THE EFFECTS OF LEAD EXPOSURE AT DIFFERENT STAGES OF LIFE ON NEUROBEHAVIORAL AND METABOLIC OUTCOMES

by

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DEDICATION

This dissertation is dedicated to my grandma, who has been and always will be the biggest inspiration to my pursuit of knowing the universe.

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Family has always been oceans away. Yet it has been the biggest inspiration for my pursuit. I would like to express my gratitude to these people who never stopped planting hopes and keeping faith in me. Their love fulfills my heart. Their faith in me filled me with strength to carry on through out these years. I love them, deeply. This is the greatest milestone I ever achieved in my life to share with them. Yet, my gratefulness to these people is beyond any achievement I could make.

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Lead Exposure and Public Health Significance

Lead poisoning is a well-recognized public health concern, and lead pollution remains a serious issue worldwide. In 2003, WHO estimated that there were 120 million people worldwide who had blood lead levels greater than 10 μg/dL, and 240 million people had blood lead levels exceeding 5 μg/dL[1]. A meta-analysis[2] mapped 242 populations from 1011 studies and datasets worldwide and identified 57 populations that had average blood lead levels exceeding 10 μg/dL, the CDC level of concern in children[3]. The main sources of lead pollution vary across countries; the most common sources of lead exposures come from metal smelters, leaded gasoline, lead paint, battery recycling and lead-glazed pottery. For instance, lead gasoline is the main source of lead pollution in Bangladesh and Senegal; family-based battery lead recycling is the main problem in Mexico, the Caribbean Islands, and India; lead contaminated food containers such as lead glazed pottery and lead solder in aluminum cans are major sources of lead pollution in Mexico and Honduras [2, 4]. In China, the lack of environmental emission control of industrial wastes results in regional lead contamination at exceeding levels. Between 1994 and 2004, about 34% of Chinese children had blood lead levels higher than the WHO's recommended limit [5].

The main sources of lead pollution in the United States came from leaded gasoline, lead paint from buildings built before 1980, and lead water pipes[6]. The

concentrations of lead in occupational settings are much higher than those in the living community [7]. Having recognized the detrimental effects of lead, the US Environmental Protection Agency (EPA) in 1973 initiated a series of actions to phase out lead from the daily use products, e.g., the removal of lead from gasoline and paint [8]. It was not until 1996 that the final step was taken by the EPA to eliminate lead from gasoline[8]. Yet, the lead the residuals in urban soil in the US remains a source of concern for lead poisoning[6].

In general, lead levels at or above 150 μg/dL in whole blood may cause irreversible and fatal consequences[9, 10]. At lower levels such as 30-40 μg/dL, lead poisoning results in the impairment of multiple systems such as the nervous system in the susceptible populations, including in children and elderly[11]. Lead poisoning is also correlated with premature birth and intrauterine growth retardation[12-14]. Chronic lead poisoning is associated with renal function impairment[15], cardiovascular diseases[16], infertility[17, 18] and pathological neurodegeneration [19]. The clinical symptoms of lead poisoning are not distinct, which make the diagnosis difficult. The long-term effects from cumulative lead exposure are intertwined with aging process, posing a great challenge for scientific investigation.

Lead poisoning causes adverse outcomes not only limited to human health, but also to economies by virtue of loss in workforce, compensation in medical care, and long term reduction in intelligence[20]. Reducing environmental lead contaminants can result in economic benefits. It is estimated that a lowering the mean blood lead in the population by 1 μg/dL could save approximately $3.5 billion per year in reduced healthcare costs from lead poisoning in the US [21, 22]. In summary, environmental lead

exposure still poses current public health concern. Research and actions should be taken in order to better characterize and control this persistent hazard.

Lead Toxicity

In the body, lead reacts with a variety of molecules at the cellular level. According to Nieboer and Richardson's classification (1980), lead belongs to Class B (Pb^{2+}) and Borderline class (Pb^{4+}) [23]. This thermodynamic characteristic of lead suggests lead ions have a high binding affinity with a broad range of ligands. In particular, lead binding selectively favors nucleophilic ligands such as sulfhydryl, amine, phosphate and carboxyl groups. Thiolate, which is abundant in many functional proteins, has highest the affinity with lead.

One way that lead interferes with the cellular machinery is by inducing oxidative stress. This has a direct impact on cell membranes, causes changes in with the enzyme δ-aminolevulinic acid dehydratase (δ-ALAD) and inhibits reductase. When binding with proteins, lead can cause changes in conformation or occupy a variety of binding sites, thus preventing subsequent reactions. Copropohyrinogen oxidase (CO) is one of the proteins that are affected by lead. When binding with Pb^{2+}, CO enzyme activity is inhibited through structural change, which results in instability of membrane integrity [24]. Alternatively, in other proteins, lead can outcompete other essential metals cofactors at the bioactive binding sites, deactivating proteins. For example, Pb^{2+} displaces Zn^{2+} from the metallothionein (MT) and inhibits synaptic membranes functions maintained by the Zn^{2+}- MT binding structure [25].

Lead can also induce oxidative stress with the involvement of mitochondrial distress. With a higher binding affinity, Pb^{2+} outcompetes Ca^{2+} at the plasma membrane Ca^{2+} transport channel. The permeability of Pb^{2+} at the cell membrane is tenfold of Ca^{2+} [26]. Unlike Ca^{2+}, Pb^{2+} uptake does not appear to reach saturation [26, 27]. Pb^{2+} replaces Ca^{2+} at both of the Ca^{2+}-ATPase sites and at voltage sensitive Ca^{2+} channels, depletes Ca^{2+} in the mitochondria by impeding Ca^{2+} uptake into mitochondria and by stimulating Ca^{2+} efflux from mitochondria [28, 29]. Alternatively, lead can impose mitochondrial stress via inhibition of heme production. Pb^{2+} can block catalytic sites that contains vicinal thiol groups in the enzyme heme synthetase [25] and result in the reduction in ATP production. Insufficient ATP production in mitochondria in turn creates free radical oxygen species (ROS).

Independent of mitochondrial stress pathways, lead-induced oxidative stress can be observed in the peripheral tissues [30, 31]. For instance, lead can result in the elongation of arachidonic acid in fat tissue [32]. Over 80% of lead in erythrocytes binds to δ-ALAD. Pb^{2+} replaces Zn^{2+} at SH sites and inhibits the subsequent binding with δ-aminolevulinic acid (δ-ALA). This leads to accumulation of δ-ALA in the cell cytoplasm. δ-ALA is auto-oxidative at pH 7.0-8.0 and can generate free radicals [33]. δ-ALAD bioactivity can be affected in blood lead levels as low as 5 μg/dL. Approximately 50% of δ-ALAD activity inhibition occurs at 16 μg/dL in whole blood, whereas 90% of the enzyme activity inhibition is observed at 55 μg/dL [34]. Antioxidant molecules, such as glutathione (GSH), glutathione reductase (GR), glutathione peroxidase, and superoxide dismutase are also susceptible to lead. Lead binds at sulfhydryl sites in GSH and GR

and reduces the amount of bioactive enzymes [35, 36]. Reduction in these bioactive antioxidants weakens defenses against oxidative stress.

Excessive elevation in oxidative stress can have severe consequences to the cell. Free radicals have a wide range of intracellular targets, such as nuclear and plasma membranes, which are needed to maintain cell function and survival [37 38]. The destruction of these structures can lead to cell apoptosis or necrosis[35]. For instance, lipid peroxidation at cell membrane results in structural derangement and alters phospholipid bilayer fluidity[32]. Additionally, oxidative stress affects enzyme activities, such as Na^+-K^+-ATPase, cytochrome oxidase, and succinic dehydrogenase. The dysfunction of these enzymes causes increase in permeability of membranes, which makes the organelles unable to contain constituents [39]. For instance, the instability of lysosome membranes causes the release of acid hydrolases, which can degrade intracellular constituents and cause cell lysis[40].

In addition, lead disrupts intracellular electrolyte gradients, which could attract water fluxing into the cell and cause osmotic lysis[41]. For example, in the endoplasmic reticulum, the presence of Pb^{2+} induces Ca^{2+} release from cytoplasmic organelles and results in elevated Ca^{2+} concentrations in the cytoplasm[27]. This leads to acute elevation of intracellular osmotic pressure and formation of cell edema. Cellular edema is especially critical clinically when it occurs in the central nervous system. Severe cerebral edema can result in the herniations in the midbrain, a life threatening condition.

In summary, lead can interfere cellular machinery by interacting with proteins, mimicking calcium activities and creating oxidative stress.

Biomarkers for Lead Exposure Measurement

Lead Absorption and Distribution in the Body

Primary routes of lead exposure in humans are inhalation and ingestion. In general, 40 to 50% of inhaled lead deposits in the lung. A very small fraction of inhaled lead is trapped in the upper respiratory tract and can be swallowed. Compared with intestinal absorption, the lung absorbs lead more efficiently[42]. However, the absorption rate in the lung or intestines increases to approximately 50% in infants and in people who are fasting[43, 44]. Deficiency of other electrolytes also facilitates lead absorption [45,46]. Around 95% of lead in circulation is sequestered in red blood cells, leaving a small fraction of inorganic lead in the plasma that can be transferred into surrounding tissues[46]. The mean biological half-life of blood lead is about 40 days [47].

Lead from blood is incorporated into calcified tissues such as bone. The fraction of lead deposited in bones can persist for years [48]. Approximately 70% of the total body burden of lead in children is carried in the skeletons and up to 95% in adults [49]. Lead in bones can be released back into circulation. Depending on bone turnover rates, the half-life of cortical bone lead is several decades and about eight years in trabecular bones [50]. Bone lead release is more prominent during intensive bone turnover periods, such as during skeletal growth, pregnancy and osteoporosis[51].

Throughout the years of lead toxicity investigations, many biomarkers have been developed and tested for the purposes of screening, biomonitoring or clinical diagnosis. Given the biokinetics of lead in the body, biomarkers from different tissue types can be used to characterize lead exposure as a function of the timing of exposures. Lead can

be detected in blood, urine, nails, hair, soft tissues and bones. Blood, urine and bone lead measurement are discussed below, given these comprise specific exposure measurements used in the projects in the following chapters.

Biomarkers for Short-term Lead Exposure

Blood Lead Measurements

Whole blood is the primary biological fluid that has been used to assess lead exposure assessment in lead studies. Whole blood lead reflects both intracellular and extracellular lead levels. It does not serve as an index for immediate lead exposure but can reflect recent exposure for up to 30 days [52]. Whole blood lead levels also reflect lead mobilization from bone to blood, dominantly from trabecular bone [53]. Many analytical approaches have been applied for whole blood lead assessment across lead studies. A recent widely adopted method is inductively coupled plasma mass spectrometry (ICP-MS). This method uses commercially available standard blood sample as the reference for quality controls. The intra-individual variation is controlled at less than 5% with detection limit at 1μg/dL. The use of whole blood lead measurement approach is relatively resistant to contamination issue at the pre-analytical phase and during laboratory sample processing. With this assay, the limitation of detection is relatively low and concentrations of lead in whole blood are highly measurable. It is cost efficient with a cost of 4 to 64 USD per sample.[54].

Plasma lead levels, on the other hand, reflect the bioactive fraction of lead that can be transferred across cell membrane and can have direct toxic effects on cells. The ICP-MS method allows the measurement of lead in plasma with detection limit at 0.1

μg/dL. Theoretically, plasma lead would be an ideal biomarker for studies of lead toxicity. However, it is difficult to achieve satisfactory accuracy in the fieldwork setting due to many technical issues arising from this measure. First, plasma separation has to be performed soon after the blood specimen has been collected because intracellular lead can be released into the plasma via hemolysis that occurs shortly after blood collection. Even mild hemolysis could increase plasma lead level up to 30% [55]. Secondly, lead concentrations in plasma make up less than 1% of whole blood lead, which imposes greater requirements for the lower detection limit and stringent sample handling procedure in the pre-analytical phase to avoid background contamination. Thus, characterizing lead levels in plasma from low environmental exposure levels may not provide valid results and may lead to exposure misclassification. Thirdly, measuring plasma lead requires the highest purity grade of analytical reagents and special metal-free collecting tubes. Currently, many of these issues have not been completely resolved[56]. These issues also increase the cost for sample processing and make it more expensive compared to the cost of whole blood measurement (approximately 38- 127 USD), especially when more sensitive detection limit is demanded [54].

Urinary Lead Measurement

Urinary lead reflects the lead component that has diffused from plasma and is filtered out through kidneys. Lead mobilized from bones contributes to urinary lead [57]. Urinary lead concentration is subject to biological variations involving glomerular filtration functions and plasma lead levels. Urinary lead may serve as a proxy for plasma lead after adjustment for creatinine excretion. The procedure of urine collection for lead measurement is non-invasive and samples are easy to obtain for epidemiologic studies.

However, contamination of urine samples from dust particles and the precipitation of urate salts can affect the estimation of urinary lead levels [56].

Biomarkers for Cumulative Lead Exposure

The half-life of bone lead ranges from years to decades. Bone lead as biomarkers for retrospective lead exposures is well recognized in research settings for studying the toxicity from cumulative lead exposures. Bone lead concentration can be measured in a variety of approaches. Among these methods, X-ray fluorescence (XRF) spectroscopy is the primary choice in the studies of human subjects.

XRF Photon-physics Mechanism

X-ray fluorescence is a non-invasive bone lead measurement procedure using low dose radiation (30mCi) [58]. The current generation of XRF uses ^{109}Cd isotope as a point excitement source [59]. It generates silver X-ray at an energy spectrum of 88 keV. This energy level provokes photoelectric interaction that can knock off a K electron or an L electron. Subsequently, an outer shell electron fills the vacancy and emits X-ray fluorescence. Depending on the metal-specific inter-shell transition, the energy level of X-ray varies. For example, for a lead K electron transition, the most common energy levels are 72.8 keV, 75keV, and 85 keV, which correspond to Pb $K_{\alpha 2}$, Pb $K_{\alpha 1}$ and Pb $K_{\beta 13}$ transitions. Compared to L-XRF, K-XRF requires higher energy excitement and suffers less from signaling attenuation by skin shielding. Due to the deep penetration capability, K-XRF performs better in terms of capturing the lead concentration dynamics across bone sections compared to L-XRF[58]. In terms of safety, the absorbed organ equivalent dose for K-XRF is slightly higher than L-XRF (4.0 μSv vs.2.9 μSv). However,

the health risk from this additional radiation exposure are considered minor [60]. Given the advantages of the K-shell XRF method, the bulk of research on human subject bone lead concentration measurements preferentially adopts K-XRF method.

Working Flow of XRF Machines

Figure 1.1 and Figure 1.2 illustrates the working flow of a K-XRF machine. The photoelectric interaction is provoked by ^{109}Cd source. The Germanium HP detector captures the energy emission during machine "live time". The signal is amplified and transferred to the computer for analytical use. Photoelectric events in lead atoms are collected and counted over a fixed period of time (30 minutes in real time in our studies). Meanwhile, the signals from calcium, carbon, oxygen and Compton scattering edges constituting the reference coherent peak (coh) are also collected. Collected data are analyzed with Canberra Genie 2000 Multichannel Analyzer (MCA) software (Figure 1.3). The ^{109}Cd isotope source resolution is tested before examining human subjects. System calibration is carried out using plaster-of-Paris phantoms. The phantom acrylic tubes are ¼" thick, which mimics human skin thickness at tibia midpoint. It also contains a mixture of lead and calcium sulfate dehydrate ($CaSO_4 2H_2O$) that resembles the chemical components of bones. Nine plaster-of-Paris phantoms with lead concentrations marked as 0 parts per million (PPM), 5PPM, 10PPM, 15PPM, 25PPM, 35PPM, 50 PPM, 75PPM, and100PPM are used to construct a calibration curve. A coherent peak (coh)/reference peak is collected during the calibrations. In order to derive the lead peak, the background peak counts are subtracted from the total peak counts. Therefore, in the cases of very low lead concentrations, negative peak counts may be generated. Calibration lines for α1 and β1 peaks are calculated by plotting α/coh

and β/coh ratios against lead concentration. The subject's lead concentration is derived by fitting α/coh and β/coh ratios onto a calibration line with correction factors [59].

Measurement Procedure

The K-XRF equipment is kept in a dust-free environment in order to reduce background noise during the measurement. A metal free chair with a leg stabilization device is provided to the study subject. The study subject is asked to sit still and to wear a lead free radiation protective apron while being measured. The tibia measurement is collected at the shinbone, which is located at the midpoint between inner ankle and tibia plateau connection. Patella measurements are collected at the point where the excitement source is perpendicular to the keen cap. The point of ^{109}Cd source should be kept about two or three centimeters away from the target organ.

