have shown that exposure to Pb is associated with a wide range of neurological deficits. Neurological impairment is thought to be on the spectrum of cognitive decline that eventually leads to clinical dementia [1,2]. Early life Pb exposure is associated with neurobehavioral impairment in children [3,4], who exhibit worse global executive function, metacognition, and behavioral regulation as Pb levels increase. Although there is a paucity of epidemiologic data directly linking Pb to neurodegenerative dementias, one study has found a link between cumulative exposure to Pb and Parkinson's disease [5]. *In utero* exposure to Pb has also been shown to exert adverse effects on learning and memory ability in young rats [6].

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that environmental perturbations early in life can affect pathophysiology in adulthood [8-13]. Even when a given exposure is lifted, its effects are persistent, as seen in studies of children whose mothers were exposed to famine conditions while in the first trimester of pregnancy [14]. Although the mechanisms by which these alterations are programmed are largely unknown, the epigenetic mark, DNA methylation, has emerged as a major candidate for

investigation. For example, maternal licking and grooming behavior in rats affects offspring glucocorticoid receptor (GR) expression via differential hippocampal CpG methylation of the GR gene, altering offspring stress response [15]. These findings suggest that stable epigenetic changes, such as DNA methylation, can affect neurodevelopmental outcomes over the life-course. Recently, we found that female mice exposed to multiple levels of bisphenol A (BPA) *in utero* display dose-dependent differences in DNA methylation in tail tissue at 3 weeks of age [16] and significantly increased hyperactivity later in life [17].

While it is crucial to acknowledge that environmental toxicants may cause permanent damage to brain structures early in life, there is also ample evidence that exposure to a heavy metal such as Pb can cause latent molecular changes that manifest much later. Rats [18] and monkeys [7] administered physiologically relevant levels of Pb early in life had an increase in a marker for oxidative stress in the brain when measured later in the life. Oxidative stress is thought to be a significant mechanism in Alzheimer's Disease (AD) development [19]. Although Pb may act as a general oxidative agent, its ability to create 8-oxoguanine lesions may also alter the binding of transcription factors, such as SP1, and other regulatory factors that are mediated by CpG methylation, such as MECP2 [20]. A decrease in DNA methyltransferase activity (DNMT) was also observed in monkey brains. Several genes in the AD pathway have been shown to be differentially expressed in monkeys exposed to Pb early in life [7]. *ADAM17* [21] and *APH1a* [22] are genes associated with amyloid precursor protein processing. These genes, and others, are implicated in AD [23-27]. Given the potential for Pb to modify DNA methylation at genes in a pathway relevant to neurodegeneration, we investigated the association of early life exposure to lead (Pb) with differential DNA methylation patterns in cortical neurons.

4.2.2 Neuronal Epigenetics – It has been traditionally accepted that once established in a post-mitotic cell, DNA methylation patterns are highly stable epigenetic markers that govern expression and tissue specificity [28,29]. Neuronal cells are peculiar in that they have approximately the same life span as the individual, and make and break synaptic connections well into adult life [30,31]. Neuronal activity modulates the DNA methylation landscape [32,33] and reciprocally, DNA methylation modulates synaptic transmission and excitability of neurons [34,35]. Given their distinct function, isolated neuronal cells have a markedly distinct methylation profile from bulk cortical tissue [36]. Thus, it is highly plausible that neurons, which make up a small percentage of the brain cell population, may accumulate changes in methylation in response to environmental exposures that are distinct from changes seen in bulk cortical tissue. These changes would be particularly relevant, in light of the finding that there are modest methylation differences among human AD cases and controls [37].

Here, we have isolated the mouse cortex because beta-amyloid plaque deposition has been noted in the frontal cortex of AD patients using biopsies as well as PET imaging [38]. This study uses an established protocol to separate $NeuN^+$ (a specific marker for neuronal nuclei) neuronal cells from $NeuN^-$ non-neuronal cells in mice exposed to Pb perinatally and measures neuron-specific genome-wide promoter DNA methylation patterns at 10 months of age.

4.3 Methods

4.3.1 Mouse Study Population – All mating mice were obtained from a C57BL/6J background agouti A^{vy} strain [39]. This A^{vy} strain has been maintained in forced heterozygosity through the male line for over 200 generations, resulting in an isogenic population that is ~93% similar to that of the background C57BL/6J strain [40,41]. Virgin dams (*a/a*) were assigned randomly to one of four treatment groups with exposure to Pb-acetate through the drinking water. Exposure was started two weeks prior to mating with viable yellow agouti male mice (A^{vy}/a), and continued throughout gestation and three weeks after birth. Exposure groups included: 2.1 ppm (low), 16 ppm (medium) and 32 ppm (high), corresponding to peak blood levels of approximately 2.5, 10, and 25 μg/dL, respectively. We chose to focus our analysis on the high Pb exposure (32 ppm) in comparison to low (2.1 ppm) or no (0 ppm) Pb exposure.

Breeding is designed to produce 50% A^{vy}/a offspring and 50% *a/a* offspring. The ectopic expression of the *agouti* gene in A^{vy} mice is known to affect adult onset obesity and tumorigenesis [42], which may confound the relationship between Pb and DNA methylation. Therefore, we conducted the mouse cortex epigenetic analysis on *a/a* offspring only. The developmentally exposed Pb and control offspring used in this study were sacrificed at 10 months of age to extract the cortex from whole brain in a manner that allowed for neuron-specific DNA extraction and epigenetic analysis.

4.3.2 Sample Ascertainment and Preparation – The cerebral cortex was carefully dissected from the rest of the brain on dry ice. Approximately ~200 mg of cortical tissue was obtained for each mouse, consistent with previously described methods for neuronal nuclei separation [36,43]. However, if more than 200 mg was obtained, treatment was

scaled appropriately. In order to prepare for fluorescence-activated cell separation (FACS), we first minced cortical tissue using razor blades. In order to create a single cell suspension, each 200 mg portion of resulting tissue was incubated with 1 mL Accutase [Sigma-Aldrich] for 30 min. The Accutase solution was then centrifuged briefly and supernatant was replaced with 1.0 mL of Hibernate A (Life Technologies). This was subsequently triturated through an ~0.8 mm glass pipette once, and a ~1.0 mm glass pipette twice (10 times up and down over 45 seconds for each trituration). This mixture was strained once through a 100-micron strainer and once through a 40-micron strainer (BD Falcon). In order to separate cells from extracellular matrix and other connective tissue, the mixture was then layered on a discontinuous density gradient of Percoll (Sigma-Aldrich) with layers that were 1 mL each 12%, 18%, and 24% Percoll, diluted in Hibernate A and 22 mM NaCl. This was centrifuged at 500 rcf for 10 min. The top layer was then pulled out, and fixed and permeabilized by incubation in a 1:1 ratio of ice-cold 100% Ethanol for 20 min at 4^{o}C. The EtOH solution was then replaced by Hibernate A.

Following fixation and permeabilization, the nuclei were ready for immunolabeling. We utilized a monoclonal mouse anti-NeuN (clone A60, from Millipore) antibody that was pre-conjugated with Alexa Fluor 488. All samples were blocked in 1 mL of a 1% BSA/10% goat serum solution for 1 hour prior to immunolabeling. The main sample to be sorted via FACS was then incubated in a 1:20,000 solution of Anti-NeuN 488 antibody in the dark for 30 minutes. This was then washed extensively in PBS solution five times at approximately 450 rcf for 5 minutes per wash to get rid of unbound antibody. Two control samples were implemented to ensure proper interpretation of FACS scatter diagrams. Approximately 100 µL of the nuclei solution was unlabeled in order to determine baseline scatter profile of cell

relative to any remaining debris. An additional 20 µL was used as a saturated binding control. Non-fluorescent Anti-NeuN was added in a 10:1 ratio prior to addition of fluorescent anti-NeuN. This was in order to saturate specific binding sites. Specific binding sites can be saturated, but non-specific binding sites cannot be. Under the assumption that non-specific binding is less fluorescent than specific binding, we used the fluorescence from non-specific binding control to set the gate for Alexa-Fluor 488 fluorescence at a level beyond which we could be certain that only neurons were specifically bound to Anti-NeuN. The antibody we employed was pre-conjugated, so there was no need for a secondary binding control.