Measurement Accuracy

There are several factors during preparation measurement that may contribute to measurement uncertainty levels. In the calibration stage, analytic choices in constructing calibration lines may slightly affect accuracy and precision of the measurement later taken in human subjects [59]. During the measurement, an increased proportion of dead time during data collection period results in the loss of photoelectric event counts that can be received by the detector and greatly impacts the uncertainty levels. Depletion of ^{109}Cd source can affect background peak distributions [61-63]. Additionally, excessive movement by the study subject during measurement may contribute to considerable amounts of uncertainty.

Measurement quality characteristics are related to biological specimens. Because bone lead concentration is derived from the background peak that mainly contains calcium, the measurement uncertainty is largely related to the features of bones, such as bone density and bone structure. Less mineralized bone, often seen in active bone turnover stages, e.g., during rapid skeletal growth, pregnancy and osteoporosis, renders weak signaling of the calcium reference. Furthermore, due to differences in mineral kinetic activities between trabecular and cortical bones, the measurement variability of these two types of bones behaves differently. Because cortical bones tend to be more mineralized than trabecular bones, the uncertainty levels for cortical bone are narrower than those of trabecular bone. Even within the same type of bone tissue, the bone lead density exhibits concentration gradient cross-sectionally. Newly formed bone layer itself (close to bone marrow side) tends to be more similar to low lead components rather than peripheral surface bone [64, 65]. The variability of K-XRF measurement increases with true bone lead concentrations regardless of calibration and calculation approaches [66]. With the current improvements in technology, the uncertainty arising from these factors can be greatly reduced, yet they cannot be eliminated.

Estimated measurement uncertainty is equivalent to standard deviation from repeated measures of same subject at same site and is derived by goodness of fit calculation of scatter in the XRF peaks. For quality control, estimates with uncertainty larger than 10 μg/g for tibia lead measurements and 15 μg/g for patella lead measurements are considered invalid. Each estimate is reported as a point of measurement ± the uncertainty. If the true bone lead concentration is close to zero, the

estimate varies above and below zero, in which case, at a single measurement could produce a negative point estimate. When reporting results back to the study subject, a detection limit (3-fold of standard deviation of blank phantom) is used. However, considering the statistical bone lead level distribution in a study population, negative values of point estimates are preserved [67, 68].

Decisions on Biomarker Selections

Biomonitoring for lead exposure reflects the toxico-dynamic nature of lead burden as a function of recent and/or retrospective exposures. Thus, the appropriate selection and measurement of lead exposure biomarkers is of particular importance for better understanding of the health outcomes from lead exposure with regard to inter-relationship among biomarkers and the timing of exposures.

Interrelationships between Lead Biomarkers

Plasma and urinary lead levels are linearly correlated with each other and both are exponentially associated with whole blood lead level [69]. Due to the limit of renal excretion, however, as the exposure level elevates, urinary lead is disproportionally associated with plasma lead [56]. It is also observed in occupationally exposed populations that plasma and urinary lead levels are linear associated with bone lead levels[69].

There are several factors that can affect the lead exchange between plasma and whole blood as reflected by ratio of plasma to whole blood lead levels (P-Pb/ B-Pb). For instance, ALAD gene variants modify the binding affinities between lead and red blood cells. Meta analysis has shown that ALAD minor allele carriers in occupationally

exposed populations have higher lead levels as measured in whole blood [70]. Another study found that minor allele carriers presented with higher P-Pb/ B-Pb ratios [71]. Additionally, pregnant woman with ALAD wild type, relative to non-pregnant women, had a 2-fold increase in plasma lead and 3-fold increase in the percentage of Pb-P/Pb-B ratio[72]. This could be due to the fact that lead is released back into circulation during active bone mobilization in pregnancy. As lead-erythrocyte binding reaches saturation, the excess lead flows into the extracellular plasma space.

Short-term vs. Long-term Lead Exposure Measurement

As our understanding of lead's effects on health outcomes grows, the timing of exposure is of particular focus. Therefore, the choice of biomarkers in the context of timing of exposures is critical. Biomarkers mentioned in the foregoing sections such as blood and urinary lead measurements are recognized as reliable indicators of recent lead exposure. A critical application of short-term exposure measurement is the construction of an exposure matrix that offers a higher resolution of cumulative exposure levels with serial short-term measurements [52]. This method may outweigh the bone lead by providing more accurate long-term exposure dose [73] and by identifying the critical windows of exposures.

Under conditions that lack historical blood lead assessment bone lead serves as a good indicator of retrospective lead exposures. By virtue of distinct toxicokinetic features in cortical and trabecular bones, one can characterize the temporal pattern of exposures. Specifically, tibia lead is more indicative of lead accrued from environmental exposure, whereas patella lead is more relevant to the secondary endogenous lead exposure[53, 74]. Studies integrating information from biomarkers of bone resorption

activities, such as urinary type I collagen cross-linked N-telopeptides (NTx) levels[75], can be used to confirm of endogenous exposure levels[74, 75] .

Strength of Long-term Monitoring Lead Exposures

Cumulative measures of lead exposure dose may be the most crucial determinant of some forms of toxicity. When using short-term exposure indicators to predict health outcomes, the effect estimates may be confounded by previous unmeasured episodes of exposures. Additionally, the exposure at a specific time point itself may have a weak the biological relevance with the health outcome. These issues are particularly prominent when studying diseases that occur in late life[73]. Integrating exposure history would not only be beneficial in gaining statistical power but also provide evidence of long-term effect from accrued lead exposure that cannot be observed in a short period of time. Further examples with respect to the strength of cumulative lead exposure indicators are illustrated in the three projects in this dissertation.

Dissertation Overview

This dissertation focuses on the health effects from lead exposure at different stages of life, as graphically represented in figure 1.4. Despite the impressive body of evidence showing the toxic effects of lead on neurodevelopment in early life[76-79] and chronic conditions in late life[15, 16, 19, 80], my goal is to further deepen my understanding of how the timing of exposures influence health. In particular, I am focusing on the impact of lead on behavioral development, interaction of lead with genetic components on neurological degenerative disease development and a novel mechanism relating lead to

metabolic disorders, such as Type 2 Diabetes Mellitus (T2DM). Three individual projects from three epidemiological studies listed in the following chapters comprise this dissertation: Early Life Exposure in Mexico to ENvironmental Toxicants study (ELEMENT), Normative Aging study (NAS), and Gene-Environmental Metal exposures on Parkinson's Disease study (GEM-PD).

First, the purpose of Chapter 2 is to explore the influence of lead exposure *in utero* and in early life (birth to early adolescence) on psychobehavioral development. This analysis is embedded in the previously established Harvard-Mexico Project on Fetal Lead Exposure, Risks and Intervention Strategies (FLERIS) study in Mexico City. This analysis examines the associations between early life lead exposures and psychobehavioral outcomes by taking advantage of longitudinal exposure measurements, accounting for the intercorrelations among consecutive lead exposures. The analysis models the tendency of internalizing and externalizing problems, social behavioral problems and attention deficit hyperactivity disorder (ADHD)-like behaviors as behavioral outcomes.

Next, Chapter 3 studies on lead effects on neurodegenerative process in the central motor control system. It explores the etiology of Parkinson's disease (PD) from the perspective of gene-environment interactions. This analysis utilizes a case-control study conducted in Boston, Massachusetts. Both the main effects and interaction effects of the genetic variants of *SNCA* gene were examined in this analysis. The research questions whether *SNCA* genetic variants modify the effect of lead on the odds of developing Parkinson's disease.

Finally, the aim of Chapter 4 is to explore the effect of cumulative lead exposure on the risk of acquiring T2DM. The study tests the association between cumulative lead exposure and T2DM among middle-aged and elderly men. In order to better understand the mechanism underlying such an association, an additional stratified analysis is conducted with respect to skeletal bone resorption activities.

Together, the three studies of this dissertation constructed the picture on lead impacting human health at the different stages of life.

Figures

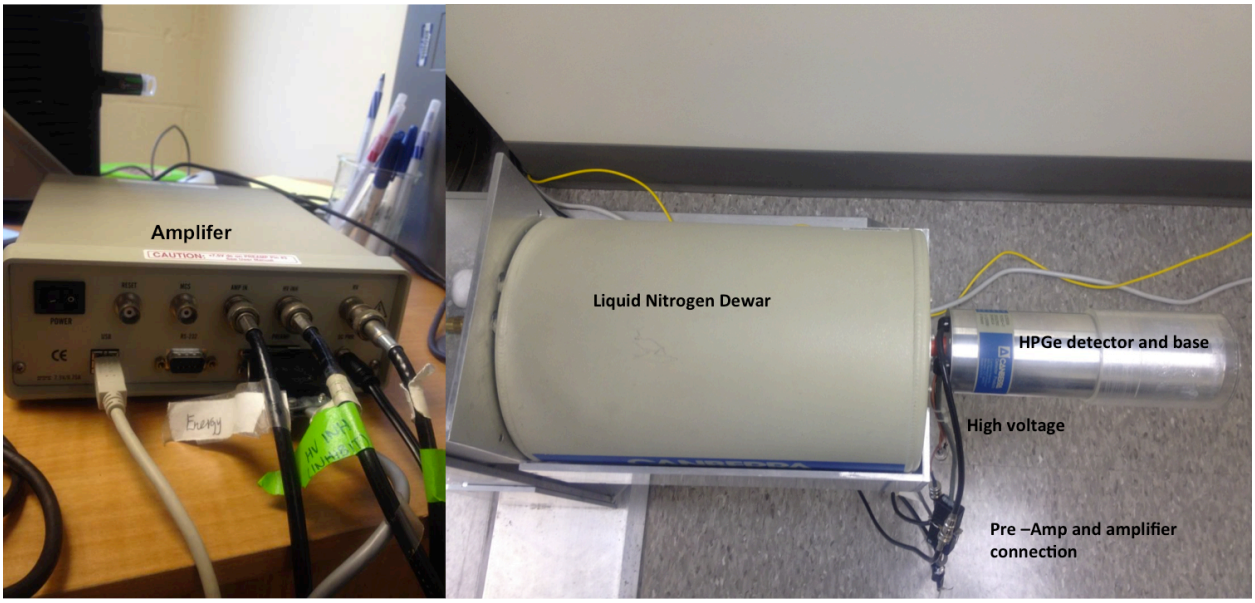

Figure 1.1 Structure of X-Ray Fluorescence Machine

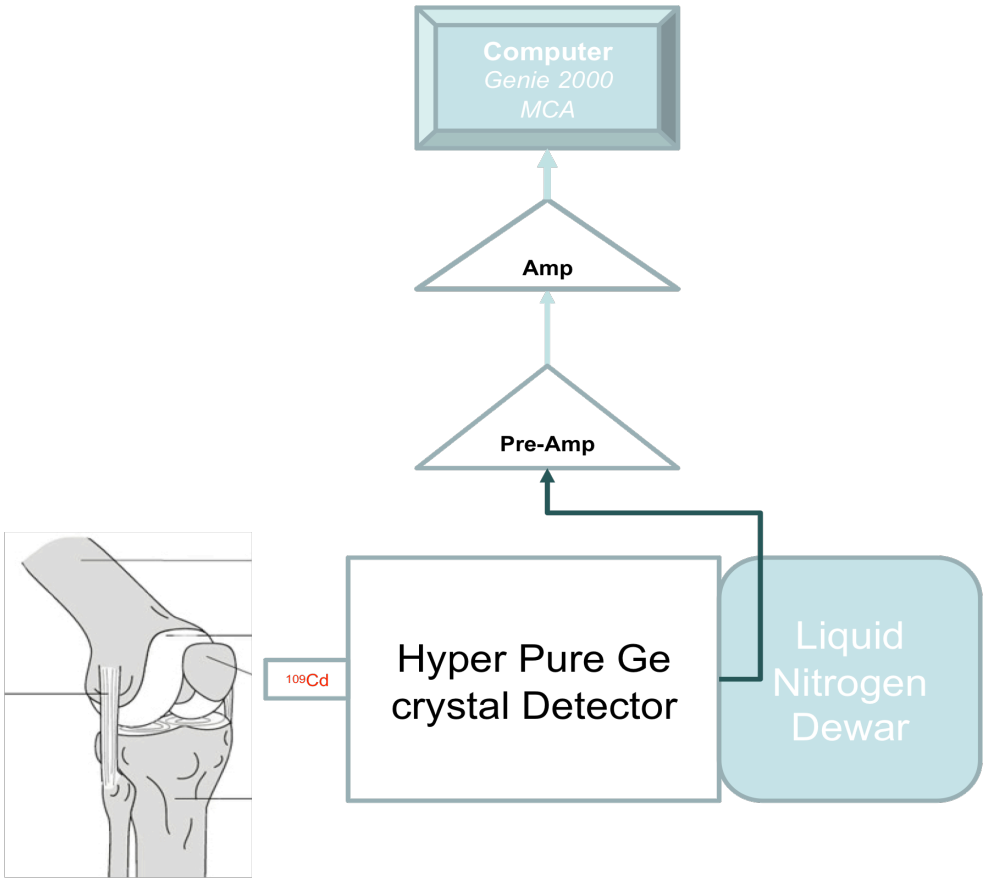

Figure 1.2 Configuration of K-Shell X-Ray Fluorescence Machine

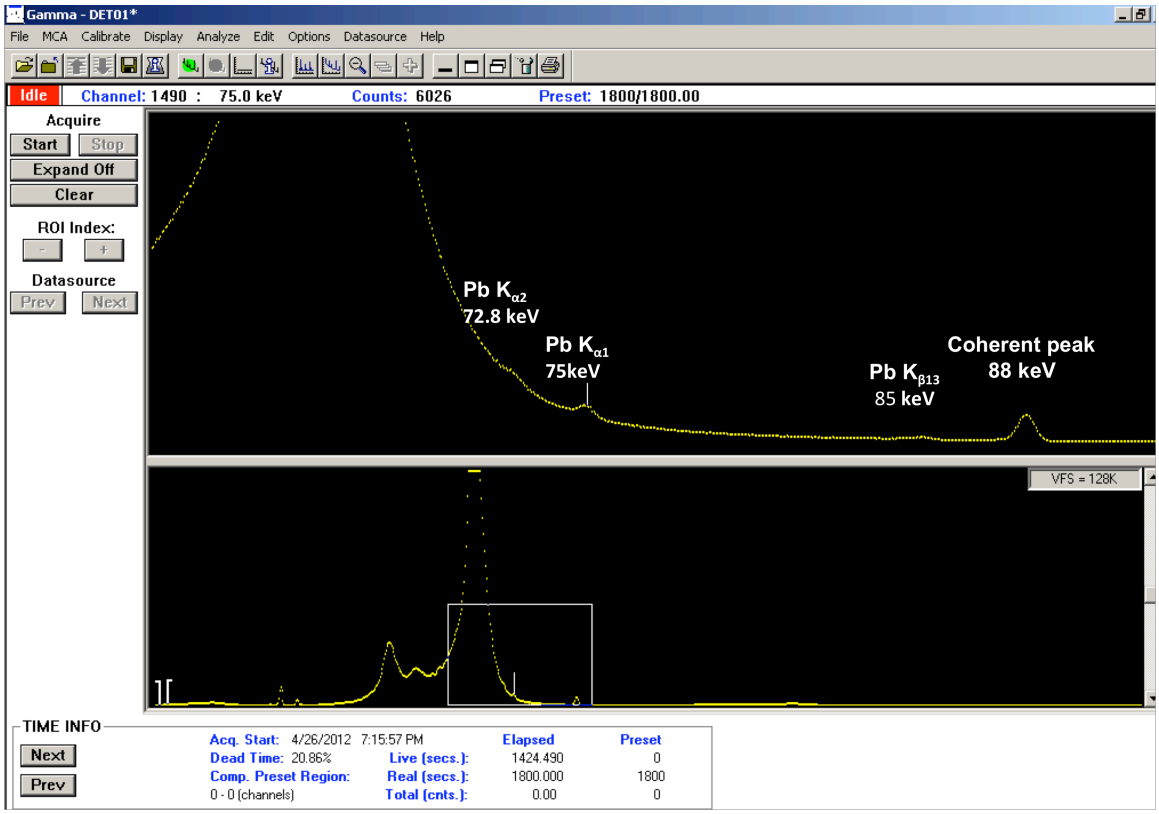

Figure 1.3 Working Interface of Genie 2000 MCA

Caption: This measurement was taken using Pb phantom at 100PPM. Three lead peaks can be observed at energy channel 72.8 keV, 75keV, 85keV. The peak at energy channel 88eV refers to the coherent peak.