FACS sorting was performed on either FACS Aria or MoFlo Astrios, with assistance from the Flow Cytometry Cores at the University of Michigan Biomedical Research Science Building and Cancer Center. Before fractionation into $NeuN^+$ samples and $NeuN^-$ debris was removed based on the profile of forward scatter-area/side scatter-area (FSC-Area vs SSC-Area). Doublets were removed by first using scatter profile of forward scatter-width vs forward scatter-height (FSC-Width vs FSC-Height), then by side scatter-width vs side scatter height (SSC-Width vs SSC-Height). Fractionation gates were set using profiles of 525/50 488-Area fluorescence vs SSC-Area.

DNA was extracted from cells by modifying previously established methods for DNA extraction from blood cells, described in Qiagen protocols (Qiagen). Briefly, all reactions were scaled appropriately, but are reported here for 1 mL of FACS-sorted $NeuN^+$ or $NeuN^-$ sample. Each sample was first incubated with 100 µL of Proteinase K and 1.2 mL of proprietary Qiagen lysis buffer at 50^oC overnight. One equivalent of 100% frozen ethanol was added, followed by centrifugation through a Qiagen DNA binding column. The solution

was then washed three times with Qiagen wash buffer, and eluted in ~200 µL of [10 mM Tris/0.5 mM EDTA buffer. This DNA was then utilized for tiling array hybridization (only neuronal NeuN$^+$ genomic DNA).

For tiling array analysis, sample pooling was necessary to reach the amount of genomic DNA necessary to perform hybridization, approximately 9 µg. The pooling design is shown in Table 4.1. Briefly, 3 pooled samples were used for each control, low, and high Pb exposure group, resulting in 9 total pooled samples. Each pool consisted of DNA from at least two isogenic mice.

4.3.3 Nimblegen Tiling Array Sample Preparation – Genomic DNA from NeuN$^+$ samples was sonicated to fragment sizes ranging from 200-1000 bp using an Episonic 1100-series sonicator (Farmingdale, NY) as described previously (Caren Weinhouse, unpublished data). Briefly, 7.5-9 µg of DNA was sonicated in cycles of 15 seconds-ON and 30 seconds-OFF for a total of 15 minutes in 8-20^{o}C water. After every 5 minutes of processing time, water temperature was monitored and cooled as necessary. Figure 4.1a shows sonication fragment sizes for all nine samples. Pooled samples 3 and 7 were re-sonicated to achieve appropriate fragment size range (Figure 4.1b). For each pool, one sonicated sample was enriched for methylated DNA, while another sample was used as a control genomic input for co-hybridization.

Methyl-CpG binding domain-based capture (MBD-Cap) was used to enrich fragmented samples for methylated DNA (EpiMark Methylated DNA Enrichment Kit, New England Biolabs, Ipswich, Massachussets). The EpiMark kit contains MBD2-Fc, which is the methyl-CpG binding domain of human MBD2 fused to the Fc tail of human IgG1. This

protein was then coupled to protein A magnetic beads via incubation at room temperature for 15 minutes, followed by two washes. Fragmented DNA was then added to the MBD2-Fc/protein A mixture and incubated at room temperature for 20 minutes, and washed three times to remove unbound DNA. The methylation-enriched DNA was eluted by incubating in water at 65^{o}C for 15 minutes. In order to obtain sufficient amounts of DNA for hybridization, 10 ng of the captured DNA was subject to whole genome amplification using the GenomePlex Complete Whole Genome Amplification Kit (Sigma-Aldrich, St. Louis, MO).

The control, un-enriched sample and CpG methylation-enriched sample – henceforth referred to as the "experimental" sample – were labeled with Cy3 and Cy5 dye, respectively, using the NimbleGen Dual-Color Labeling Kit (Roche NimbleGen, Madison, WI) following the protocol outlined in the NimbleGen Array User Guide (NimbleGen Array User Guide *DNA Methylation Arrays*, Version 7.2). Briefly, Cy3 and Cy5 were incubated with control and experimental sample, followed by sample amplification via incubation in a Klenow fragment and dNTP mixture. These labeled fractions were then pooled together in equivalent amounts and co-hybridized to the Roche NimbleGen Mouse DNA methylation 3x720K CpG island Promoter Array for 16-20 hours. Each array slide contains three subarrays, each of which holds 720,000 probes scanning 15,980 CpG islands in 20,404 murine gene promoters. Each promoter is covered by probes spanning from 2,960 bp upstream of the promoter transcription start site (TSS) to 740 bp downstream of the TSS. The length of each probe ranges from 50-75 bp with median probe spacing of 100 bp. After hybridization, each array was washed and scanned using a 2 μn-resolution scanner (NimbleGen MS 200 Microarray Scanner, used courtesy of Dr. Thomas Glover, Department of Human Genetics).

4.3.4 Bioinformatics Pipeline – A brief overview of the bioinformatics pipeline is shown in Figure 4.2. Scanned images were uploaded to Nimblegen DEVA Software, version 2.3. Each image was overlaid onto a pre-specified alignment grid in order to extract location and raw Cy3 and Cy5 intensity – corresponding to control and experimental sample, respectively – of each feature. Locally weighted polynomial regression (LOESS) spatial correction was performed in order to correct for position-dependent non-uniformity of signals within the sub-array. After position-dependent normalization, the DEVA software calculated $\log_2$(Cy5-labeled experimental/Cy3-labeled control) ratios for each probe. Probes were centered around Tukey's biweight mean for all probes. Briefly, the biweight mean is a measure of central tendency that is resistant to outliers. In principle, it is calculated by assigning weights to data values based on their departure from the median of the data cluster. Thus, $\log_2$(ratio) values that are closer to the median of the data are assigned higher weights than those that are further away. Quantile normalization was used to normalize the data across arrays, using the *preprocessCore* package in Bioconductor [44] in R Statistical Software (version 3). After quantile normalization, each probe-associated $\log_2$(ratios) was fit to a basic linear regression model, as shown in Equation 4.1 below. The $\beta_{\text{Pb dose}}$ coefficient signifies the value of change in methylation ratio associated with having the given category of Pb exposure relative to non-exposed mice.

Equation 4.1

$$\textbf{Normalized BWR}_{probe\_i} \sim \beta_0 + \beta_{Pb\ dose}(\textbf{Pb dose})$$

We applied empirical Bayes (e-Bayes) smoothing to the standard errors obtained from the regression in Equation 4.1 using the *limma* Bioconductor package. The e-Bayes method is

used to better estimate variance for t-tests performed on a small sample size. The moderated t-statistic produced by e-Bayes is a result of a weighted average of each sample gene's variance and the background variance of the whole set of probes.

4.3.5 Bumphunt analysis – We utilized a previously described statistical approach termed bumphunting [45] to generate associations between Pb exposure and gene promoter methylation at the regional level instead of at the individual probe level. Briefly, this statistical analysis uses four steps to summarize the information from consecutive probes in any designated promoter region to generate a region-wide methylation association with Pb exposure. First, methylation measurements for each probe are plotted against the exposure level, which in this case, is categorical variable of control, low, and high Pb dose. Next, a regression coefficient is generated for each probe. For consecutive probes, these probe-level coefficients are then plotted against their genomic location. Once the coefficient values are smoothed along the genomic locations, the area under the smoothing line is tested to see if the difference in methylation reaches a pre-defined threshold absolute value; in this case, it was set as |0.005|. The area is then tested against null distributions that are generated from 1000 bootstrap permutations from the data to determine statistical significance of each bump.

4.3.6 Pathway analysis – Individual probes were ranked based on p-values from the linear regression model and read into two pathway analysis programs: *Pathway Analysis using Logistic Regression* (*LRpath*) [46]and *Database for Annotation, Visualization and Integrated Discovery* (*DAVID*, *version 6.7*) [47,48]. All probes were input into LRpath,

regardess of p-value. For pathway analysis using DAVID, the top 499 differentially methylated probes ranked by p-values served as the input set of probes.

4.4 Results

4.4.1 Neuronal separation – Fluorescence-activated cell sorting was performed in several steps. As shown in Figure 4.3A (Gate 1), labeled nuclei that were not residual debris comprised 63.12% of the initial sample. Figures 4.3B and 4.3C indicate FSC-Area vs FSC-Height and SSC-Area vs SSC-Height, respectively. These were used to separate doublets out of the sample. There was an auto-fluorescence rate of 0.08% for a sample of 10,000 cells (Figure 4.3D, Gate R4). The R5 gate was further adjusted as shown in figure 4.3E, the sample that was pre-labeled with non-fluorescent anti-NeuN antibody. Figure 4.4F shows that 74.36% of the sample was $NeuN^+$, whereas the $NeuN^-$ population comprised 22.83% of the sample. These proportions were similar throughout the samples that were sorted. The average number of NeuN+ nuclei per sort was $1.21x10^6$ nuclei and $4.65x10^5$ $NeuN^-$ nuclei. The average amount of DNA extracted was 4.72 ug per 1 million nuclei.

4.4.2 Overall Differential Methylation – Mice exposed *in utero* to 32 ppm Pb had 11,517 (1.7%) probes with differential methylation as compared to non-exposed mice at $p<0.005$, (lowest FDR 0.30). Of these, 7554 (65.6%) were hypomethylated (lowest $\log_2(\beta_{Pb\ dose})= -2.22$) and 3963 were hypermethylated (highest $\log_2(\beta_{Pb\ dose})= 1.93$). Mice exposed to 2.1 ppm Pb had 2793 probes with differential methylation at a p-value <0.005, with 1249 hypomethylated probes (44.7%) and 1544 hypermethylated probes (55.3%). However,

only 3 differentially methylated probes in mice in the low dose Pb exposure group reached an FDR cutoff of 0.3.

4.4.4 Bumphunt Analysis – Probe-level data was combined using a bumphunting approach in order to find larger differentially methylated regions in the genome. The six regions that had the lowest p-values and met an FDR and Family-wise error rate cutoff of 0.3 are shown in Figure 4.4. The region that was most highly differentially methylated was most closely linked with retrotransposed gene for the heterogeneous nuclear ribonucleoprotein C (*retro-Hnrnpc*). The regions associated with *Retro-Hnrnpc,* Olfactory receptor 1085 (*Olfr1085*), and Histamine N-methyltransferase (*Hnmt*) were all hypermethylated in the mice exposed to 32 ppm Pb in drinking water relative to the control and low dose (2.1 ppm Pb). Methylation change in the region associated with Selection and upkeep of intraepithelial T-Cell 5 (*Skint5*) showed more of a monotonic response with increasing Pb dose. Mice exposed to both high and low doses of Pb were hypomethylated at a 635 base-pair region upstream of Xylosyltransferase (*Xylt1*). The differentially methylated region in Protocadherin 19 (*Pcdh19*) suggested a non-monotonic association with Pb dose, with the highest dose being hypomethylated relative to the low dose, but hypermethylated in relation to control.

4.4.3 Pathway Analysis – Pathway analysis was done using two online programs to detect which differentially methylated regions in the exposed mice were enriched for certain pathways. DAVID analysis utilized 499 differentially methylated regions ranked by p-value in the e-Bayes analysis. The top two annotation clusters are shown in Table 4.2A-B.

The top annotation cluster had an enrichment score of 2.06, and was enriched for genes in the GTPase activator pathways. The next most significant DAVID annotation cluster, with an enrichment score of 1.89, was predominantly enriched mainly in neurological systems processes and olfactory sensation processes. Although most p-values reported were less than 0.01, multiple-comparison corrections of p-value including false discovery rate (FDR), Bonferroni, and Benjamini corrections all failed to reach significance.

Because DAVID pathway analysis only uses a limited number of probes from the whole array analysis, we also analyzed all array probes using LRpath. As shown in Table 4.3, the pathways enriched using LRpath were "Myofibril assembly", "Determination of left/right symmetry", and "Negative regulation of BMP signaling pathway". Although the p-values associated with these pathways were less than 0.01, the FDR method to correct for multiple comparisons indicated that these pathways did not reach statistical significance as differentially methylated pathways.

4.5 Discussion

This is the first neuron-specific genome-wide DNA methylation analysis of a group of mice that was environmentally exposed *in utero* to Pb. We demonstrated clear separation of anti-NeuN$^+$ cells from anti-NeuN$^-$ cells, as shown in Figures 4.3A-E. We isolated approximately 0.63-1.74 x 10^6 NeuN+ nuclei and 0.27-1.26 x 10^6 NeuN- nuclei, yielding DNA on a scale of micrograms for each sample. Although the NimbleGen platform used in the current study demanded much of this DNA, the input genomic DNA required for genome-wide methylation studies is decreasing with advancing methylation-sequencing technologies.

Overall, we did not see evidence for an abundance of large changes in percent methylation among Pb dose levels. Analysis of consecutive probes as performed in the bumphunting approach provided opportunity to identify significant regions (ranging from 600-2900 bp in size) on whole regions rather than the 100 base-pairs covered by individual probes. This analysis yielded a handful of promising sites showing a relationship with Pb dose: hypomethylation with increasing Pb dose in some genes (*Xylt1*), hypermethylation in others (*Hnmt*, *Olfr1085*, and *retro-Hnrnpc*). However, given the small sample size and FDR level of 0.30, we interpret these results with caution, emphasizing the need for orthogonal validation [49], such as bisulfite sequencing of target regions. Despite this limitation, given that the direction of methylation varied with gene region, it is possible that Pb exposure modifies DNA methylation in a gene- or pathway-specific manner.

Probe-level analysis did not suggest an association of Pb exposure at low and high doses with differential methylation in neuron-specific cell populations, as the DAVID and LRpath pathway analyses failed to reach statistical significance after adjusting for multiple comparisons. Both pathway analyses were limited in that they utilized information at the probe-level. It is possible that several probes that do not reach statistical significance may not be picked up by such an analysis; hence our emphasis on using the bumphunt approach. Reasons we did not detect a robust effect on DNA methylation by Pb dose include: a small sample size with limited power, the assessment of only promoter and CpG island regions, and the assessment of only neuronal cells. While our study provides evidence that there is a strong overall shift in DNA methylation pattern in neurons due to *in utero* Pb exposure, it does not rule out the possibility of a targeted set of regions or genese affected in a dose-dependent manner.