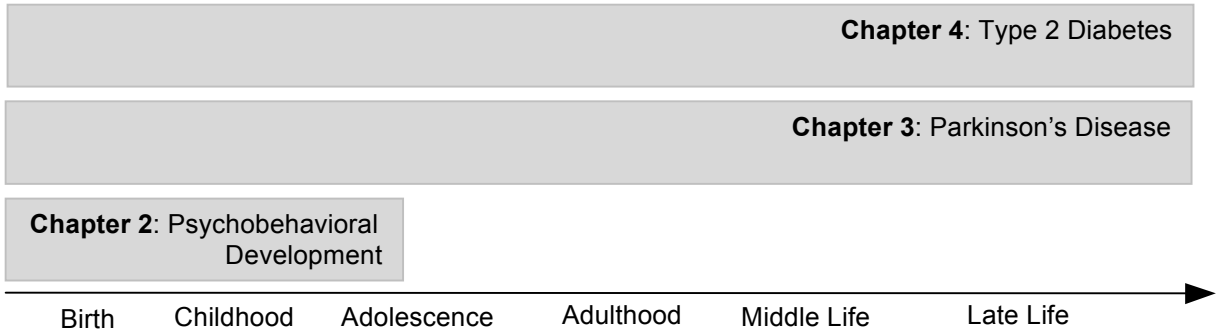

Figure 1.4 Scope of the Dissertation

Caption: The shaded areas represent the lead exposure periods that were of interest in the three projects. Chapter 2 studies the effects of lead exposures *in utero* and in early life (birth to early adolescence) on psychobehavioral development. Chapter 3 focuses on modifying effect of genetic variants on the effect of cumulative lead exposures in the etiology of Parkinson's disease (PD). Chapter 4 focuses on the effect of cumulative lead exposure on the risk of type 2 diabetes (T2DM).

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CHPATER 2 EFFECT OF TIMING OF LEAD EXPOSURE IN EARLY LIFE ON CHILDHOOD NEUROBEHAVIORAL OUTCOMES

Introduction

Recent epidemiological studies have shown an increasing trend in the prevalence of serious emotional and behavioral disorders in early childhood. [1]. Behavioral disorders are often underreported and they are given less attention compared to intelligence problems. The consequences of underestimating the public health significance of childhood emotional and behavioral disorders can be serious. In the long run, lack of proper management of emotional and behavioral disorders would not only aggravate the quality of life of the suffering individuals, but it could also lead to public safety concerns. Psychobehavioral development in early life is shaped by the child-rearing environment. Nevertheless, behavioral problems cannot be solely explained by the factors related to parenting. In recent years, studies on environmental chemical exposures from the living environment have shed light on the etiology of behavioral problems in childhood [2-4].

The toxic effects of lead have been well studied. Several cross-sectional studies have shown the associations between lead exposures and behavioral problems. Studies have linked concurrent blood lead levels to anxiety, social problem[5], inattentive and hyperactive- impulsive behaviors[6, 7], aggression[8] as well as attention deficit hyperactivity disorder (ADHD)[9-14]. Lead effects on behavioral problems can be observed as early as age of 3[5] through adolescence[15].

A handful of studies have explored the timing of lead exposure on long-term neurobehavioral outcomes. Current knowledge suggests that the effect of lead exposure in the early life could extend to later life[15, 16]. For instance, exposure to lead between the ages of 12 to 33 months in humans was found to be associated with externalizing behaviors and school problems at age of seven[17]. The Cincinnati lead study also found that prenatal lead exposure has the strongest impact on attention deficit at the age of 6.5 years compared to the lead exposure in early childhood[16]. Needleman et al[18] used K-shell X-ray Fluorescence Spectroscopy to measure and explore the relationship between retrospective lead exposure and neurobehavioral outcomes at the ages of seven and eleven. They found that children with a higher concentration of lead were more likely to be delinquent, aggressive, anxious or depressed, and they were more likely to have externalizing and internalizing problems.

Despite evidence from these studies suggesting a relationship between early life lead exposure and behavioral problems, some questions have not yet been explicitly addressed in the literature. First, the current knowledge is primarily based on cross-sectional studies, that show the association between lead exposure and behavioral problems at one time point. This level of evidence is insufficient to establish a causal relationship between lead exposure and psycho-behavioral outcomes. It is important to address the chronological order of lead exposure in studies in order to gain an in depth understanding of the mechanisms of the effect of lead on neurobehavioral development. It is of special importance to note that bone lead can serve as an endogenous source of lead exposure during rapid skeletal growth in early life[19, 20]. Therefore, the observed lead levels in blood can be a reflection of previous exposures. Ignoring this relationship

may result in inaccurate or biased effect estimates at the time-point of interest[21, 22]. Second, even though a handful of longitudinal studies have shown that early life lead exposure could have long-term impacts on behavioral development, the effect of the timing of lead exposure on behavioral development is not clear and is not well-discussed in the literature. This study fills this gap in the literature and reports on the critical windows of lead exposure in early life by measuring lead exposure during gestation, during the first four years of life and during childhood and early adolescence and the impact of these lead exposure measurements on neurobehavioral development. Specifically, the primary aim of this analysis was to investigate the programming influence of exposure to lead in early life and to evaluate the toxic effects of timing of lead exposures on the neurobehavioral performance from childhood to early adolescence. We hypothesized that increasing levels of lead exposures in early life would be associated with an increasing propensity for developing behavioral problems.

Methods

Study Design

This analysis is embedded in the parent birth cohort of Early Life Exposure in Mexico to ENvironmental Toxicants (ELEMENT) study[23]. The study is constituted of three mother-infant pair birth cohorts recruited in Mexico City, Mexico starting in 1994, 1997 and 2001. The overall goal of this study was to explore the associations between early life environmental exposures and a cascade of health outcomes in the offspring. Upon the completion of the first stage of this study, subjects from three cohorts were selected and combined into a cohort of subjects for this study. 204 mother-infant pairs from the 1994 cohort, 367 pairs from the 1997 cohort (223 in *Biomarker* cohort (BI) and

144 in *Plasma* cohort (PL)) and 216 pairs from the 2001 cohort were eligible, yielding a total 787 mother-infants pairs for this study.

Subject Selection

Subjects were recruited at one of three clinics (Mexican Social Security Institute, Manuel Gea Gonzalez Hospital and the National Institute of Perinataology) in Mexico City. When pregnant women were screened for initial recruitment into the parent birth-cohort study, they were excluded if they exhibited any of the following conditions: any factor that could interfere with maternal calcium metabolism; reported intention not to breastfeed; preeclampsia, kidney or cardiac diseases, gestational diabetes, history of urinary infections, family or personal history of kidney stone formation, seizure disorder requiring daily medications; ingestion of corticosteroids, or a single-parent household. They were later excluded from the study if they were given a physical diagnosis of multiple fetuses of if their child had one of these conditions: a gestational age which was less than 37 weeks, a birth weight less than 2000g, an infant Apgar score of 6 or under at 5 minutes, a condition that required admittance to the NICU or a serious birth defect. When reconstituting the current cohort, subjects were preferentially selected based on availability of information regarding past exposure, questionnaires, health status and demographic characteristics. The study subjects in the current cohort represent low to middle-class people in the local region. The behavioral test results vary by age therefore created a pronounced cohort difference in the dataset. Additionally, gestational blood samples were only available in cohort 2 PL and cohort 3. Therefore, we decided to use cohort 2 and cohort 3 in this analysis. As a result, a total of 583 subjects, 223 from

cohort 2 BI, 144 from cohort 2 PL, and 216 from cohort 3 were entered into the final dataset.

The research protocol was approved by the Institutional Review Boards (IRB) of the National Institute of Public Health of Mexico (Instituto Nacional de Salud Publica (INSP)), the Harvard School of Public Health, the Brigham and Women's Hospital, the University of California, the University of Michigan School of Public Health, and the participating hospitals. Written informed consent and/or assent were obtained from all participants.

Lead Exposure Measurements

Prenatal Lead Exposure

Prenatal lead exposure was estimated by measuring lead in maternal venous blood in cohort 2 PL and cohort 3, in cord blood, and in maternal bone lead. Maternal peripheral venous blood samples were collected once during each trimester of pregnancy using trace-metal free tubes after sanitizing the lancet site. Umbilical cord blood was collected at delivery for lead measurement. Atomic absorption spectrometry was used to measure lead in whole blood.

Maternal bone lead is a proxy for early lead exposure stemming from mobilization of maternal bone lead stores. Although bone lead is not trimester-specific and it does not take into account ongoing external exposure, it still can be considered a biomarker for early life lead exposure *in utero*. Between one to 30 days post-partum, maternal bone lead levels were measured using K-shell X-ray fluorescence spectroscopy at the tibia and the patella.

Postnatal Lead Exposure

Postnatal exposure was assessed by collecting offspring blood from peripheral veins and fingertip capillaries at 3, 6,12,18,24, 30, 36, 48 months of age. Blood was also collected from the children during their neurobehavioral tests. During these neurobehavioral tests, the youngest of these children was six years old and the oldest was 13. All of the blood samples were collected in trace metal-free tubes after thorough sanitation at lancet sites. Blood samples from birth to 48 months were analyzed using atomic absorption spectrometry instrument at the Metals Lab of the American British Cowdray Hospital in Mexico City. External blinded quality control samples were provided throughout the study period by the Maternal and Child Health Bureau of Mexico City and by the Wisconsin State Laboratory of Hygiene Cooperative Blood Lead Proficiency Testing Program. Precision and accuracy with a correlation coefficient of 0.99 and a mean difference of 0.17 μg/dL were achieved. Due to the systematic difference between fingertip capillary and venous blood measures, only venous blood levels were used in this analysis. Blood samples collected during neurobehavioral visits were analyzed at University of Michigan and Michigan Department of Community Health using the Inductively Coupled Plasma Mass Spectrometry (ICP-MS) method. The quality control showed satisfactory accuracy and precision with a detection limit of 1.3 μg/dL.

Outcome Measurements

The psychobehavioral outcomes were scored using the Behavioral Assessment System for Children, second edition (BASC-2)[24], Conners Rating Scales Revised(CRS-R) [25], and Conners' ADHD/DSM-IV Scales Parents (CADS-P)[26] completed by both

children and parents. These tests were administered by trained examiners who were in-turn were supervised by psychometricians or psychologists in Mexico City. We chose these tests in order to fully capture the effect of lead on psychobehavioral development. These tests measure behavior domains of inattention, hyperactivity, internalizing problems, externalizing problems, emotional controls, compulsive behavior, social skills and adaptive skills. BASC-2 is a coordinated system of psychological assessment that evaluates the behavioral and emotional problems in children and adolescents and the Self-Reported of Personality (SRP), and Parent Rating Scales (PRS) subscales were applied to this study. The BASC-2 questionnaires contained clinical scales and adaptive scales covering both adaptive and maladaptive behaviors. There were four composites of PRS: externalizing problems, internalizing problems, behavioral symptoms index and adaptive skills. Five composites were evaluated on SRP: Clinical Maladjustment Composite, Personal Adjustment Composite, Emotional Symptoms Index, and Suicidal Risk. Higher scores on clinical scales indicate disruptive or internalizing problems whereas lower scores on the adaptive scale indicate lower adaptability, social skills, functional communications, leadership skills and study skills. CRS-R was applied to parents or caregivers. Scales in CRS-R covered behavioral problems on opposition, cognitive problems, inattention, hyperactivity, anxious-shy, perfectionism, social problems, psychosomatic, Conners' Global Index, DSM-IV Symptom Subscales, ADHD Index. Conners' ADHD/DSM-IV Scales Parents (CADS-P) is featured by DSM-IV Symptom subscales that distinguishes the inattentive and hyperactive-impulsive subtypes of ADHD. All scales were standardized into T-scores. A higher score on CRS-

R and CADS-P indicates increased tendency to have corresponding behavioral problems.

Measurement of Confounders

Maternal age, marital status, parental education levels, social economic status of family, and maternal smoking behavior during pregnancy was obtained from a questionnaire administered to the mothers. Birth weight, which can be consequent from prenatal lead exposure [27] is also a risk factor for behavioral problems[4, 28, 29] so, this was obtained from clinical records.

Statistical Analyses

Univariate and Bivariate analyses

All statistical analyses were performed in R version 2.15.2 [30]. All significance testing was two-sided and was determined at $p< 0.05$. Univariate analysis was performed to examine the distribution of the variables of interest. Extreme observations that were at least four standard deviations away from the means were removed from the analysis.

All lead exposure variables were treated as continuous variables. Prenatal lead exposure variables included cord blood lead levels, gestational maternal venous blood lead levels, and maternal bone lead levels. Maternal blood lead levels during gestation were integrated as a cumulative blood lead level (CBLL) calculated using equation 2.1. Postnatal lead exposures were divided by three time points: the first and the second two years post-delivery and lead exposure at the time of neurobehavioral testing. Cumulative blood lead level index at each time point was integrated using blood lead

level at each follow up time point using equation 2.1. The strength of the correlation coefficients among lead exposure variable pairs were calculated and tested for significance.

$$BLL = \int_j Pb_j = \tfrac{1}{2}\sum_j (Pb_j + Pb_{j+1})t_j \qquad (2.1)$$ [1]

The agreement between parental rated scales and children's self-reported scales was assessed with Pearson's correlation coefficients and was tested for statistical significance. All the behavioral assessment scores were treated as continuous variables. Maternal marital status, mother's smoking status during pregnancy and child's sex variables were categorized. Maternal age and educational years, child's age at behavioral tests, socioeconomic status (SES) levels, and birth weight were treated as continuous variables. Age, SES level and birth weight were centered for the purpose of interpretation.

Statistical Modeling

Our analytic strategy consisted of two stages: residual extraction from pair-wise lead exposures regression and multivariate regression modeling adjusted for covariates. First, in consideration of adjusting for the correlations among lead exposures, residuals from each consecutive pair of lead exposure regression model were extracted and referred as X_j' in equation 2.2. This approach is commonly seen in nutritional epidemiology for controlling correlated nutrition intake[21, 22]. The rationale in the environmental exposure assessment context is illustrated in the Appendix. Except for prenatal lead exposure levels, all other observed blood lead levels were regressed on

[1] Detailed captions are provided in the Appendix

lead exposure in the previous window. In the second stage, all residuals were fitted simultaneously into the covariate-adjusted models.

$$X_j = \alpha_{j-1} X_{j-1} + X_j^{'} \qquad (2.2)^{2}$$

Gestational lead exposures were approximated by maternal blood lead, umbilical cord blood lead and maternal bone lead separately. Due to the sample availability, gestational blood based modeling was performed in cohorts PL and SF. The original intent for models using cord blood lead as prenatal lead exposure indicator was to include cohort 2 (BI and PL) as well as cohort 3 in the dataset. But cohort 3 was excluded due to tremendous missing data therefore only cord blood lead from cohort 2 was used. Maternal bone lead models were collected from women in cohorts 2 and 3 and they were combined. Cohort-specific analyses were also performed as sensitivity analyses. In these analyses, prenatal blood lead models were the primary focus. We chose not to report the results from the bone lead models as the primary findings due to following reasons: maternal bone lead was taken one month post-delivery and evidence has shown that bone turnover after delivery is higher than that in pregnancy[32]. In addition, cohort 3 was involved in a calcium supplement randomized trial[33] and shown to have lower bone lead mobilization during pregnancy. Therefore, post-delivery bone lead levels may less accurately reflect fetal exposure levels and could result in biased effect estimates due this cohort difference.

[2] Detailed captions and proof are provided in the Appendix

All other biologically relevant confounders were selected into the model at the initial step. Multicollinearity diagnosis and *F*-statistics of model fitting were used for covariates selection. Coefficient estimates were standardized as in one standard deviation increase in exposure to the amount of change in standard deviation in outcomes. Maternal age, years of education, marital status, smoking behavior during pregnancy, family SES level, and children's sex, age and birth weight were included in the final models.

Model diagnosis was performed with regard to linearity assumption, constant variance assumption, identifying influential points and collinearity. The penalized spline smoothing method was applied for linearity examination. Linearity diagnosis showed very limited number of models that followed non-linear relationships (<5%). Therefore, multivariate linear regression model was applied to the final models. Homoscedasticity was examined by Breusch-Pagan test. Variance inflation factor (VIF) at 1.56, which corresponded to r=0.6, was used as collinearity index. Influential points were identified by Cook's distance at 0.5 or larger. Sensitivity analysis was performed to compare coefficient estimation upon removal of the influential point(s).