Although the present research is unique in its analysis of a cell-specific population, neurons are a particularly confounding set of cells in which to study DNA methylation. Additionally, as mentioned previously, neuronal activity modifies DNA methylation [32,33], suggesting that each individual cell will be differentially methylated depending on its activity. Thus, it is difficult to tell which regions of the methylome are activity-dependent, prone to direct methylation modification by Pb, or prone to modification by Pb through its independent effect on neuronal activity. Future studies would have to differentiate between the activity-dependent neuronal methylome and the part of the methylome that may be less susceptible to neuronal firing. An additional limitation of the study was that our genome-wide analysis platform was a promoter tiling array. Although it provides excellent coverage of the genome, it focuses only on promoters. Other literature on the association between DNA methylation and various cancers clearly suggests that functional CpG methylation may be taking place in CpG shores, and not necessarily associated with promoter regions [50,51].

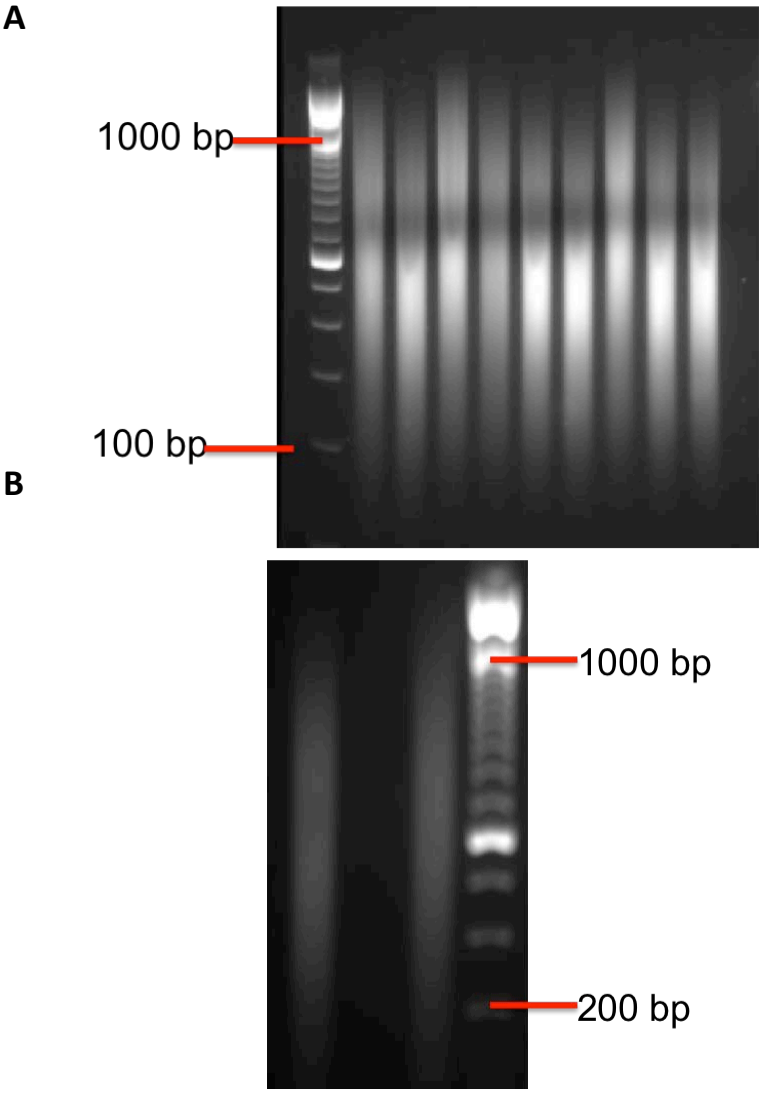
Despite the limitations of this study, it is the first to look at a neuron-specific population of cells of mice exposed to Pb *in utero*. The window of susceptibility to exposure was critical: all mice in our study were exposed to Pb via the maternal drinking water, though gestation and weaning. This unique exposure paradigm allowed us to look at the influence of environmental exposures on the methylome during neuro-development. The tiling array platform used in these studies was intended as a discovery tool to identify pathways and regions that may be differentially methylated *in utero* dependent on environmental exposure. The present study has generated several targets for further downstream study, the most interesting of which is *Hnmt*, due to its clear role in

neurotransmitter metabolism. *Pcdh19* is also of interest due its expression during neurodevelopment in the mouse embryo [52]. *Pcdh19* is a cadherin molecule that is also associated with epilepsy in females [53]. Future directions of study would include correlation of DNA methylation with expression of the *Hnmt* and *Pcdh19* as well as other top hits found in the bumphunt analysis. This study was done on a subset of males only, so future studies should include an analysis of females to examine potential gender-specific effects of Pb. Our results are consistent with prior work published on the effect of early life exposure to Pb on the mouse methylome by Dosunmu *et al* [54]. The authors observed a correlation between expression and methylation, but they noted only a small percentage of genes were differentially methylated as a result of Pb exposure. The Dosunmu study was not performed on a specific population of neurons, nor were mice exposed *in utero*. Combined with results from our study, the sum of results suggests a weak association between early-life exposure to Pb and DNA methylation.

Table 4.1: Number of mice used in pooling scheme

	Pooled Sample 1	Pooled Sample 2	Pooled Sample 3
0 ppm	2 mice	2 mice	2 mice
2.1 ppm	2 mice	2 mice	2 mice
32 ppm	3 mice	2 mice	2 mice

Figure 4.1 – Fragmentation of NeuN(+) DNA via sonication



A) Pooled samples 1-9. Samples 3 and 7, corresponding to pool 3 for control group and pool 1 for high dose group, did not reach appropriate fragment size.
B) Pooled samples 3 and 7, corresponded to lanes 1 and 3, respectively. After re-sonication, the samples reached appropriate fragment size.

Figure 4.2 – Bioinformatics Pipeline. Image alignment and data extraction, Loess correction, and ratio calculation and centering were all performed in DEVA software. Quantile normalization across arrays was performed using *preprocessCore* in R Bioconductor. Cross-array analysis includes individual probe-level analysis, region-level analysis, and pathway analysis using individual probe-level analysis

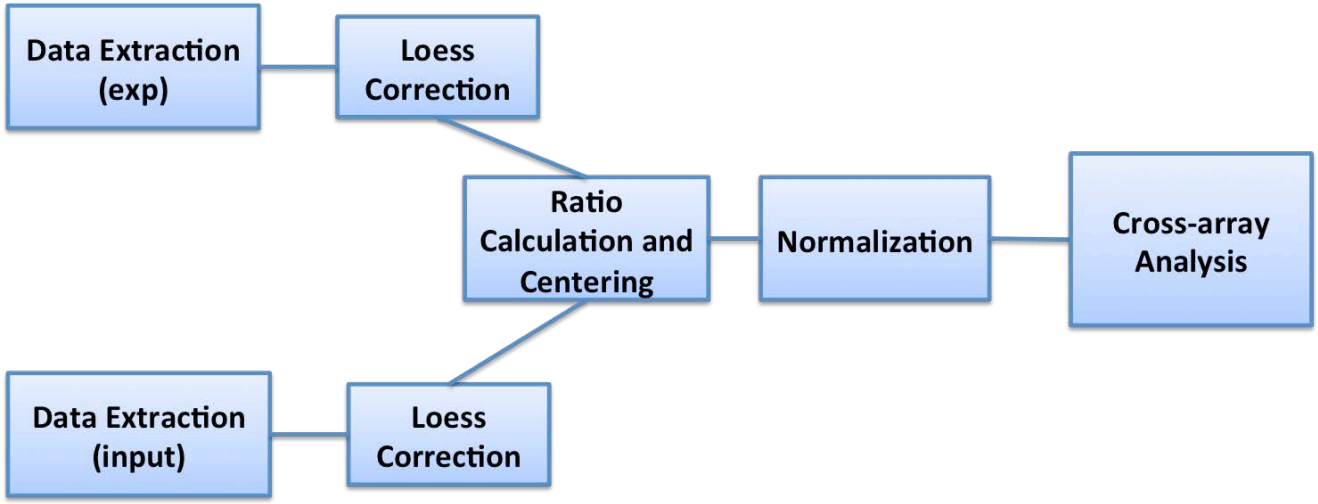
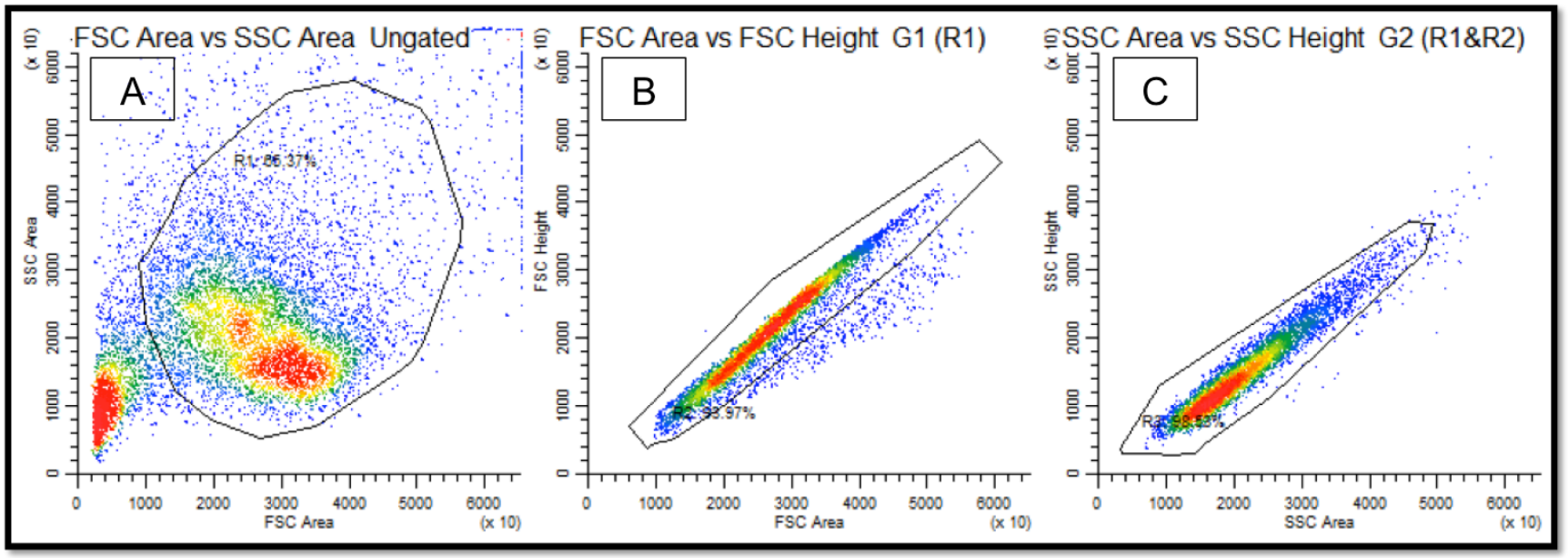
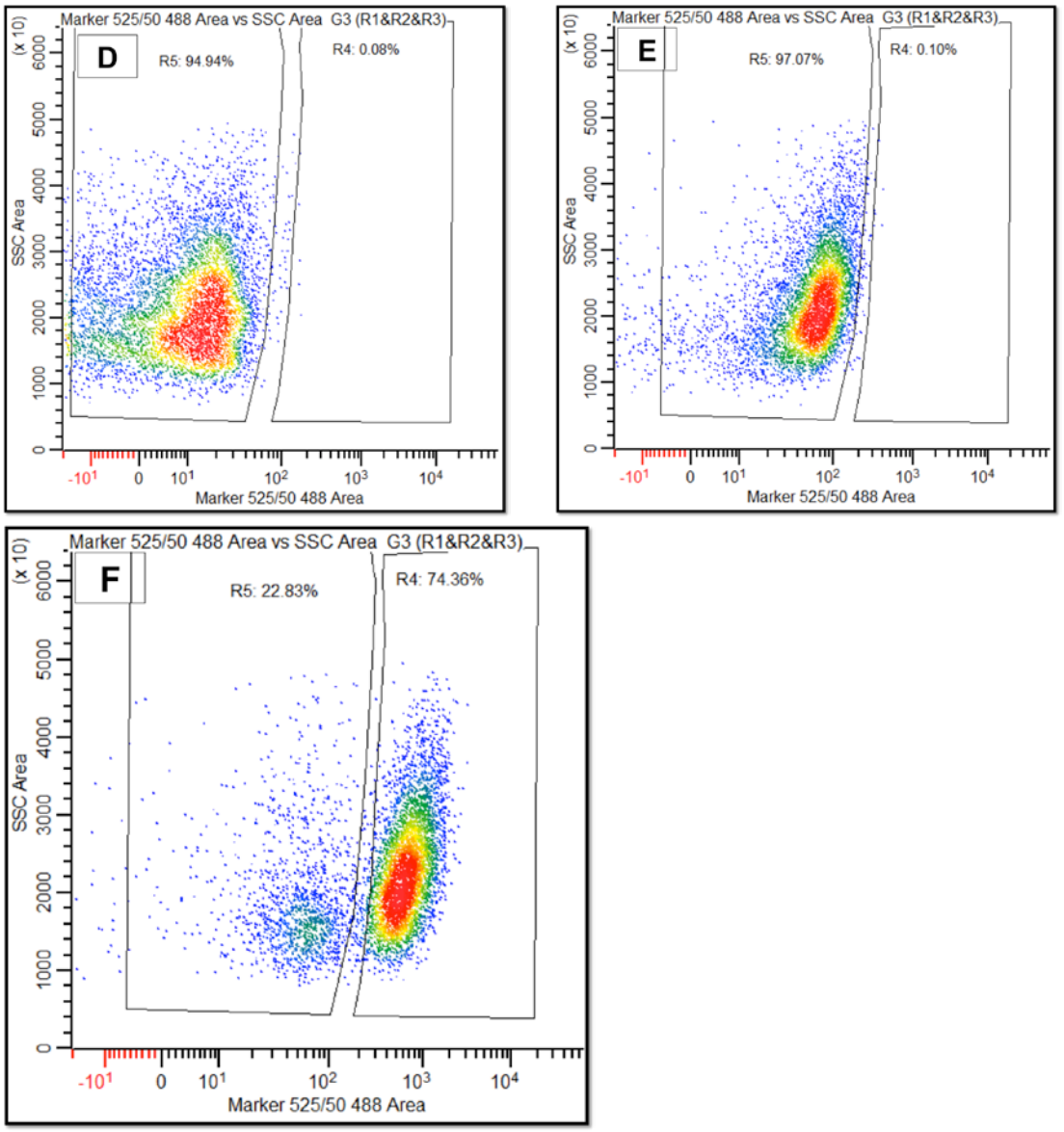