Results

A total of 583 subjects entered the final study with different amounts of missing information on major variables of interest. The mean ages of children were 9 years in cohort 2, and 7 years in cohort 3. Table 2.1 summarizes the exposures and psychobehavioral outcomes in the three cohorts. Umbilical cord blood levels were 4.31 μg/dL, 6.37 μg/dL and 3.22 μg/dL in cohort 2 BI, PL and cohort 3, respectively. Cumulative blood lead levels from maternal gestational blood on average were 7.58

μg/dL and 4.83 μg/dL in cohort 2 PL and cohort3. Cumulative blood lead levels from the first two years exceeded 5 μg/dL in cohorts 2 and 3, and cumulative blood lead levels from the second two years were slightly higher than that of the first two years. Weak to moderate Pearson's correlations among lead exposures in different windows were found (Figure S 2.2 and Figure S 2.3). No significant pair-wise correlations were found in pair-wise extracted residuals. The distributions of behavioral outcomes are shown in Table 2.1. In total there were 23 behavioral scales and all were scored within the normal range. ANOVA tests showed significant cohort differences on BASC-2 SRP scales with cohort 3 (SF) scoring slightly higher (worse) than cohort 2 (BI and PL). This difference was not detected in most of parental rated scales. The concordance between children's self-rated scales and parental rated scales was lower (data not shown).

In the bivariate analyses, lead exposure did not show significant correlations with major behavioral outcomes. Maternal educational level and SES level showed significant negative correlations with children's blood lead levels after birth and with behavioral outcomes. Gestational age and birth weight showed significant negative correlations with internalizing problems and inattention. Table S 2.1 and Table S 2.2 showed the comparison of the exposure and outcome characteristics between groups with complete and incomplete data in cord blood lead models subjects and in gestational blood lead models subjects. The included and excluded groups showed comparable lead exposure levels, but were slightly different in some behavioral outcomes such as hyperactivity and impulsive behavior. In general, the excluded subjects groups scored higher (worse) compared with included subjects.

Table 2.2 shows adjusted cord blood lead models. A significant association was observed between Perfectionism on CRS-R and cord blood lead at $p<0.05$. Otherwise, cord blood lead was not significantly associated with measured behavioral outcomes. In the majority of behavioral outcome models, however, the tendency toward behavioral problems increased with elevated cord blood lead. Increases in blood lead levels in the first two years post-delivery were generally associated with higher (worse) scores. The effect estimates of the first two years exposure tend to be stronger than those of exposures in any other exposure windows. Except for Inattention/Hyperactivity on BASC-2 self-reported scales, none of the behavioral outcomes showed statistical significant associations with blood lead during the first two years. Blood lead in the third and fourth years and at the tests (around 9 years in cohort 2) did not show strong significant associations with any behavioral outcome.

Gender differences were observed; females were rated worse by parents on attention problem scales, but they were rated better on the Personal Adaptive Skills and Internalizing Problem scales on BASC-2 PRS. Higher maternal age, education level and SES level were associated with better behavioral outcomes in children.

In the gestational blood models, no statistical significant associations were found between lead exposures and behavioral outcomes (Table 2.3). Blood lead levels at the time of the behavioral test (9 years in cohort 2 PL and 7 years in cohort 3) were negatively associated with the Psychosomatic scale on CRS-R. On the other hand, the magnitude of effects of lead exposure during the gestational period and in the first two years post-delivery tend to be greater on inattention and hyperactivity scales (e.g. Inattention/Hyperactivity on BASC-2 SRP, Cognitive Problems/Inattention, Global

Restless-Impulsive Index, and ADHD Index on CRS-R, Hyperactive-Impulsive, Inattention on CADS). In addition to the concordant observations on the effects of children's sex, maternal age and educational levels, the results in gestational blood models showed that an increase in birth weight was associated with better behavioral outcomes. Cigarette smoking during pregnancy was significantly associated with worse outcomes related to Externalizing Problems, Opposition and to the Behavioral Symptom Index.

In maternal bone lead models, maternal bone lead levels in tibia and patella showed significant protective effect on inattention, impulsive behaviors. None of the lead exposures in other windows were significantly associated with behavioral outcomes (data not shown).

Discussion

In these analyses, we did not find statistically significant evidence of any deleterious effects of early lead exposure on psychosocial status of children aged 6 to 13 years. When weighing the direction and magnitude of lead effects among the early life exposure windows, however, the data suggests that lead exposures in the prenatal period and in the first two years after birth has a greater impact on behavioral outcomes like inattentive and hyperactive behaviors between the ages of 6 and 13 years. The results also suggest that lead exposure in these windows could affect control of emotions and somatic perception development in children. Compared to perinatal lead exposure, lead exposure later in life showed a weaker impact on children's behavioral development. Our results suggest that lead exposure during the first two years after of life is a critical window of exposure as it shows a strong and long lasting impact on

behavioral development. It also suggests that the effect of prenatal lead exposure was mediated via secondary endogenous lead exposure from maternal bone lead released during active bone turnover in pregnancy. Consistent with current knowledge on the deterministic factors on behavioral development, we found that parental factors that may indicate a good child-rearing environment such as higher maternal educational levels and social economic status[34] are associated with better outcomes on psychosocial behavioral development. In addition, low birth weight showed strong association with inattention and hyperactivity behaviors in gestational blood lead models.

Unlike findings from studies with a cross-sectional study design, our results indicate that concurrent lead exposure was not the major contributor to the behavioral problems during the childhood-early adolescence period, but exposure during the first two years of life had a greater impact These results could be attributed to the more detailed and refined analysis in this longitudinal study; we fitted lead exposures from all exposure windows simultaneously and adjusted for inter-correlations using the residual method. This has two implications for lead effect estimates. First, the pair-wise residual method solved the correlation issue among lead exposures, and it modeled the endogenous lead circulation between bone and blood during the rapid skeleton growth in the early life. Therefore, the observed blood lead levels reflected both historical and current lead exposures. Second, fitting lead exposure variables simultaneously constrained the potential mediating effect from earlier exposures that can be reflected by the current exposure. The detailed proof is provided in the appendix. This method, compared to the models fitting lead exposure at each window separately, increased the

magnitude of the effect estimates. The effect estimates, however, from this method did not differ greatly in terms of the directions and the magnitude of effects compared to the models fitting observed lead levels simultaneously (result not shown). Yet, in several models the residual method slightly improved efficiency than the models that did not adjust correlations among exposures (results not shown). The other explanation for the weak impact from blood lead measured at the time of the behavioral tests is that the levels of exposure were low (<5 μg/dL on average in all cohorts). It is possible that the amount of lead reaching the brain is even lower given that most of children may have developed protective mechanisms from lead intoxication in the central nervous system at the age of 5 or older [35]. Therefore, the lead can hardly exhibit strong effects with this low exposure range.

Our study result is compatible with the findings from the Cincinnati lead study[16]. In this study, the researchers examined effects of lead exposure using prenatal maternal blood lead, children's blood lead averaged from first five years and at 78 months on cognitive, behavioral and motor functions at age15 to 17 years. The 20th and 70th percentile of the average five-year blood level distribution was 15 μg/dL and 25 μg/dL, respectively. They found that 78-month blood lead was related to attention problems. However, the effect of lead on attention and visuoconstruction functions tended to be the strongest in the prenatal period compared to lead exposure in other age periods. We examined psychobehavioral functions in children in our study at younger ages (6- 13 years) than the Cincinnati cohort. The 20th and 70th percentile of the first four years cumulative blood lead levels were 14.4 μg/dL, 23.45 μg/dL in cohort 2 and 12.72 μg/dL, 22.79 μg/dL in cohorts 2 PL and 3 combined. Our analyses explored

the critical windows of lead exposures in shorter time intervals. Our results indicated that the exposure to lead in prenatal period as well as the first two years post-delivery could contribute to the risk of having attention problems, hyperactivities, and compulsive behaviors in children aged 6 to 13 years. However, other behavioral problems such as internalizing problems, externalizing problems and school problems were not under strong influence of lead exposures in these periods. Our findings on the critical exposure windows were inconsistent with current knowledge on the brain structure and behavioral development[36-38].

Timing of Psychobehavioral Development

In humans, the neuronal infrastructure development mainly occurs during the prenatal period, while the neuronal reorganization and functional gains occur rapidly after birth and reach to full maturation in early adulthood [39, 40]. Psychosocial development can be traced as far back as the first three years of life. Newborns, in the first few hours of life, start to look for simple face-like patterns and this may suggest that they are attempting to establish a bond with adult caregivers [37]. The major psychosocial development in infants involves the limbic nuclei, and it is mainly through the 'experience-expectant' mechanism. Experience-dependent plasticity is a type of behavior-learning approach whereby infants gain functions and develop psychosocial affections through their interactions with the child-rearing environment. This plays a crucial role in the psychosocial development in the first few years of life, so, it could play a role in behavioral problems in adulthood[3]. If deprived of environmental stimuli, infants exhibit various degrees of functional deficits with the severest damage being permanent. For instance, infants who were separated from the caregiver (e.g. mother)

repeatedly or for a long period in the first three years of life, exhibit social withdrawal, pathological shyness, aggressive and inappropriate emotionality and they are incapable of forming normal emotional attachment [41, 42]. Depending on the window that stimuli deprivation occurred, children manifest dissimilar subtypes of behavior problems which reflects different brain regions that were affected [3]. Our results also show that the child-rearing environment plays a crucial role in children's psychobehavioral development. Higher maternal age and educational levels and social economic status level implies better capabilities in supporting and promoting the physical, emotional, social, and intellectual development of a child. These variables were shown to reduce the probability of developing negative behavioral problems.

Intrinsic and Extrinsic Factors Affecting Biosocial Development

Both intrinsic and extrinsic environments are involved in guiding the development of psychosocial behaviors. For example, dopamine is one of the most important neurotransmitters that is highly involved in reward learning and it is associated with neuronal branching and outgrowth[43, 44]. Sex is a major determinant of patterns of neurobehavioral development. During the peri-adolescent period, dopamine receptor 2 in males is overproduced and is subsequently eliminated by around 40%. However this fluctuation is not observed in females[40] . Females in general reach grey matter and white matter peaks, which mark the brain maturation, ahead of males. Yet regional differences have been observed between sexes. It is observed that during adolescent period amygdala volume bumped up greatly in males, whereas right hippocampus volume increases in females [40].

The extrinsic environment has a strong and direct impact on behavior development. In this study, we found the suggestive effects of lead exposure during the prenatal period and during the first two years after birth to be the strongest on inattentive and hyperactive behaviors. This can be explained with two possible biological mechanisms. First, the fetal brain lacks protective mechanisms to defend against lead poisoning. Studies show that adult endothelial cells in the blood brain barrier have a lower permeability to lead, and mature astroglias are capable of pumping lead back into blood stream against a concentration gradient [45-47]. Without a fully developed blood brain barrier structure, neurons and glias are directly exposed to lead transported from the blood. This explains why this window is particularly vulnerable to a low concentration of lead from the circulation and why pronounced neuronal impairments can be observed [48]. Previous studies confirm that the developing brain is a target organ for lead poisoning [48, 49]. In rat studies, animals chronically exposed to lead sequestered lead in zinc rich regions like the hippocampus [50], while animals with acute exposure showed more lead, in the pons medulla, cerebellum, midbrain and cortex striatum [49]. Second, as previously mentioned, the limbic system which is involved in emotional memory and socialization, develops and matures quickly in the first three years of life [51]. Our evidence suggests that the damage occurred during this neurodevelopment period cannot be reversed and the functional deficits can be long-lasting.

Furthermore, our findings shows that birth weight is a strong factor in predicting inattention and hyperactivity problems at age 6 to 13. This again suggests that the perturbation occurred in early life, especially during the gestational period could result in

permanent behavioral deficits. Obstetric complications, such as anoxia, forceps delivery, pre-eclampsia can result in brain damage that is related to psycho-behavioral development [52]. Our data also suggests that maternal smoking behavior is related to increased tendency on externalizing problems, opposition, atypicality and withdrawal behaviors. Smoking behavior in pregnant women would expose the fetuses not only to nicotine, but also to carbon monoxide. Nicotine exposure during the prenatal period interferes with the development process in the cerebral cortex, and the early disturbance from smoking exposure has a long-term impact on behavioral development. Offspring who had been exposed to maternal smoking during pregnancy are twofold likely to have a criminal record than negative controls, even after adjusting for socio-economic status, childrearing behavior, parenting behavior and birth complications [52]. However, neurodevelopment is constantly shaped by the interactions between intrinsic cues and the extrinsic environment. When accounting for criminality and antisocial personalities of the parents, the magnitude of the association attenuated. Yet, the smoking effect was stronger in subjects who were born with complications, born to teenage mothers, born into single parent families, or showed motor development lags.

We believe that our approach, compared to others, resulted in comparisons that were more reliable, and it builds validity by testing on multiple behavioral scales and by covering diverse behavior domains. Even though most of the literature favors parental rated results to self-reported results in young children, our result shows good validity of children's responses. The responses from BASC-2 SRP were more sensitive for detecting age effect in response to lead exposure. Our data showed that BASC-2 SRP captured the cohort difference introduced mainly by age (average 9 in cohort 2 and 7 in

cohort 3), while parental rating scales did not detect this feature. Furthermore, even though the results were all non-significant, the magnitudes of the lead effects were stronger in children's self-rated scales than in parent-rated scales. A discrepancy in gender effects between SRP and PRS was observed; females tended to rate themselves better than males while parents rated females worse on inattention and hyperactivity scales. Parental rating is contradictory to the current knowledge that ADHD is more prevalent in males[53]. However, our results show that the consistency of lead effects on overall behavior problems disregard responders. Therefore, we believe the results are valid.

On the other hand, these results should be placed in the appropriate context. First, this study was conducted in the low-middle class Mexican population so, the lead effects cannot be directly applied to other ethnic populations or social classes. Moreover, sensitivity analysis showed cohort specific patterns of the effects of lead. We observed strong and significant effects of gestational blood and first-two-year blood lead on inattention/ hyperactivity scales in cohort 2 PL, while in cord blood lead models using cohort 2 PL, blood lead in the first two years showed a strong and significant detrimental impact on ADHD related scales. Cohort 2 BI showed that the first two years blood lead have the strongest and significant influence on personal adjustment and perfectionism, but they have a weak impact on ADHD-like behavior scales. Given these findings, results from combined cohort analyses should be cautiously interpreted due to the diverse responses in the sub-populations. Secondly, neurobehavioral measurement was examined at one time. We do not have data to show the behavioral changes overtime as a function of early lead exposures. Hormonal levels also change in

adolescent period and it is another important factor affecting behavioral formation. It would definitely provide new understanding on the effect of timing of lead exposure on psychobehavioral development as a dynamic growing process. Third, excluded subjects can also impact the effects estimates. A comparison of groups with and without complete information showed the similar distributions in lead exposure, but not in several behavioral outcomes. Slightly worse scores were observed on inattention scales in the excluded group. This may indicate a minor differential missing issue so that our models only captured subjects with slightly better behavioral outcomes, and it could be that the excluded group can be more vulnerable to lead or less likely to follow protocol due to attention problems. Therefore, the lead effect estimates could be deflated based on complete observations.

In conclusion, this is the first analysis comparing the effects of lead exposure at different windows in early life on neurobehavioral outcomes. Unlike previous findings on concurrent lead effects on these outcomes, our data implied that lead exposure during the gestational period and the first two years after birth have strong and long-lasting impacts on behavioral problems in childhood or early adolescence. The main effect of lead implicated a deleterious effect on behavioral development, but child-rearing environment factors such as mother's education, SES levels had even greater impact on a child's psychobehavioral development. Future studies may need to clarify the effects of the temporal exposure patterns on a large scale, as well as to provide a new understanding of the effect of timing of lead exposures on psychobehavioral development as a dynamic process.