Figure 4.3 – Fluorescence-activated Cell Sorting



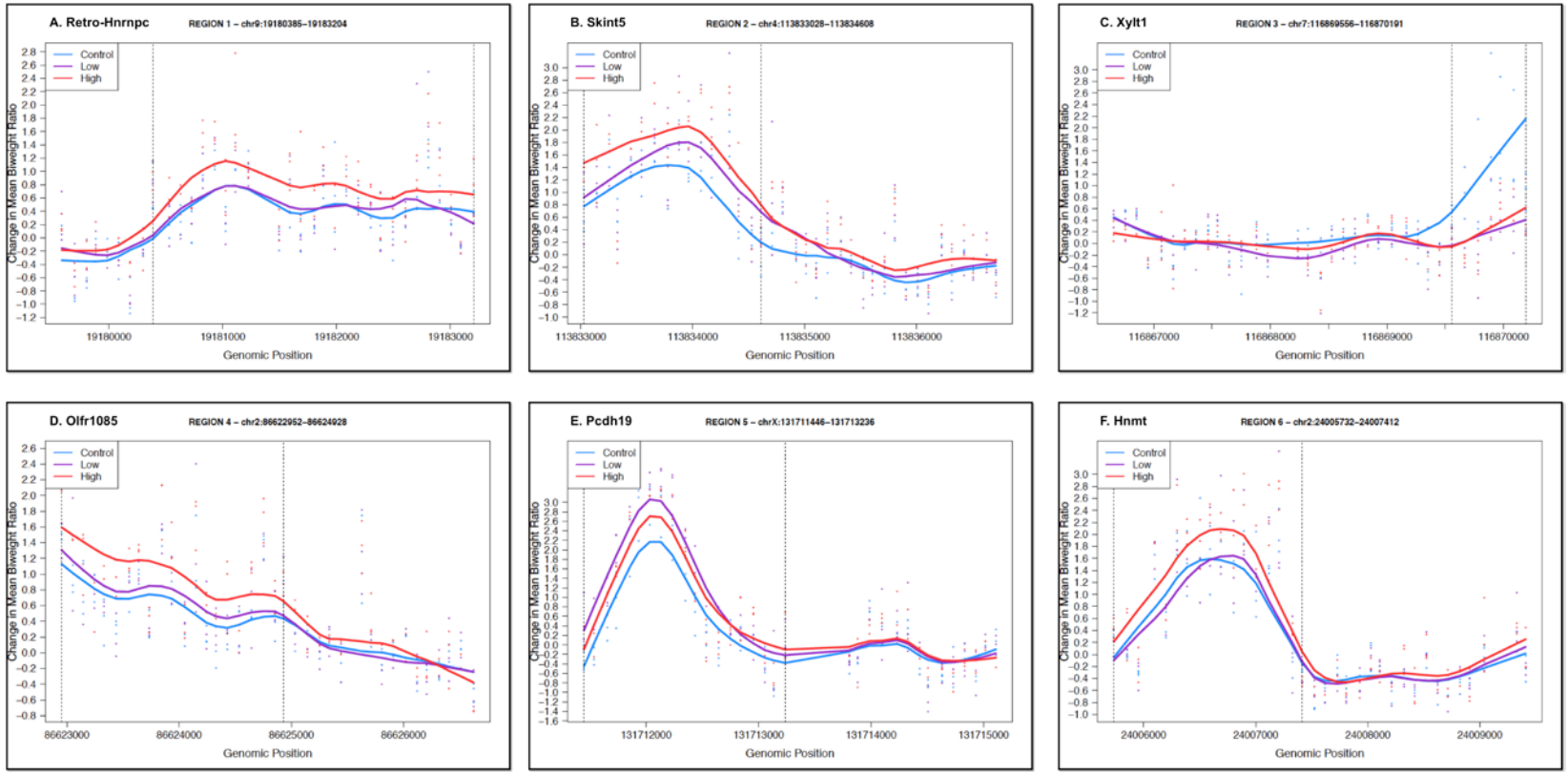
A) Plot of FSC-Area vs SSC-Area to filter out debris from nuclei population (encircled area, Gate R1). **B and C**) – Plots of FCS-Area vs FCS-Height **(B)** and SSC-Area vs SSC-Height **(C)** to remove doublets. Abbreviations – FSC: Forward Scatter, SSC: Side Scatter

Figure 4.3 (cont) – Fluorescence-activated Cell Sorting



D) Unstained control. **E)** Saturation control, pre-incubated with untagged Anti-NeuN, to determine non-specific binding gate. **F)** Separation of AlexaFluor488-Anti-NeuN^{+} neuronal nuclei in Gate R4 vs non-neuronal Anti-NeuN^{-} nuclei in Gate R5.

Figure 4.4: Top hits from Bumphunt analysis. Top hits reaching Family-wise error rate ~ 0.3, False discovery rate ~ 0.29. Regions within dashed lines indicate the significant differentially methylated "bump", regions outside of boundary are plotted to show trend outside the region of bump.



A) *Retro-Hnrpc*, chromosome 9:19,180,385-19,183,204. **B)** Selection and upkeep of intraepithelial T-Cell 5 (*Skint5*), chromosome 4:113833028-113834608 **C)** Xylosyltransferase 1 (*Xylt1*), chromosome 7:116869556-116870191. **D)** Olfactory receptor 1085 (*Olfr1085*), chromosome 2:86622952-86624928. **E)** Protocadherin 19 (*Pcdh19*), chromosome X:131711446-131713236. **F)** Histamine N-methyltransferase (*Hnmt*), chromosome 2:24005732-24007412.

Table 4.2: Top pathway hits from Individual Probe-level DAVID Analysis

4.2A - Annotation Cluster 1: Enrichment Score: 2.055				
Category	**Pathway**	**PValue**	**Count**	**Fold Enrichment**
GOTERM_MF_FAT	GTPase activator activity	0.0025	13	2.794
GOTERM_MF_FAT	GTPase regulator activity	0.0067	18	2.058
GOTERM_MF_FAT	Enzyme activator activity	0.0077	14	2.320
GOTERM_MF_FAT	Nucleoside-triphosphatase regulator activity	0.0080	18	2.024
SP_PIR_KEYWORDS	GTPase activation	0.0525	8	2.371

Table 4.2 (*cont*): Top pathway hits from Individual Probe-level DAVID Analysis

4.2B - Annotation Cluster 2: Enrichment Score: 1.889				
Category	**Pathway**	**PValue**	**Count**	**Fold Enrichment**
GOTERM_BP_FAT	Neurological system process	0.0022	58	1.474
PIR_SUPERFAMILY	G protein-coupled olfactory receptor, class II	0.0023	35	1.681
PIR_SUPERFAMILY	Rhodopsin-like G protein-coupled receptors	0.0029	43	1.551
KEGG_PATHWAY	Olfactory transduction	0.0033	38	1.577
GOTERM_BP_FAT	Sensory perception	0.0042	49	1.493
INTERPRO	Olfactory receptor	0.0109	40	1.499
GOTERM_BP_FAT	Cognition	0.0114	49	1.415
GOTERM_BP_FAT	Sensory perception of smell	0.0118	39	1.492
GOTERM_MF_FAT	Olfactory receptor activity	0.0158	40	1.454
GOTERM_BP_FAT	Sensory perception of chemical stimulus	0.0193	40	1.434
GOTERM_BP_FAT	G-protein coupled receptor protein signaling pathway	0.0208	58	1.320
SP_PIR_KEYWORDS	G-protein coupled receptor	0.0228	48	1.371
INTERPRO	7TM GPCR, rhodopsin-like	0.0245	47	1.370
GOTERM_BP_FAT	Cell surface receptor linked signal transduction	0.0278	73	1.250
SP_PIR_KEYWORDS	Transducer	0.0285	49	1.346
INTERPRO	GPCR, rhodopsin-like superfamily	0.0514	48	1.295

Table 4.3: Top pathway hits from probe-level LR Path analysis

Name	ConceptType	# Genes	Coeff	OddsRatio	P-Value	FDR	Direction
Regulation of cell size	GO Bio Process	273	-0.195	0.298	6.60E-04	0.588	depleted
Myofibril assembly	GO Bio Process	18	0.387	11.099	9.97E-04	0.588	enriched
Regulation of cell growth	GO Bio Process	205	-0.222	0.251	0.001	0.588	depleted
Determination of left/right symmetry	GO Bio Process	32	0.313	7.005	0.001	0.588	enriched
Cell growth	GO Bio Process	257	-0.190	0.306	0.001	0.588	depleted
Negative regulation of BMP signaling pathway	GO Bio Process	20	0.369	9.930	0.001	0.588	enriched

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Chapter 5

Conclusion

5.1 Summary of Results – This summary section is included for convenient reference to the overall results of aims 1-3.

Aim 1 – Among men 51-89 years of age at baseline, and interquartile range increase (IQR=21 μg/g)in Pb was associated with a lower baseline MMSE (β=-0.128,p=0.04) and a faster decline in the rate of MMSE (β=-0.016,p=0.04). We also found a suggestive association between patella Pb and the risk of cognitive impairment defined as MMSE score dropping below 25 using Cox proportional hazard models (hazard ratio=1.21, 95% CI:0.99-1.49). Patella Pb was not associated with summary scores of global cognition at baseline or longitudinally. Tibia Pb was not associated with baseline measures or changes in MMSE or global cognition. Associations between Pb and baseline level or change in individual tests of cognition varied by domain.

Aim 2 – The hazard ratio (HR) associated with an interquartile range (21 μg/g) increase in patella lead was 2.15 (95% confidence interval (CI), 1.36-3.41) for subjects heterozygote at the *CR1* locus, but it was 1.11 (95% CI, 0.85-1.44) for subjects with no *CR1* minor allele ($p_{interaction}$=0.01). For subjects who were homozygous for minor allele at *PICALM*, the HR associated with and IQR increase in patella lead was 3.54 (95% CI, 0.95-13.25), whereas a

null association was found for men with no minor allele ($p_{interaction}$=0.08). We found no significant effect modification by ApoE.

Aim 3 – In a cohort of adult male mice exposed via the maternal drinking water to 0 ppm, 2.1 ppm, or 32 ppm of Pb two weeks before mating, throughout gestation, and three weeks after birth, we analyzed genome-wide DNA methylation levels in a neuron-specific cell population. Using the bioinformatics bumphunting method and a combined false-discovery rate cutoff of 0.3 and family-wise error rate cutoff of 0.3, we report 6 novel exposure-dependent differentially methylated regions associated with the following genes: retro-*Hnrnpc*, *Skint5*, *Xylt1*, *Olfr1085*, *Pcdh19* and *Hnmt*.

5.2 Synthesis of findings

There is a growing body of evidence that environmental exposures play a role in cognition. This thesis adds weight to that evidence in several ways: it strengthens evidence for a causal effect of Pb by establishing significance in a longitudinal model, and it provides data to support the hypotheses that the Pb interacts with the genome in two ways: 1.) it modifies the effect of single-nucleotide polymorphisms at the epidemiologic level and 2.) it alters of DNA methylation.

In conclusion, I hope to place these findings in the context of the current state of knowledge of how environment modifies disease. Does exposure to Pb truly pre-dispose humans to accelerated cognitive decline and neurodegenerative disease? If so, does the epidemiologic and mouse data help us in delineating mechanisms by which Pb exerts its

effect? In answering these critical questions, it is important to look at alternative mechanisms and weigh these explanations against the literature and our own results.

5.3 Use of Longitudinal Models to Track Trajectory of Environment-associated Cognitive Decline

Cognitive Decline – There is substantial literature to correlate Pb with a broad spectrum of cross-sectional cognitive markers in community dwelling older adults. In the Normative Aging Study (NAS), blood lead levels in a small sample significantly predicted scores on the MMSE [1] as well as tests of speed, memory, and visuospatial ability [2]. However, in a larger subset of the same study population in the NAS, blood lead levels were not strongly associated with cross-sectional or longitudinal MMSE scores [3] or any tests of visuospatial, language, or memory domains, with the exception one cross-sectional association with a vocabulary test [4]. Similar findings were reported in the Baltimore Memory Study (BMS), in which BLL's were not associated with any of seven cognitive domains [5]. On the other hand, in the NAS, patella Pb was found to be associated with cross-sectional scores as well as a decrease between first and second visits in MMSE. Similar results of cross-sectional [6] and studies of change were noted in tests of pattern comparison and spatial copying scores [4]. Tibia Pb analysis yielded similar, yet slightly less statistically significant results. In the BMS tibia lead was associated with lower cross-sectional test scores in 7 cognitive domains [5]. Taken together, these findings suggest that at a cross-sectional level, there is consistent association with bone Pb levels and impaired cognition.

Our study adds a crucial component to this body of literature in community-dwelling older adults. In our population, mean patella and tibia Pb levels were respectively 30.6 ug/g (SD=19.4) and 21.6 ug/g (SD=13.3), similar to prior studies. We have been able

to analyze trajectories of cognition over a longer follow up time of 12-15 years. This allows us to move beyond the learning effect that is inherent in tests of cognition. In the general literature on MMSE scores and other tests of cognition, the most pronounced cognitive decline is seen over a time-scale similar to that reported in this thesis [7-9]. We have been able to model the chronic effects of cumulative Pb dose on alteration of cognitive trajectory utilizing mixed effects longitudinal regression as well as a Cox proportional hazards analysis. This can help public health researchers track the efficacy of preventive interventions as well as stratify populations that are at risk for cognitive decline [10,11]. This type of analysis is dependent on markers of cumulative exposure, as well as on repeat measures of neurological outcomes that span approximately 10 years. Overall, there is enough evidence to say that Pb exerts a causal effect on cognitive decline.

There is one prominent limit on our ability to interpret the effect of Pb on clinical outcomes. We have not correlated blood or cumulative bone Pb with neurodegenerative disease. To my knowledge, only Parkinson's dementia has been found to be associated with Pb exposure [12,13]. An epidemiologic study that establishes the association between Pb and AD would be the most relevant experiment to establish Pb as a general neurodegenerative toxicant. A researcher might run a Cox proportional hazards model in two possible studies: 1.) A study of the association between Pb measured by inductively coupled mass spectrometry (ICP-MS) of post-mortem bone with AD brain specimens or 2.) A study of living patients that analyzes the association between K-XRF bone Pb with Pittsburgh compound B-assisted brain scans of at-risk patients [14].

5.4 The Utility of Discovering Gene-Environment Interactions

The interplay of genetics and environmental exposure is of key interest in this thesis. The basis of gene-environment interaction can be thought of from an evolutionary perspective. A randomly selected trait may not influence fitness of a species until external conditions pressure the selection for that trait. Similarly, any given genetic variant may truly be associated with a disease, but we cannot observe that association until we coax it out with environmental exposure. Thus, gene-environment interactions have driven selection of species, and are important in modern medicine. SNP gene variants explain only up to 33% of the variability in AD. It is possible that studies incorrectly calculate the population attributable risk (PAR) explained by genes simply because they ignore the joint effect of the interaction between genes and environment [15]. Another benefit of such studies is that they may one day allow for preventive advice that takes into account an individual's genetic susceptibility to environmental exposures.