Tables

Table 2.1 Characteristics of Study Participants by Cohorts

	BI			PL			SF [3]			
	N	Mean	SD	N	Mean	SD	N	Mean	SD	
Maternal Blood Pb 1st Trimester (µg/dL)	0	-	-	120	7.58	3.51	213	4.83	3.14	**[4]
Maternal Blood Pb 2nd Trimester (µg/dL)	0	-	-	134	6.89	3.32	211	3.88	2.72	**
Maternal Blood Pb 3rd Trimester (µg/dL)	0	-	-	121	7.15	3.49	203	4.61	2.95	**
Gestational Blood Pb Level (µg/dL)[5]	0	-	-	101	4.43	1.77	197	2.81	1.64	**
Patella Pb (µg/g)	221	8.93	9.88	109	13.68	11.23	182	7.05	9.02	**
Tibia Pb (µg/g)	212	8.7	10.05	96	11.62	9.51	88	5.04	8.21	**
Cord Blood Pb (µg/dL)	170	4.31	2.52	73	6.37	4.16	38	3.32	2.54	**
Children Blood Pb Level 1-2year (µg/dL)	222	8.54	5.1	141	10.21	6.01	209	9.14	5.73	**
Children Blood Pb Level 3-4year (µg/dL)	220	11.36	4.27	143	12.85	5.81	177	9.75	4.37	**
Children Blood Pb Level 4years (µg/dL)	223	20.04	8.08	143	23.34	10.26	215	18.85	9.77	**
Children Blood Pb Level at tests (µg/dL)	167	3.13	3.09	109	3.21	2.13	138	3.75	3.1	
BASC SRP: School Problems	223	51.57	10.25	138	51.2	9.8	50	53.3	10.32	*
BASC SRP: Internalizing Problems	222	50.27	7.88	138	50.93	7.88	50	55.54	10.4	**
BASC SRP: Inattention/Hyperactivity	223	52.94	10.23	138	53.31	10.46	50	56.7	11.38	
BASC SRP: Emotional Symptoms Index	223	50.72	8.29	138	51.3	8.28	50	55.72	9.52	**
BASC SRP: Personal Adjustment	222	48.55	8.21	138	47.72	9.27	50	43.88	9	**
BASC PRS: Externalizing Problems	217	49.8	9.45	136	50.84	9.72	211	48.03	9.35	*
BASC PRS: Internalizing Problems	217	53.4	10.77	136	52.88	10.08	211	51.47	9.68	
BASC PRS: Behavioral Symptoms Index	216	51.34	9.61	137	51.37	10.43	210	49.98	8.9	
BASC PRS: Adaptive Scale	217	46.43	10.11	137	47.79	10.41	212	47.02	10.22	
CRS-R: Opposition	217	51.01	8.23	136	50.55	9.69	211	50.04	9.31	
CRS-R Cognitive Problems/ Inattention	217	54.49	10.32	137	54.35	11.32	212	53.52	10.48	*
CRS-R: Hyperactivity	217	56.05	11.23	137	56.35	12.18	212	54.55	9.03	
CRS-R: Anxious- Shy	217	57.17	11.59	137	56.2	10.26	212	54.45	10.68	*
CRS-R: Perfectionism	217	51.89	8.07	136	52.01	7.98	211	51.64	7.65	
CRS-R: Social Problems	216	53.47	10.26	137	52.64	10.2	212	53.6	10.79	
CRS-R: Psychosomatic	217	54.03	11.72	137	54.04	11.13	210	52.84	9.94	
CRS-R: ADHD Index	216	54.69	10.72	137	54.35	11.17	212	53.45	9.84	
CRS-R: CGI Restless-Impulsive	217	54.68	9.87	137	55.55	11.5	212	53.75	9.89	
CRS-R: CGI Emotional Lability	215	50.47	8.61	137	51.71	10.69	212	49.42	8.61	
CADS: DSM IV Inattentive	217	53.62	9.93	137	53.66	11.67	212	52.97	9.66	

[3] Cohort BI, PL constitute cohort 2. Cohort 3 is labeled as cohort SF.

[4] *p<0.05, ** p<0.01 from either Fisher's exact tests or ANOVA tests

[5] Gestational blood lead level was calculated as cumulative blood levels from three trimesters

CADS: DSM IV Hyperactive-Impulsive	217	57.58	10.77	137	57.94	11.65	212	56.08	9.91	
CADS: DSM IV Total	217	55.85	10.41	137	56.11	11.23	212	54.67	9.73	
Maternal Age	223	24.92	5	144	26.68	5.25	216	26.81	5.74	**
Years of Education (Maternal)	223	10.92	2.75	144	10.62	2.86	216	10.99	2.9	**
Years of Education (Paternal)	205	10.9	3.16	144	9.95	4.31	190	10.81	2.94	**
Social Economic Levels	216	8.75	3.12	137	9.07	3.46	204	8.49	3.21	*
Ever Smoked during Pregnancy [N (%)]	222	16	-	144	7	-	216	1	-	
Birth Weight	223	3.17	0.47	141	3.11	0.48	216	3.17	0.51	
Birth Height	223	49.87	2.29	143	49.53	2.71	210	50.18	2.23	**
Gestation Length	223	39.05	1.05	140	38.66	1.63	213	38.75	1.41	**
Children's Age	222	9.24	0.73	143	9.68	1.02	195	7.24	0.5	**

Table 2.2 Lead Effects from Cord Blood Lead Models in Cohort 2[6]

	Cord Blood Lead		Blood Lead in the 1st Two Years		Blood Lead in the 2nd Two Years			Blood Lead at Test	
Models	Std.Coeff	Std.SE	Std.Coeff	Std.SE	Std.Coeff	Std.SE		Std.Coeff	Std.SE
BASC SRP: Inattention/ Hyperactivity	0.06	0.08	0.04	0.09	0.18	0.08	*	-0.02	0.08
CRS-R: Cognitive Problems/ Inattention	-0.09	0.08	0.07	0.09	0.02	0.09		-0.06	0.08
CADS: DSM IV Inattentive	-0.07	0.08	0.06	0.09	-0.04	0.09		-0.06	0.08
CRS-R: Hyperactivity	-0.02	0.08	0.12	0.09	0.03	0.09		0.02	0.09
CRS-R: CGI Restless-Impulsive Index	0.01	0.08	0.07	0.09	0.07	0.09		0.04	0.08
CADS: DSM IV Hyperactive-Impulsive	0.01	0.08	0.11	0.09	0.00	0.09		0.05	0.09
CRS-R: ADHD Index	-0.08	0.08	0.11	0.09	0.05	0.09		-0.02	0.09
CADS: Total	-0.04	0.08	0.11	0.09	0.01	0.09		-0.02	0.09
BASC SRP: Emotion Symptoms Index	0.13	0.08	0.03	0.09	0.02	0.09		0.04	0.08
BASC SRP: Personal Adjustment	0.07	0.08	0.03	0.09	0.04	0.09		0.04	0.09
BASC PRS: Adaptive Scale	0.06	0.08	0.07	0.08	0.10	0.08		0.05	0.08
CRS-R: Anxious-Shy	-0.12	0.08	0.06	0.09	-0.10	0.09		0.02	0.09
CRS-R: Social Problems	0.00	0.08	0.14	0.09	0.06	0.09		-0.02	0.09
BASC SRP: School Problems	-0.01	0.08	-0.01	0.09	-0.01	0.09		0.07	0.09
BASC PRS: Behavioral Symptom Index	0.07	0.08	0.13	0.09	0.03	0.08		0.01	0.08
CRS-R: Psychosomatic	-0.06	0.08	0.11	0.09	0.11	0.09		-0.15	0.09 .
CRS-R: CGI Emotion Lability	0.12	0.08	0.10	0.09	0.06	0.09		0.04	0.09
BASC SRP: Internalizing Problems	0.13	0.08	0.02	0.09	-0.02	0.09		0.00	0.08
BASC PRS: Internalizing Problems	0.07	0.09	0.11	0.09	-0.07	0.09		0.01	0.09
BASC PRS: Externalizing Problems	0.04	0.08	0.03	0.09	0.04	0.09		0.02	0.08
CRS-R: Opposition	0.03	0.08	0.11	0.09	0.10	0.09		-0.01	0.09
CRS-R: Perfectionism	0.17	0.08	*[7] 0.09	0.09	-0.12	0.09		-0.06	0.08

[6] All models adjusted for maternal age, educational level, marital status, smoking behavior during pregnancy, socioeconomic status levels, children's sex, age and birth weight.

[7] * $p<0.05$, .$p<0.1$;

Table 2.3 Lead Effects from Gestational Blood Lead Models in cohorts PL and SF[8]

	Gestational Blood Lead		Blood Lead in the 1st Two Years		Blood Lead in the 2nd Two years		Blood Lead at the Test	
Models	Std.Coeff	Std.SE	Std.Coeff	Std.SE	Std.Coeff	Std.SE	Std.Coeff	Std.SE
BASC SRP: Inattention/ Hyperactivity	0.05	0.13	0.14	0.13	0.06	0.12	0.13	0.13
CRS-R: Cognitive Problems/ Inattention	0.03	0.10	0.06	0.10	-0.15	0.10	-0.05	0.10
CADS: DSM IV Inattentive	-0.01	0.10	0.06	0.10	-0.16	0.10	0.00	0.10
CRS-R: Hyperactivity	0.15	0.11	0.13	0.10	0.08	0.10	0.06	0.10
CRS-R: CGI Restless-Impulsive Index	0.10	0.10	0.12	0.10	0.04	0.10	0.02	0.10
CADS: DSM IV Hyperactive-Impulsive	0.15	0.11	0.07	0.10	0.07	0.10	0.04	0.10
CRS-R: ADHD Index	0.07	0.10	0.12	0.10	-0.08	0.10	-0.05	0.10
CADS: Total	0.07	0.10	0.07	0.10	-0.07	0.10	0.01	0.10
BASC SRP: Emotion Symptoms Index	0.15	0.13	0.17	0.13	0.02	0.12	0.18	0.13
BASC SRP: Personal Adjustment	0.00	0.13	0.15	0.13	-0.05	0.12	0.10	0.13
BASC PRS: Adaptive Scale	0.01	0.11	-0.07	0.10	-0.06	0.10	0.09	0.10
CRS-R: Anxious-Shy	0.03	0.11	0.11	0.10	-0.01	0.10	-0.11	0.10
CRS-R: Social Problems	0.03	0.11	-0.08	0.10	0.05	0.10	-0.12	0.10
BASC SRP: School Problems	0.00	0.13	0.16	0.14	-0.11	0.13	0.02	0.14
BASC PRS: Behavioral Symptom Index	0.06	0.11	-0.06	0.10	0.01	0.10	0.06	0.10
CRS-R: Psychosomatic	0.11	0.10	-0.11	0.10	-0.15	0.10	-0.18	0.10
CRS-R: CGI Emotion Lability	0.02	0.11	-0.02	0.10	0.08	0.10	0.08	0.10
BASC SRP: Internalizing Problems	0.10	0.12	0.14	0.13	0.03	0.12	0.17	0.13
BASC PRS: Internalizing Problems	0.02	0.11	-0.17	0.10	-0.07	0.10	-0.06	0.10
BASC PRS: Externalizing Problems	0.06	0.11	0.04	0.10	0.07	0.10	0.16	0.10
CRS-R: Opposition	-0.03	0.11	-0.03	0.10	0.06	0.10	0.04	0.10
CRS-R: Perfectionism	0.04	0.11	0.01	0.10	-0.11	0.10	-0.02	0.10

[8] All models adjusted for maternal age, educational level, marital status, smoking behavior during pregnancy, socioeconomic status levels, children's sex, age and birth weight

*p<0.05, .p<0.1

Table S 2.1 Comparison between Included and Excluded Subjects in Cohort 2

	Pooled			Included			Excluded			
	N	Mean	SD	N	Mean	SD	N	Mean	SD [9]	
Maternal Blood Pb 1st Trimester (μg/dL)	120	7.58	3.51	41	7.48	3.16	79	7.63	3.69	
Maternal Blood Pb 2nd Trimester (μg/dL)	134	6.89	3.32	50	6.56	3.44	84	7.09	3.25	
Maternal Blood Pb 3rd Trimester (μg/dL)	121	7.15	3.49	48	7.27	3.22	73	7.08	3.68	
Cumulative Gestational Blood Pb Level (μg/dL)	101	4.43	1.77	38	4.31	1.56	63	4.51	1.89	
Patella Pb (μg/g)	330	10.50	10.57	160	11.11	10.22	170	9.93	10.89	
Tibia Pb (μg/g)	308	9.61	9.96	150	9.83	10.17	158	9.41	9.79	
Cord Blood Pb (μg/dL)	243	4.93	3.24	170	4.99	3.28	73	4.80	3.16	
Children Blood Pb Level 1-2year (μg/dL)	363	9.19	5.52	170	9.01	5.27	193	9.35	5.74	
Children Blood Pb Level 3-4year (μg/dL)	363	11.95	4.98	170	12.06	4.73	193	11.85	5.21	
Children Blood Pb Level 4years (μg/dL)	366	21.33	9.13	170	21.23	8.24	196	21.42	9.85	
Children Blood Pb Level at tests (μg/dL)	276	3.16	2.75	170	2.97	2.07	106	3.48	3.56	.
BASC SRP: School Problems	361	51.43	10.07	170	51.09	9.72	191	51.72	10.39	
BASC SRP: Internalizing Problems	360	50.52	7.88	170	50.09	8.17	190	50.91	7.61	
BASC SRP: Inattention/Hyperactivity	361	53.08	10.31	170	52.75	10.85	191	53.38	9.82	
BASC SRP: Emotional Symptoms Index	361	50.94	8.28	170	50.72	8.36	191	51.14	8.23	
BASC SRP: Personal Adjustment	360	48.23	8.63	170	48.81	8.48	190	47.72	8.75	
BASC PRS: Externalizing Problems	353	50.20	9.55	167	49.44	9.21	186	50.89	9.83	.
BASC PRS: Internalizing Problems	353	53.20	10.50	166	52.36	10.55	187	53.94	10.43	.
BASC PRS: Behavioral Symptoms Index	353	51.35	9.92	167	50.22	9.56	186	52.38	10.15	*
BASC PRS: Adaptive Scale	354	46.96	10.24	167	47.49	10.08	187	46.49	10.38	
CRS-R: Opposition	353	50.84	8.81	168	50.51	8.65	185	51.14	8.97	
CRS-R: Cognitive Problems/Inattention	354	54.44	10.70	168	53.61	10.11	186	55.18	11.19	.
CRS-R: Hyperactivity	354	56.17	11.59	168	55.88	11.47	186	56.42	11.72	
CRS-R: Anxious- Shy	354	56.80	11.09	168	55.73	10.91	186	57.76	11.20	*
CRS-R: Perfectionism	353	51.93	8.03	167	51.65	8.08	186	52.19	7.99	
CRS-R: Social Problems	353	53.14	10.23	167	53.60	10.27	186	52.74	10.21	
CRS-R: Psychosomatic	354	54.03	11.48	168	53.38	11.73	186	54.63	11.24	
CRS-R: ADHD Index	353	54.56	10.88	168	54.44	11.01	185	54.66	10.79	
CRS-R: Restless-Impulsive	354	55.01	10.53	168	55.58	11.08	186	54.51	10.01	
CRS-R: Emotional Lability	352	50.95	9.48	166	50.34	9.07	186	51.51	9.81	
CADS: DSM IV Inattention	354	53.64	10.62	168	52.46	10.02	186	54.70	11.05	*
CADS: DSM IV Hyperactive-Impulsive	354	57.72	11.10	168	57.30	10.81	186	58.10	11.37	
CADS: DSM IV Total	354	55.95	10.72	168	55.07	10.34	186	56.75	11.01	.
Maternal Age	367	25.61	5.16	170	25.79	4.84	197	25.46	5.44	
Years of Education (Maternal)	367	10.81	2.79	170	10.92	2.90	197	10.71	2.70	
Years of Education (Paternal)	349	10.51	3.71	164	10.74	3.65	185	10.30	3.75	
Social Economic Levels	353	8.87	3.26	170	8.91	3.16	183	8.84	3.36	
Ever Smoked during Pregnancy [N (%)]	366	13	-	170	15	-	196	11	-	
Birth Weight	364	3.15	0.47	170	3.14	0.43	194	3.15	0.51	
Birth Height	366	49.74	2.46	170	49.96	2.23	196	49.55	2.64	
Gestation Length	363	38.90	1.31	169	39.05	1.12	194	38.77	1.45	
Children's Age	365	9.41	0.88	169	9.46	0.82	196	9.38	0.93	

[9] * $p<0.05$, . $p<0.1$ by Student's t-test between the included and excluded groups

Table S 2.2 Comparison between Included and Excluded Subjects in Cohort 2 PL and Cohort 3 Combined

	Pooled			Included			Excluded			
	N	Mean	SD	N	Mean	SD	N	Mean	SD	[10]
Maternal Blood Pb 1st Trimester (µg/dL)	333	5.82	3.53	158	5.83	3.59	175	5.82	3.48	
Maternal Blood Pb 2nd Trimester (µg/dL)	345	5.05	3.31	159	5.05	3.46	186	5.05	3.18	
Maternal Blood Pb 3rd Trimester (µg/dL)	324	5.56	3.39	145	5.67	3.47	179	5.47	3.33	
Cumulative Gestational Blood Pb Level (µg/dL)	298	3.36	1.85	134	3.37	1.88	164	3.35	1.83	
Patella Pb (µg/g)	291	9.53	10.39	129	9.53	10.87	162	9.54	10.03	
Tibia Pb (µg/g)	184	8.47	9.48	82	9.72	9.83	102	7.48	9.12	
Cord Blood Pb (µg/dL)	111	5.33	3.95	50	4.82	3.28	61	5.74	4.41	
Children Blood Pb Level 1-2year (µg/dL)	350	9.57	5.86	163	9.27	5.23	187	9.84	6.36	
Children Blood Pb Level 3-4year (µg/dL)	320	11.14	5.29	150	11.21	5.44	170	11.07	5.17	
Children Blood Pb Level 4years (µg/dL)	358	20.65	10.20	168	21.06	10.99	190	20.28	9.46	
Children Blood Pb Level at tests (µg/dL)	247	3.51	2.73	118	3.49	2.40	129	3.53	3.00	
BASC SRP: School Problems	188	51.76	9.96	87	52.17	10.55	101	51.41	9.46	
BASC SRP: Internalizing Problems	188	52.16	8.83	87	52.17	9.45	101	52.15	8.32	
BASC SRP: Inattention/Hyperactivity	188	54.21	10.79	87	53.97	11.42	101	54.43	10.26	
BASC SRP: Emotional Symptoms Index	188	52.47	8.82	87	52.29	9.36	101	52.63	8.37	
BASC SRP: Personal Adjustment	188	46.70	9.33	87	46.61	10.03	101	46.77	8.74	
BASC PRS: Externalizing Problems	347	49.13	9.58	161	48.83	9.88	186	49.39	9.33	
BASC PRS: Internalizing Problems	347	52.02	9.85	161	52.07	10.05	186	51.98	9.69	
BASC PRS: Behavioral Symptoms Index	347	50.53	9.54	161	50.14	9.23	186	50.87	9.82	
BASC PRS: Adaptive Scale	349	47.32	10.29	161	47.19	10.42	188	47.44	10.20	
CRS-R: Opposition	347	50.24	9.45	160	49.20	9.01	187	51.13	9.75	*
CRS-R: Cognitive Problems/Inattention	349	53.85	10.81	162	54.02	11.24	187	53.70	10.45	
CRS-R: Hyperactivity	349	55.26	10.40	162	54.20	9.92	187	56.17	10.74	*
CRS-R: Anxious- Shy	349	55.14	10.54	162	55.10	10.93	187	55.18	10.22	
CRS-R: Perfectionism	347	51.79	7.77	162	52.05	7.85	185	51.56	7.72	
CRS-R: Social Problems	349	53.22	10.56	162	52.38	9.03	187	53.95	11.70	.
CRS-R: Psychosomatic	347	53.31	10.43	160	52.45	10.07	187	54.05	10.69	.
CRS-R: ADHD Index	349	53.81	10.38	162	53.50	10.88	187	54.07	9.94	
CRS-R: Restless-Impulsive	349	54.46	10.57	162	53.60	10.68	187	55.20	10.45	.
CRS-R: Emotional Lability	349	50.32	9.53	162	49.89	9.43	187	50.70	9.62	
CADS: DSM IV Inattention	349	53.24	10.48	162	53.23	10.81	187	53.25	10.22	
CADS: DSM IV Hyperactive-Impulsive	349	56.81	10.65	162	55.70	10.46	187	57.77	10.74	*
CADS: DSM IV Total	349	55.23	10.35	162	54.80	10.57	187	55.61	10.17	
Maternal Age	360	26.76	5.54	168	27.09	5.46	192	26.47	5.61	
Years of Education (Maternal)	360	10.84	2.88	168	10.90	2.88	192	10.79	2.89	
Years of Education (Paternal)	334	10.44	3.62	158	10.35	3.99	176	10.52	3.26	
Social Economic Levels	341	8.72	3.32	158	8.87	3.44	183	8.60	3.22	
Ever Smoked during Pregnancy [N (%)]	360	3	-	168	2		192	5	-	
Birth Weight	357	3.15	0.50	167	3.15	0.52	190	3.14	0.48	
Birth Height	353	49.92	2.45	165	49.93	2.40	188	49.91	2.51	
Gestation Length	353	38.71	1.50	164	38.74	1.56	189	38.69	1.44	
Children's Age	338	8.27	1.43	154	8.32	1.46	184	8.23	1.40	

[10] * $p<0.05$, . $p<0.1$ by Student's t-test between included and excluded groups

Figures

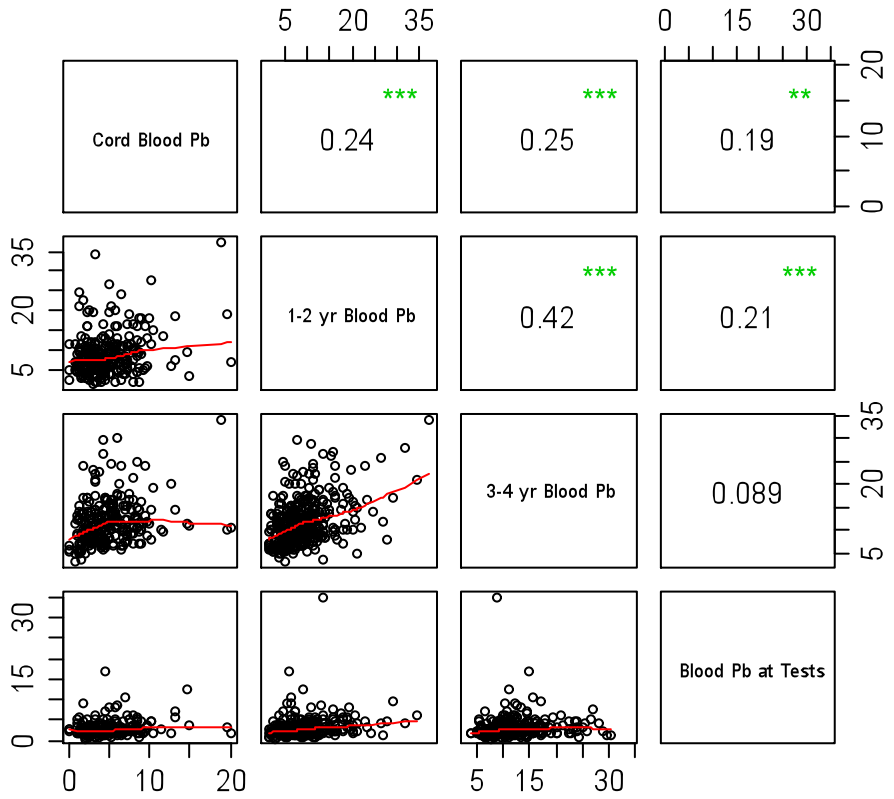

Figure S 2.1 Correlations among Lead Exposures in Cohort 2 (BI and PL)

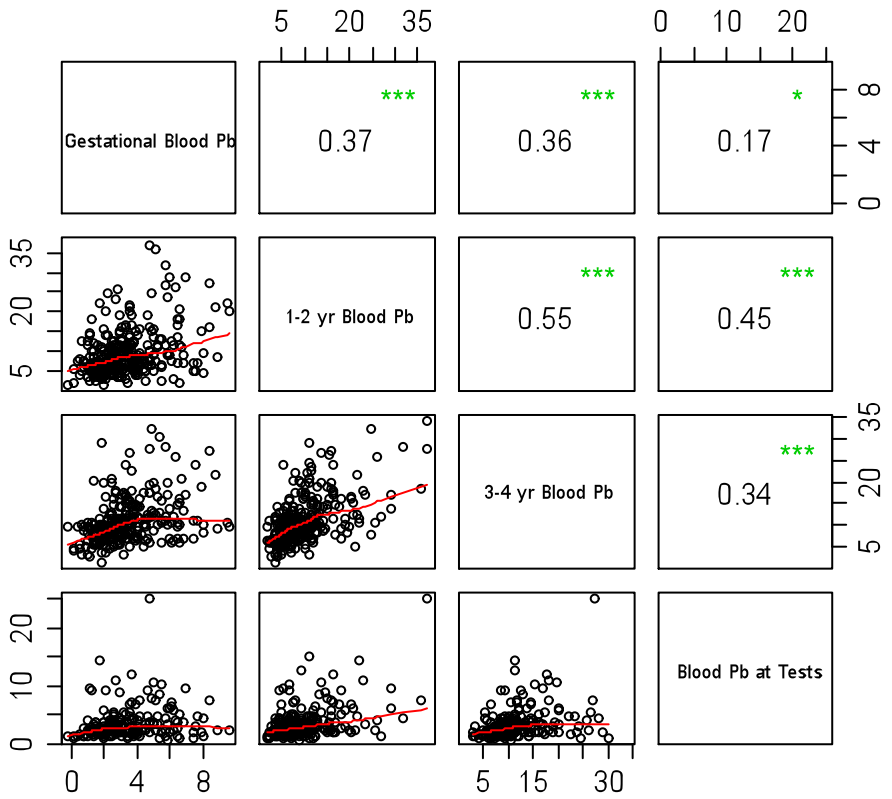

Figure S 2.2 Correlations among Lead Exposures in Cohorts PL and SF combined

Appendix

Equation 2.1

$$BLL = \int_j Pb_j = \tfrac{1}{2}\sum_j (Pb_j + Pb_{j+1})t_j \qquad (2.1)$$

Caption: *j*- observation time; Pb_j - observed lead level at time *j*; *t*- time interval between *j* and *j*+1

Equation 2.2

$$X_j = \alpha_{j-1}X_{j-1} + X_j^{'} \qquad (2.2)$$
$$X_2 = \alpha_1 X_1 + X_2^{'}$$
$$X_3 = \alpha_2(X_1 + X_2^{'}) + X_3^{'}$$
$$X_4 = \alpha_3(X_1 + X_2^{'} + X_3^{'}) + X_4^{'}$$

Caption: *j*- observation time points *j*≥1; X_j- observed lead level; X_j'- unobserved additional lead exposure

The observed blood lead consists both historical and current lead exposures. The effect estimates of observed current blood lead level can be confounded by chronologically remote exposures. We used the residual method to differentiate the effect from additional lead exposures in the current windows. The residuals serve as latent exposure variables at each post-delivery window that could not be directly observed. Specifically, the current lead exposure can be decomposed as a summation of previous exposure and current exposures as expressed in equation 2.2. This is biologically relevant to the lead mobilization from bone to blood during active bone turnover period, such as rapid skeletal growth. In equation 2.2, X_j and X_{j-1} denote observed lead levels; α_{j-1} characterized the fraction of current blood lead that can be explained by previous blood lead. $X_j^{'}$ indicates the additional blood lead that cannot be explained by the previous blood lead. This model assumes the contribution to current

blood lead from previous lead exposure is fixed and indicated by α_{j-1} for every individual, while the additional exposure ($X_j^{'}$) in current window varies across individuals.

When fitting regression models with observed lead levels separately, the previous exposures have been counted due to the intercorrelations among exposures, as illustrated in Equation 2.3.

Equation 2.3

$$E[Y] = \gamma_j(\sum_{k=1}^{j-1}\{X_k^{'}\prod_{k=1}^{j-1}\alpha_k\} + X_j^{'}) \qquad (2.3)$$

$$E[Y] = \gamma_1 X_1$$

$$E[Y] = \gamma_2 X_2 = \gamma_2\alpha_1 X_1 + \gamma_2 X_2^{'}$$

$$E[Y] = \gamma_3 X_3 = \gamma_3\alpha_2\alpha_1 X_1 + \gamma_3\alpha_2 X_2^{'} + \gamma_3 X_3^{'}$$

$$E[Y] = \gamma_4 X_4 = \gamma_4\alpha_3\alpha_2\alpha_1 X_1 + \gamma_4\alpha_3\alpha_2 X_2^{'} + \gamma_4\alpha_3 X_3^{'} + \gamma_4 X_4^{'}(*)$$

Caption: *k*- observation windows, *k*= 1,2…*j-1*; *j*- observation time points *j*≥1; X_j- observed lead level; X_j'- unobserved additional lead exposure; Y- the outcome measured at or after the window max(*j*); γ_j- effect estimate of observed lead exposure at window *j*

The estimated lead effect at each following window *j* integrates the effects of exposures from both current window and past windows via secondary endogenous lead exposure. As illustrated in Equation 2.3, the effect of X_1 on Y at *j*=4 (*) is mediated through the bone- blood exchange over next three windows (α_1, α_2, α_3). Similarly, X_2, X_3 also impact on Y via α_2, α_3.

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CHAPTER 3 THE POTENTIAL INFLUENCE OF VARIANTS OF THE *SNCA* GENE ON THE IMPACT OF CUMULATIVE LEAD EXPOSURE ON RISK OF PARKINSON'S DISEASE

Introduction

Parkinson's disease (PD) is one of the most common degenerative neurological disorders. The incidence of PD increases with age, with annual incidence rates from 20 out of 100,000 persons at age 60 to 120 out of 100,000 persons at age 70 [1]. Males have a higher risk of developing the disease than females [2]. Around 70% of PD patients suffer from motor symptoms such as resting tremor, slow movement (bradykinesia) and postural instability. The characteristic pathological finding in PD is loss of dopaminergic neurons in the basal ganglia and, in particular, in the substantia nigra pars compacta (SNpc) region. PD has a silent onset. By the time clinical symptoms arise, neuronal losses have occurred in around 77% of the posterior putamen, 68% of the anterior putamen, and 50% of the substantia nigra (SN) [3]. The prevailing view is that the loss of dopaminergic (DAergic) neurons in PD is a consequence of increased oxidative stress level and protein aggregation[4-6]. Both genetic and environmental components are involved in the disease's development and progression. Genome-wide association studies (GWAS) have provided evidence of genetic contributions to the disease [7-16]. *SNCA*, *UCH-L1, PRKN, LRRK2, PINK1, DJ-1, and ATP13A2* have been found to be significantly associated with PD [16]. However, family history and twin studies suggest that the contribution of inheritance to PD is minor[17-22]. Low concordance- 15% or lower

in monozygotic twins and 11% or lower in dizygotic twins- was reported in the majority of twin studies of PD [21, 22].

With increasing attention to effect of the environmental exposures on health, studies have been conducted assessing the potential contribution to the etiology of PD of environmental chemical exposures. For example, 1-methyl-4-phenyl-1, 2,3,6-organophosphates (MPTP) [23]and paraquat [24, 25] exposures are well studied as inducers of Parkinsonism in animal models. A few studies have identified associations between metal exposure and PD[26-29]. Our group has previously found that cumulative exposure to lead (as reflected by measurements of bone lead levels) is associated with an increased odds of PD [30].

In the current study, we continued the investigation of lead impacts on PD by examining lead effect in subjects with different levels of genetic susceptibilities to PD. In particular, we chose to study the potential modifying effect of the *SNCA* gene, which has been found to be linked to PD in many GWAS studies[9, 10, 12, 14, 15]. In addition, evidence from animal studies showed that lead can induce *SNCA* gene expression and the formation of inclusion bodies, which is commonly found in PD brain tissue[31, 32]. This gives rise to our hypothesis that lead interacts with *SNCA* genetic variants to create a synergistic effect on PD. We examined the main effect of genetic markers of the *SNCA* gene as well as the interaction with cumulative exposure to lead as reflected by bone lead levels in tibia and patella in this study.

Methods

Study Design

Parkinson's patients were recruited from several clinics in Boston, Massachusetts area: the Boston University Medical Center (BUMC), the Brigham and Women's Hospital (BWH), the Beth Israel Deaconess Medical Center (BIDMC) and Harvard Vanguard Medical Associates (HVMA). The initial proposed recruitment was 800 PD cases and controls, with cases made up of recently diagnosed PD populations (40% have symptomatic PD less than 5 years, 80% have symptomatic PD less than 10 years). Controls were recruited from the same hospital sites as well as from participants in the Harvard Cooperative Program on Aging (HCPOA), participants in Normative Aging study (NAS) who were of the same age as PD cases and who have had bone lead measurements within 1 year of that of a PD case. The controls were intended to match PD cases on age, sex, race and geographic distributions.

Subject Selections

The majority of the participants in this study were American Caucasians (86.8%); 63.2% of the total participants were males. A total of 330 PD cases were identified by two clinical neurologists and confirmed with neuroimaging diagnosis (CT or MRI scan) showing abnormal structure in basal ganglia or brainstem and exhibiting the following symptoms: resting tremor, cogwheel rigidity, bradykinesia. Medical charts was reviewed by a study neurologist and additional neurologist affiliated with our collaborating clinics. Specifically, cases were selected based on the following criteria: (1) complete history and clinical evaluation by an attending neurologist; (2) the presence of at least two of

three symptoms: resting tremor, cogwheel rigidity, bradykinesia; (3) asymmetry; (4) none of the following: supranuclear gaze palsy out of proportion for PD, unexplained cerebellar findings, unexplained hypperreflexia, definite absence of response to levodopa 600mg/day, clearly nonprogressive course, findings strictly unilateral after 5 years, MRI or CT showing infarcts in the basal ganglia or brainstem with either stepwise clinical progression, lower-body predominance or pyramidal signs, neuroleptic use in the past 6 months; (5) at least two clinical evaluations at a minimum of six months apart, the last one of which must be within twelve months prior to recruitment for this study, in which preceding criteria were met; and (6) symptoms of PD for fewer than ten years.