5.4.1 Potential Mechanisms of Interaction – This thesis analyzed the interaction between Pb and several genes associated with cognition and/or AD to modify the longitudinal trajectory of MMSE scores. A biological interpretation of gene-environment interactions found from epidemiologic data must be done on a case-by-case basis. For the case of Pb and *APOE* interaction, Pb has previously been shown to be associated with APOE expression levels. Interestingly, the *APOE* variants at rs7412 and rs429358 are CpG switches [16], suggesting that methylation may play a role in how *APOE* modifies the effect of Pb on cognition. Another way in which Pb may be interacting with genes is that SNPs may serve as proxies for methylation sites. One cannot ignore the fundamental role of Pb as a highly oxidative, divalent cation [17-19]. Pb may differentially interfere with the gene

product of *APOE* variants in comparison to wildtype *APOE*. What are the potential mechanisms of interaction of Pb with *PICALM*, *CR1*, and *CLU*? The SNPs we utilized for these genes are intronic, and so the mechanism of their effect on cognition is unclear. *PICALM*, *CR1*, and *CLU* are involved in processing and trafficking APP cleavage products, but the SNPs we examined are non-coding. Perhaps these SNPs, too, are proxies for epigenetic regulators that can be modified by Pb. Future studies would need to determine if Pb differentially modifies the expression of these genes. We did not observe any association of Pb-CELF1 interaction with cognitive decline. Despite the non-significance (p=0.35), we did observe an additive effect of the minor allele on the Pb-associated hazard ratio. CELF1 is interesting in its association with cognition [20] as well as plasma homocysteine [21]. Plasma homocysteine levels are also shown to increase with cumulative Pb exposure [22]. If a true interaction existed, it would show that Pb and *CELF1* may converge on similar pathways to modify cognition. However, we were not able to observe this in our population.

In summary, do gene-environment interactions truly modify the rate of cognitive decline? Our data, combined with prior literature supporting biological plausibility, suggest that an interaction is possible. Given the broad-acting effects of Pb, each gene-environment interaction must be interpreted on its own merit. To validate such effects, one would have to reproduce these findings in other populations. Our study is among the first to propose a gene-environment interaction that modifies longitudinal trajectories of neurological outcomes.

5.5 Epigenetics as a Mediator of Pb Effects on Cognition

Bone Pb is an excellent proxy for long-term cumulative exposure. However, data suggests that very early life exposure to toxicants – even when the exposure is removed – can pre-dispose an organism to adverse outcomes. The evidence for this developmental origin of health and disease is quite strong in some exposures. For example, the maternal licking and grooming behavior infant rats are exposed to early in life modifies their stress response much later [23,24]. In this example, DNA methylation changes at two CpG sites were crucial in mediating the differential stress response. In monkeys exposed to Pb in the first year of life, researchers have noted amyloid plaques in post-mortem brain analyses [25]. Thus, it is plausible that DNA methylation is a mechanism by which early life exposure to Pb leaves a stable mark that modifies neurological outcomes later in life.

There is ample evidence that Pb is associated with changes in DNA methylation in various tissues in the body. Epidemiologic evidence in the NAS shows that bone Pb levels are associated with hypomethylation of LINE-1 retrotransposable elements in the DNA of peripheral blood cells [26]. In a separate population of mother-infant pairs in Mexico, Alu retrotransposons were also hypomethylated in association with maternal bone Pb [27]. Both Alu and LINE-1 are repeat elements, and therefore, markers of global DNA methylation. Thus, the epidemiologic data presented suggests that Pb may play a role in global hypomethylation, and our overall genome-wide data corroborates this, where we see hypomethylation in 65.6% of significant differentially methylated genes ($p<0.005$, FDR<0.30). However, a follow up study of the mother-infant pairs in Mexico noted hypermethylation with higher bone Pb at the insulin-like growth factor 2 (IGF2) gene [28].

This suggests that although there may global hypomethylation, Pb may act variably on individual gene regions.

A few pieces of toxicologic data are particularly relevant to the role of Pb in influencing methylation in the brain. First, human embryonic stem cells display altered neuronal differentiation in response to Pb, and these changes may be mediated by DNA methylation as measured by the Illumina HumanMethylation450 BeadChip [29]. Second, Zawia *et al* demonstrated that early life exposure to Pb decreases DNA methyltranferase 1 activity (DNMT1) [25]. The same group has shown modest changes in methylation with early life Pb exposure in rats [30]. Our data particularly strengthen this evidence for two reasons: First, our exposure paradigm was *in utero*, where the offspring were exposed via maternal drinking water. Therefore, the differential epigenetic modifications observed are a result of the actions of Pb on the developing embryo. Secondly, we isolated a neuron-specific population. This is of key importance because a mixed cell population may not be representative of the differential states of methylation of its constituent cell types [31].

Combining the toxicologic and epidemiologic literature, there seems to be a modest association between early life exposure to Pb and DNA methylation. This association may be functional, but further research is needed. First, a limitation of our study as well as the Zawia studies is the lack of correlation to tests of cognition. Although a memory test such as the water maze learning test would allow for us to link exposure, DNA methylation, and phenotype along a causal pathway, many of the behavioral tests may not be sensitive enough to detect minute differences in the effect of low Pb doses [32]. Aside from validation of neurological phenotypes, direct mechanistic studies of how Pb modifies DNA methylation will be crucial. Since Pb has a broad number of targets, one might expect it to

act in a diffuse manner. There are two potential mechanisms that deserve further exploration. First, *de novo* DNA methylation and maintenance are dependent on substrates in the 1-carbon metabolic pathway. Specifically, homocysteine is a key molecule in the pathway for transfer of a methyl group to CpG dinucleotides. Since epidemiologic studies indicate that Pb elevates homocysteine levels, animal and biochemical studies investigating the mechanism by which that occurs would shed light on the interaction between Pb and the epigenome. Secondly, the action of Pb as an oxidative molecule may increase the level of 8-oxoguanine [33], which is associated with hypomethylation at adjacent CpG sites [34]. Pb may also directly oxidate methyl-CpG sites as part of a pathway to demethylation [35]. These are all only speculative hypotheses and need to be tested in mice as well as via direct biochemical binding studies.

5.6 Public Health Implications

In summary, human disease is a result of a complex interplay of the genome and the environment. Despite advances in sequencing and large-scale data analysis of genetic variance, medicine has only been able to explain a fraction of variability in multi-factorial diseases such as neurodegeneration. This dissertation was aimed at studying how to increase the ability to explain variability in disease by modeling the effect of the environment. Despite advances in decreasing exposure to toxicants, there are still new, harmful environmental exposures arising every day. Modern demands of technology and mass consumption of energy have devastating consequences that release toxic materials into the environment [36,37]. The effects of destabilization in war leads to exposure to

pollution, buried toxicants that are uplifted due to explosive damage, and even heavy metals such as uranium [38]. It is imperative that we have a basis with which to study the long-term effects of these toxicants, particularly in the brain. This dissertation uses Pb as a model toxicant to support the evidence that it is, indeed, a contributing cause of cognitive decline. This work has the potential to further modify policy to reduce Pb exposure by showing that bone Pb is associated with longitudinal decline. Current policy focuses only on blood lead levels, but given the findings of this dissertation and other work, it may be useful to track bone Pb in potentially exposed populations [39]. We hope that this finding allows researchers to model the longitudinal effects of other environmental exposures to better identify groups that are at risk of progressing from normal cognition to MCI to full-blown dementia. We also recognize that such work has public health implications by strengthening the argument to abate Pb use and mitigate Pb exposure. However, simple association studies are not enough. This dissertation also examined the genetic and epigenetic mechanisms by which long-term exposure to Pb may alter cognition.

5.7 References

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