A total of 354 control subjects were mainly recruited from two populations. Spouses and in-laws were the first consideration of controls due to the little likelihood of being genetically associated with cases. Subjects were free from PD or did not exhibit any PD like symptoms, did not have 1st or 2nd degree blood relative with confirmed or suspected PD case. Subjects who were younger than 50 years old and/or living more than a 2-hour drive from Boston were not considered. Given the lack of an adequate number of successfully recruited controls from the originally proposed population, subjects participating in the Normative Aging Study (NAS) who were free of PD were adopted based on matching demographic information. Of the controls, 59.6% were the spouses, in-laws and friends of the PD subjects, and 40.4% were selected from subjects participating in the long-running Normative Aging Study (NAS) and communities (HCPOA).

This study was approved by Human Research Committee of Harvard School of Public Health, the Brigham and Women's Hospital (BWH), the Beth Israel Deaconess

Medical Center (BIDMC) and Harvard Vanguard Medical Associates (HVMA). All subjects in this study had been informed and consented to participate.

Retrospective Lead Exposure

Bone lead levels were measured non-invasively using a ^{109}Cd K-shell X-ray fluorescence (K-XRF) [33, 34] instrument at the Harvard University/Channing laboratory. Subjects who consented to take lead measurements were asked to take two 30-minute *in vivo* bone lead measurements at clinic visit. The left tibia and patella were selected to represent the lead depositions in cortical and trabecular bones. The physical principle, technical specifications were described in Aro et al's paper [35]. As a quality control measure, lead exposure measurements with estimated uncertainties greater than10μg/g in tibia and greater than15μg/g in patella were excluded. Both tibia and patella lead level reflect cumulative exposure to lead. Compared to patella lead, tibia lead, which is made up primarily of cortical bone, exhibits a lower decay rate over time due to the limited bone-blood lead exchange [36]. Therefore tibia lead is considered as a reliable biomarker for lifetime lead exposure. By contrast, the patella, which is mostly trabecular bone, has a proportionately larger surface area in contact with blood and the associated lead content decays faster than that of tibia lead. Comparing and contrasting the effect of these two biomarkers helps to determine the relevant exposure interval for any associations.

Other Information

A series of questionnaires were conducted during initial recruitment. The questionnaires covered domains of family medical history, personal medical history,

lifestyle, environmental and occupational exposures, food consumption frequencies and tobacco use. Two updated versions that have been conducted with and without repetition in study subjects.

Genotyping and Quality Control

A total of 14 candidate SNPs in the *SNCA* gene from 548 study subjects' DNA samples were genotyped at Channing lab at the Harvard University. The markers from 14 loci were selected based on previous literature, showing an association between these common markers and the risk of PD pathology [7, 11, 14, 37-47]. DNA was extracted from whole blood using standard techniques. Genotyping was done by Sequenom iPLEX SNP genotyping technique.

We defined a call rate threshold at 0.9 for each marker and 0.6 for each individual sample as quality filters before analysis. We compared the call rates between cases and controls. We defined the threshold of minor allele frequency at 5% and compared allele frequency and heterozygosity rate between cases and control groups. Departure from Hardy-Weinberg equilibrium was calculated in the pooled study population and in cases and controls separately. Linkage disequilibrium (LD) examination was defined as r^2 greater than 0.8 and D' greater than 0.9.

Statistical Analyses

All of the statistical procedures were performed in R version 2.15.2 [48]. Packages *genetics* [49] and *CGEN* [50] and *visreg* [51] were installed for genetic data processing. The threshold for statistical significance were determined at $p < 0.05$. Prior to data analysis of the final dataset (genotype combined dataset), we compared the distributions of

variables of interests in cases and controls between included and excluded datasets, in order to examine the variable distribution shifts brought by data attrition from merging with genotype data. We performed logistic regressions to examine the main effect of lead in the final dataset and compared this to the effect estimates from the original exposure-only dataset, which included fewer NAS subjects compared to the dataset published[30]. Bone lead levels were categorized into quartiles. The OR of PD in each quartile was estimated using logistic regression. Comparisons were made of ORs from the final dataset and from the exposure dataset with those that have previously been published[30] to examine differences in the main effects of bone lead in the final dataset.

Univariate and Bivariate Analyses

The distributions of tibia and patella lead levels were examined for normality and variance. Lead was treated as a continuous variable in the final model. After accounting for age, age squared, sex, race, smoking status, and educational levels, bone lead distributions in the HCPOA, NAS and community controls were equivalent to the bone lead distributions in the BUMC controls (tibia difference: 1.17μg/g, p=0.59, patella difference: 0.26μg/g, p=0.92). Therefore, controls from HCPOA, NAS and community were merged into BUMC control group. Education levels were categorized into high school diploma or less, some college, college graduate, graduate school and others. Race information was dichotomized into Caucasians and others. For the covariates with missing information (< 8% for any covariates in the final models), we created separate missing categories and included them into the final models. We assigned the median value to any missing data in pack-years of cigarette smoking, and created missing indicators. The new pack-years and missing indicator variables were fitted into the final

models. Genotypes were categorized into having 0, 1, 2 copies of the dominant alleles. We then compared genotype frequency by disease status, gender, race and recruitment sites; we also examined bone lead levels, age at lead measurements, age at appearance of first PD symptoms, and age at PD diagnosis by genotypes.

Association Tests

Fisher's exact tests and Cochran-Armitage trend tests were performed among the 14 markers to examine their main effects. Statistical significance was considered but not determined using Bonferroni correction across the number of tested loci, which renders *p* value threshold at 0.05/14. Our analyses constituted two stages; before testing the gene-environment interaction effects, polytomous logistic regressions were performed to examine the degrees of independence between selected loci and bone lead levels (θ_{GE}). This step served as an alert for potential biases if any associations were found between genotype distributions and lead exposures, then unconditional logistic regression and empirical bayes logistic regression models [52, 53] were used to estimate the gene-environment interaction effects. The results from both methods are reported here. Due to the break of matching on the matching variables in the final dataset, all models included age, sex, race, recruitment sites, educational levels and cigarette smoking pack-years as covariates.

Results

All 14 *SNCA* markers reached a call rate above 0.9. Eleven individuals were filtered out due to call rates less than 0.6. The overall call rate for all individuals was 0.92. No statistical difference in call rate was found between cases and controls. The

minor allele frequencies (MAF) of the selected markers were all above 5%. Allele frequency and heterozygosity were not significantly different between cases and controls. Departure of Hardy-Weinberg disequilibrium was identified in six markers at $p<0.05$ (Figure S 3.1). Figure 3.1 shows the chromosome physical distance of 14 *SNCA* markers. Six marker pairs were detected in high LD ($r^2>0.8$, $D'>0.95$) (Figure S 3.2). A total of 537 subjects were maintained in the genotype dataset. After combining with the lead exposure dataset, 535 subjects with complete information were included in the final dataset. Prior to the main analysis, the sensitivity analysis showed that the main effect estimates of lead on risk of PD from the final dataset were not meaningfully different from the lead effect estimates from the published dataset[30](Table S 3.1).

Table 3.1 lists the distributions of each variable in the final models for the pooled, final, and excluded datasets and by disease status. In general, the controls who entered into the final dataset were younger, lighter smokers, compared to cases. In the final dataset, the average age at bone lead measurement was 66.6 years in cases (SD=9.34) and 69.13 years in controls (SD=9.72), and the mean age of subjects at time of PD diagnosis was 60 years (range 28.5-83.91, SD=10.99). After adjusting for age, age squared, educational levels, years of smoking, sex and race, the bone lead level in a White female aged 50 years with a college degree was 3.85 μg/mg in tibia and 2.46 μg/mg in patella in the final dataset, whereas in the excluded subjects an equivalent subject's tibia level was 4.46 μg/mg in tibia and 3.61 μg/mg in patella.

Allele frequencies in the final dataset are shown in Table 3.2. No significant differences in allele frequencies were found between case and control groups (Table 3.2) or among recruitment sites (data not shown). Excluding marker rs356186, no

difference was observed in allele frequency between genders (data not shown). The allele frequency in the majority of the markers was significantly different between whites and nonwhites (data not shown) at $p<0.05$. Allele frequencies of all makers were comparable to the reference population (HapMap CEU). The genotype frequencies for all 14 loci among cases and controls are given in Table 3.3. Markers rs10005233, rs2301134, rs2301135, rs356186, and rs356188 had different genotype distributions between cases and controls at $p<0.05$. Tibia and patella lead levels did not differ by genotypes for any of the 14 markers at $p<0.05$. The onset of PD was not associated with any genotype groups of all makers at $p<0.05$.

The initial association tests showed statistical significance at $p<0.05$ at loci rs356188, rs356186, rs10005233, rs2301135, and rs2301134, on Fisher's exact tests and at loci rs356188, rs356186, rs2301135, rs2301134 and rs2736994 on Cochran-Armitage trend tests (Figure S 3.3, Figure S 3.4). However, after Bonferroni correction, only marker rs2301135 reached statistical significance. The gene-environment independence assumption tests in control subjects showed that, in general, the association between bone lead levels and genotype frequency is weak (θ_{GE} range: -0.054, 0.066,Table S 3.2). However, several markers reached statistical significance at $p<0.05$, indicating significant gene-environment dependence. After Bonferroni corrections for 14 loci ($p= 0.0036$), none of the models reached statistical significance. Since under the gene-environment dependence condition, case-only estimates of interaction term are subject to bias; furthermore, empirical bayes method weighs between the effect estimates using case-only method and the effect estimates using case-control method, which renders the point of estimates in between of these two[53].

Therefore, we decided to report effect estimates based on the unconditional logistic regression method. Interaction term effect estimates were plotted in Figure S 3.5, Figure S 3.6 for comparison purposes.

After adjusting for age, educational level, race, gender, and pack-years of cigarettes smoking in the gene-environmental final models, we found no significant associations between bone lead levels and PD status in any models. The effect of bone lead with one standard deviation increase is listed in Table S 3.4. We found strong and significant main effects of markers at loci rs11931074, rs356186, rs1812923, rs10005233 rs2301135 rs2301134 and rs2736994 on PD (Table 3.4, 3.5). The ORs comparing homozygote of minor alleles are 0.494 (95%CI: 0.20,1.20) for heterozygote, 0.31 (95%CI: 0.12, 0.75) for homozygotes at rs1812923; 1.56 (95%CI: 0.77, 3.14) for heterozygotes and 4.29 (95%CI: 1.77, 10.39) for homozygotes at rs10005233; 2.07 (95%CI: 1.03, 4.15) for heterozygotes, 5.74 (95%CI: 2.26, 14.57) for homozygotes at rs2301135; and 0.38 (95%CI: 0.16, 0.89) for heterozygotes, 0.21 (95%CI: 0.08, 0.53) for homozygotes at rs2301134 in the tibia bone lead models. Similar results were found in patella bone lead models. Since rs10005233, rs2301135 and rs2301134 were in high mutual LD, we chose to report rs2301135 in this analysis, based on *p* values of main effect of the markers from both tibia and patella logistic regression models. The markers' main effects remained significant after the post-hoc adjustment.

Additionally, we found that the gene-environment interaction effects were significant at loci rs1812923, rs2301135 in tibia lead models at $p<0.05$, and marginal significance ($p<0.1$) at loci rs1193107, rs356221 and rs2736994. After Bonferroni correction (adjusted for LD pairs, $p_{BF}= 0.0056$), none of the interaction terms maintained

statistical significance. The results for the main effects of each genotype and the interactions between genetic variant and bone lead levels shown in Table 3.4 and 3.5. Figure 3.2 depicts dose-response relationships between bone lead and probability of being a PD case by genotype in markers rs1812923**,** rs2301135**,** and rs2736994**.** As shown in the plots for the genotype at locus rs1812923, subjects with homozygous A allele (G0) showed the highest odds of having PD compared to heterozygous subjects (G1) and homozygous subjects carrying the C allele (G2). However, the dose-response relationship showed that the probability of having PD in G0 subjects was not affected by an increased level of bone lead, whereas in the G1and G2 groups, an elevated bone lead level is associated with an increasing probability of being a PD case. In the tibia model, the interaction term OR for G1 was 1.09 (95%CI: 1.00, 1.18, *p*= 0.04) and OR for G2 was 1.08 (95%CI: 0.99, 1.18, *p*= 0.06). As for the locus rs2301135, the main effect of the C allele homozygote (G0) showed a protective effect compared with heterozygotes (G1) and G allele homozygotes (G2). In the G0 group, an increase in bone lead was associated with a greater probability of being a PD case; whereas in the G1 group the same direction of association was observed with lesser magnitude (OR= 1.03, 95%CI: 0.96,1.09, *p*= 0.41). In the G2 group, increased lead was associated with decreased probability of being a PD case with an OR of 0.91 (95%CI: 0.84,0.99, *p*= 0.03). Similar patterns was observed at locus rs2736994, with marginal significance (*p*= 0.08 for the interaction term in G1 (CT) and a *p* value of 0.47 for the interaction term in G2 (TT). In group G0 (CC), being a PD case was not associated with lead levels, whereas in the G1, G2 groups, increased bone lead levels were positively associated with the probability of being PD cases. We also observed weak protective effects with

an increase in education level (result not shown). However, these test results did not reach statistical significance in most models.

Discussion

This study is the first to report an analysis of the potential interactions between markers in the *SNCA* gene and cumulative environmental lead exposure risk on Parkinson's disease. In these analyses of a subset of subjects from the parent study, we found that none of the main effect estimates of bone lead was significantly related to the odds of being a PD case, which is somewhat different than what we found in the parent study. However, we found significant main effects of markers on PD at loci rs2736994, rs1812923, rs10005233, rs2301135, and rs2301134. These results indicate C allele at rs2736994, the G allele at rs2301135 and the A allele at rs1812923 detrimental effects on PD. We also found significant gene-environment interactions with respect to PD at loci rs1812923 and rs2301135. The results showed that bone lead levels were positively associated with PD in subjects who had less genetic risk of PD; whereas bone lead levels were negatively associated with PD in subjects with higher genetic risk of PD. Most of the genetic markers locate in the introns of *SNCA* gene. rs11931074 and rs2736994 located at the upstream and downstream of *SNCA* gene. The functions of these noncoding SNPs are largely unknown.

Our results are comparable to previously reported main effects of lead from our group in that lead exposure tended to increase the odds of PD, even though the effect we saw was not statistically significant. It also provides evidence of the dynamics of lead effects in subpopulations with different levels of genetic susceptibilities. This adds

to our understanding of the role of lead exposure in PD development and sheds light on population dynamics of susceptibility to PD.

Our results showed that tibia lead appeared to have stronger associations with PD in the interaction terms than patella lead. This distinction can be explained by the different decay rates of cortical and trabecular bone lead and may simply reflect the different value of these bone measurements in representing retrospective exposures [36]. The implication from this difference is that effect of lead on PD is more likely due to cumulative exposures rather than secondary endogenous exposure. This contention has been well discussed in our previous work[30].

The *SNCA* gene encodes α-synuclein, a brain-enriched neuron specific protein that binds phospholipid membranes at synaptic vesicles. It localizes at the pre-synaptic terminals in the mature neurons and can be found in both axons and dendrites in immature neurons [54].The expression of *SNCA* is involved in synaptic plasticity and synapse maturation [54]. In *SNCA* knockout mice reductions in striatal dopamine levels and in the corresponding dopamine dependent locomotory activities were observed [55]. α-synuclein can bind to tyronsine hydroxylase and inhibits dopamine synthesis [56]. It also increases the dopamine transporter (DAT) localization at cell surface by binding to DAT. This leads to an increase of dopamine reuptake, thus elevating intracellular dopamine level and creating oxidative stress [57].

α-synuclein and PD

At physiological pH α-synuclein, especially in mutated form, has the propensity to aggregate and form oligomer or fibrils [58]. Aggregated α-synuclein contributes to the

major component of the Lewy body [59], the pathological finding commonly seen in PD brains [60]. The oligomeric form of α-synuclein has a detergent-like property and forms pores in lipid membrane. This results in the increased permeability of cell membranes [61, 62], which can lead to cell death at severe extent. Aggregated α-synuclein can activate microglia and induces subsequent chronic inflammation in central nervous system, which is a well-recognized pathogenesis process of PD [63, 64]. It is also observed that aggregated α-synuclein directly induces apoptosis of dopamine neurons [65].

Previous genome-wide association studies [16 7, 9, 37, 38, 40, 41, 43, 66-70] have confirmed the role of α-synuclein in PD development. Our results are in agreement with previous knowledge. Specifically, we observed the deleterious effects at loci rs2736994, rs1812923, rs10005233, rs2301135, and rs2301134. Due to the fact that rs10005233, rs2301135, and rs2301134 were in pair wise LD, we chose to report rs2301135 to represent the genetic effects in this region. SNPs rs1812923, rs2301135 and rs2736994 exhibit additive effects, with an increase in the number of detrimental alleles (the minor allele A in rs1812923; the major allele G in rs2301135 and the minor allele C in rs2736994) associated with increasing likelihood of being a PD case in tibia lead models.

Lead Effect on PD by *SNCA* Variants

In addition to the findings on the main effect of the SNPs, we also observed significant interaction effects at loci rs1812923 and rs2301135. It is noteworthy that among all these loci, the increased odds of being a PD case from higher lead exposure were only present in subject with low genetic susceptibilities. In subjects with

homozygous deleterious alleles, elevation of bone lead level did not increase the odds of being a PD case. In some cases, increase in bone lead levels was negatively associated with odds of being a PD case. This result suggests that lead's impact on PD depends on genetic predispositions. It also suggests that lead exposure and genetic predisposition in *SNCA* gene does not have a synergistic effect on PD development. In addition, we did not observe that genotypes of *SNCA* were strongly associated with bone lead levels. This indicates that *SNCA* gene does not modify the toxicodynamics of lead.

The mechanism of lead exposure contributing to the risk of PD is not clearly understood. However, evidence suggests that lead could impact on the pathogenesis of PD via the oxidative stress mechanism, since lead is a known prooxidant. Lead induces oxidative stress by binding with δ-Aminolevulinic acid dehydratase (δ-ALAD) in erythrocytes [71, 72], inhibiting the reductase activities[73, 74], interfering with intracellular Ca^{2+} activities [75, 76] and inhibiting mitochondria functions [77, 78]. Dopamine neurons are particularly susceptible to oxidative stress in the physiological environment [79]. Dopamine and its metabolites generate highly reactive dopamine and DOPA quinones. DOPA quinnone is highly linked to current known pathogenesis of PD such as mitochondrial dysfunction, inflammation, oxidative stress[4], and dysfunction of the ubiquitin-proteasome system[5]. On the other hand, oxidative stress and dopamine itself has been shown to induce the α-synuclein aggresome, which, in turn, creates an unfavorable environment for the survival of dopaminergic neurons [56, 58, 80, 81].

On the other hand, Waalkes's et al has pointed out that the inclusion body that is mainly involved in lead-induced α-synuclein aggresomes, plays a protective role in

preventing further cellular disruptions from lead by sequestering lead in the metallothionein (MT)- lead- α-synuclein inclusion body[31, 32]. In their *in vitro* study[32], the researchers observed that in wild type MT mice cells, lead increased *SNCA* expression in a timely manner, which peaked at 24 hours, then subsided at 48 hours when lead-induced inclusion body is formed. They also observed that MT knock-out mice did not form lead inclusion bodies and accumulated less lead in kidney after lead exposures[31]. These mice exhibited dose-related nephromegaly, and their renal functions were significantly diminished after lead exposures[31].

Weighing the dual roles that α-synuclein plays in the context of lead- related PD, our results, to some extent, supported biological findings from animal studies. Our results showed an interactions between lead and *SNCA* genetic variants in such a pattern that increased lead was not associated with an increased odds of PD in subjects who were genetically susceptible to PD. We can postulate that in these subjects, α-synuclein, *per se,* may have a tendency to form aggresomes, thus creating unfriendly environments for dopaminergic neurons; while on the other hand, due to the inclusion body formation, it prevents lead interfering with cellular machineries and buffers lead's impact on neurons. Therefore, we observed that lead increased the odds of PD in subjects with less genetic susceptibility, possibly due to a decrease in inclusion body formation.

However, the interpretation of our results needs to be made with caution. Our results may not be put into direct comparisons with those from Waalkes et al's work in several respects. In the *in vitro* study, the cells were cultured with an exceedingly high dose of lead solution (200μM/L, approximately 4140.78 μg/dL), which could hardly be

achieved at physiological level in human beings. Not only is it impossible for a subject to be exposed at this high level environmentally, but it also has to be taken into consideration that due to the blood brain barrier protection, the biological dose of lead entered into neurons is substantially lower. Secondly, all observations from Waalkes et al's work were made in renal cells. Compared to neurons, inclusion bodies are more commonly observed in kidney cells. Thirdly, the researchers pointed out the crucial role of MT in the inclusion body formation process. Our study did not examine the effects of genetic variants in MT gene interacting with *SNCA* gene and lead exposure. It would provide great insights on disease mechanism if we could consider the potential modifying effect of MT gene into our study. Finally, the functions of these SNPs are unclear; we only postulated that the detrimental effect from these loci may be related to α-synuclein aggresome formation. Biological studies are needed to further confirm this contention.

In addition, our results should be placed in limited context. Over one third of the control subjects failed to enter into the genotype data. The sample size in the final dataset was substantially reduced in comparison to the parent study. Our sensitivity analysis showed lead effects contrasted by quartiles were underestimated compared to our previous report. In addition, the statistical power was substantially compromised due to the reduced sample size. Many of our results were significant only at the $p<0.05$ levels. Our results are preliminary and would need replication in other study settings.

In summary, this study considered the roles of markers from *SNCA* gene in dopaminergic system for Parkinson's disease. We examined the main influence of genotypes and lead exposures as well as their interactions on PD. Markers at

rs2736994, rs1812923, and rs2301135 significantly predicted PD status. Lead increased the odds of PD only among subjects who were less genetically susceptible. Subjects with higher genetic susceptibility were less affected by lead exposure. Our findings were consistent with current knowledge on genetic etiology of PD and our previous findings on main effect of environmental lead exposure. These results are clearly preliminary and are in need of replication. Future work is needed to better understand the pathogenesis of PD.

Tables

Table 3.1 Covariate Distributions in the Included and Exclude Subjects by PD Status

	Total Pool of Subjects[11]						Included in Gene-Environment Study						Excluded from Gene-Envrionment Study					
		Non-PD			PD			Non-PD			PD			Non-PD			PD	
	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD
Sex [N (%)]	297	0.56	-	375	0.37	-	207	0.66	-	328	0.37	-	90	0.32	-	47	0.38	-
Whites [N (%)]	281	0.84	-	354	0.96	-	192	0.85	-	307	0.96	-	89	0.83	-	47	0.96	-
Age at Pb measures	274	69.99	9.82	340	66.46	9.39	204	69.13	9.72	327	66.60	9.34	70	72.48	9.78	13	62.91	10.33
Birth Year	274	1936.90	9.77	340	1939.20	9.45	204	1937.63	9.63	327	1939.07	9.40	70	1934.77	9.94	13	1942.51	10.42
Education level	279	3.63	1.11	353	3.94	1.11	190	3.76	1.08	306	3.98	1.08	89	3.37	1.13	47	3.68	1.27
Years of Smoking	273	13.01	15.51	278	8.00	12.12	202	12.25	14.83	265	8.00	12.08	71	15.15	17.23	13	8.00	13.52
Pack -years	273	10.91	17.65	301	7.07	13.65	202	9.15	15.87	288	7.28	13.88	71	15.95	21.26	13	2.48	4.92
Age of PD Onset	0	-	-	209	57.67	12.06	0	-	-	203	57.65	12.09	0	-	-	6	58.52	12.21
Age at Diagnosis	0	-	-	224	59.93	11.07	0	-	-	217	60.06	10.99	0	-	-	7	56.21	13.66

[11] The pooled dataset contained fewer Normative Aging Study subjects compared to published data.

Table 3.2 Allele Frequency in Case and Control Groups

			Pooled		PD		Non-PD		Reference Population[12]	
Markers	A1	A2	A1	A2	A1	A2	A1	A2	A1	A2
rs356219	A	G	0.56	0.44	0.55	0.45	0.58	0.42	0.58	0.43
rs11931074	G	T	0.88	0.12	0.88	0.12	0.89	0.11	0.92	0.08
rs356221	A	T	0.54	0.46	0.55	0.45	0.53	0.47	0.47	0.53
rs356168	G	A	0.54	0.46	0.55	0.45	0.53	0.47	0.47	0.53
rs356188	A	G	0.79	0.21	0.81	0.19	0.76	0.24	0.84	0.17
rs356186	G	A	0.80	0.20	0.82	0.18	0.77	0.23	0.86	0.14
rs2737029	A	G	0.55	0.45	0.54	0.46	0.56	0.44	0.55	0.45
rs1812923	C	A	0.59	0.41	0.56	0.44	0.62	0.38	0.50	0.50
rs10005233	C	T	0.51	0.49	0.52	0.48	0.56	0.44	0.47	0.54
rs2301135	C	G	0.52	0.48	0.51	0.49	0.57	0.43	0.50	0.50
rs2301134	T	C	0.52	0.48	0.51	0.49	0.57	0.43	0.48	0.52
rs2619364	A	G	0.71	0.29	0.71	0.29	0.70	0.30	0.68	0.32
rs2583988	C	T	0.71	0.29	0.72	0.28	0.70	0.30	0.74	0.26
rs2736994	C	T	0.78	0.22	0.80	0.20	0.75	0.25	0.83	0.17

[12] Reference population source: HapMap-CEU

Table 3.3 Genotype Frequency between Cases and Controls

	Non-PD			PD			Pooled				
Markers	G0	G1	G2	G0	G1	G2	G0	G1	G2	*p* value	[13]
rs10005233	0.34	0.43	0.23	0.24	0.49	0.27	0.28	0.47	0.26	0.04	*
rs11931074	0.83	0.13	0.05	0.79	0.18	0.03	0.80	0.16	0.04	0.18	
rs1812923	0.17	0.41	0.41	0.20	0.48	0.33	0.19	0.45	0.36	0.12	
rs2301134	0.21	0.43	0.36	0.25	0.52	0.23	0.23	0.48	0.28	0.01	**
rs2301135	0.37	0.41	0.22	0.23	0.52	0.25	0.28	0.48	0.24	0.00	**
rs2583988	0.53	0.33	0.14	0.52	0.39	0.09	0.52	0.37	0.11	0.16	
rs2619364	0.52	0.34	0.14	0.51	0.40	0.09	0.52	0.37	0.11	0.16	
rs2736994	0.58	0.34	0.08	0.64	0.33	0.03	0.61	0.33	0.05	0.06	
rs2737029	0.32	0.47	0.21	0.29	0.50	0.21	0.30	0.49	0.21	0.68	
rs356168	0.24	0.46	0.30	0.23	0.43	0.34	0.23	0.44	0.32	0.76	
rs356186	0.07	0.31	0.62	0.03	0.30	0.67	0.05	0.31	0.65	0.05	*
rs356188	0.60	0.33	0.08	0.66	0.31	0.03	0.63	0.32	0.05	0.04	*
rs356219	0.37	0.41	0.22	0.33	0.43	0.24	0.34	0.43	0.23	0.60	
rs356221	0.30	0.46	0.24	0.34	0.43	0.23	0.32	0.44	0.23	0.70	

[13] *p value <0.05, ** p value <0.01 from Fisher's exact tests

Table 3.4 Markers' Main Effects and Interaction Effects from Tibia Lead Models[14]

	Main Effects of Markers			Lead x Markers Interaction Effects			No. of Observations in Each Group				
Markers	OR	LCI	UCL	OR	LCI	UCI	G0	G1	G2	PD	Non-PD
rs356219	0.86	0.43	1.75	1.04	0.98	1.11	162	205	115	298	184
	1.16	0.50	2.72	1.01	0.93	1.09	162	205	115	298	184
rs11931074	0.93	0.37	2.37	1.07	0.98	1.17	393	78	18	302	187
	6.76	0.77	59.48	0.86	0.73	1.02	393	78	18	302	187
rs356221	0.53	0.26	1.09	1.06	0.99	1.13	161	215	109	300	185
	0.68	0.28	1.66	1.04	0.96	1.12	161	215	109	300	185
rs356168	0.86	0.39	1.89	1.02	0.95	1.09	110	216	161	302	185
	1.57	0.65	3.79	0.96	0.89	1.04	110	216	161	302	185
rs356188	0.56	0.28	1.12	1.03	0.97	1.10	307	156	24	301	186
	0.35	0.09	1.41	1.01	0.88	1.16	307	156	24	301	186
rs356186	2.26	0.41	12.37	0.99	0.84	1.16	22	150	312	300	184
	4.85	0.94	25.10	0.94	0.80	1.10	22	150	312	300	184
rs2737029	1.09	0.53	2.24	1.01	0.95	1.08	144	240	104	302	186
	0.99	0.41	2.36	1.01	0.93	1.09	144	240	104	302	186
rs1812923	0.49	0.20	1.20	1.09	1.00	1.18	87	220	177	300	184
	0.31	0.12	0.75	1.08	1.00	1.18	87	220	177	300	184
rs10005233	1.56	0.77	3.14	1.03	0.97	1.10	137	228	123	302	186
	4.29	1.77	10.39	0.93	0.86	1.01	137	228	123	302	186
rs2301135	2.07	1.03	4.15	1.03	0.96	1.10	140	230	115	300	185
	5.74	2.26	14.57	0.91	0.84	0.99	140	230	115	300	185
rs2301134	0.38	0.16	0.89	1.11	1.02	1.20	113	234	140	301	186
	0.21	0.08	0.53	1.07	0.99	1.17	113	234	140	301	186
rs2619364	0.83	0.43	1.62	1.05	0.98	1.11	249	180	53	298	184
	0.55	0.22	1.38	1.00	0.91	1.09	249	180	53	298	184
rs2583988	0.85	0.44	1.64	1.04	0.98	1.10	254	179	53	301	185
	0.57	0.23	1.42	1.00	0.92	1.09	254	179	53	301	185
rs2736994	0.45	0.23	0.90	1.06	0.99	1.12	296	164	25	301	184
	0.31	0.08	1.17	1.05	0.92	1.19	296	164	25	301	184

[14] All markers were treated as categorical variables, no inherit mode was assumed, G0 as reference group. The tibia lead levels were treated as continuous variable. Unconditional Maximum likelihood (UML) method was applied.

Table 3.5 Markers' Main Effects and Interaction Effects from Patella Lead Models[15]

	Main Effect of Markers			Lead x Markers Interaction Effect			No. of Observations in Each Group				
Markers	OR	LCI	UCI	OR	LCI	UCI	G0	G1	G2	PD	Non-PD
rs356219	1.08	0.54	2.17	1.01	0.96	1.06	160	200	113	293	180
	0.86	0.37	1.99	1.03	0.97	1.09	160	200	113	293	180
rs11931074	0.87	0.36	2.10	1.07	1.00	1.14	385	78	17	297	183
	9.33	0.90	97.17	0.88	0.76	1.02	385	78	17	297	183
rs356221	0.68	0.33	1.40	1.02	0.97	1.07	158	211	107	295	181
	1.00	0.43	2.33	0.99	0.94	1.05	158	211	107	295	181
rs356168	0.80	0.37	1.71	1.02	0.96	1.07	108	212	158	297	181
	1.06	0.46	2.46	1.00	0.95	1.06	108	212	158	297	181
rs356188	0.93	0.48	1.81	0.98	0.93	1.02	301	153	24	296	182
	0.45	0.09	2.26	0.98	0.88	1.09	301	153	24	296	182
rs356186	2.79	0.46	16.77	0.98	0.87	1.10	22	147	306	295	180
	3.03	0.53	17.31	1.00	0.90	1.12	22	147	306	295	180
rs2737029	1.02	0.50	2.07	1.01	0.97	1.07	142	235	102	297	182
	0.65	0.27	1.56	1.05	0.98	1.11	142	235	102	297	182
rs1812923	0.70	0.30	1.67	1.03	0.97	1.10	87	213	175	295	180
	0.42	0.17	1.04	1.03	0.97	1.10	87	213	175	295	180
rs10005233	2.02	0.98	4.16	1.00	0.95	1.05	135	222	122	297	182
	2.80	1.17	6.70	0.99	0.93	1.05	135	222	122	297	182
rs2301135	2.96	1.43	6.10	0.99	0.94	1.04	138	224	114	295	181
	3.97	1.61	9.79	0.96	0.91	1.02	138	224	114	295	181
rs2301134	0.71	0.31	1.63	1.02	0.96	1.08	112	228	138	296	182
	0.29	0.12	0.70	1.03	0.96	1.09	112	228	138	296	182
rs2619364	0.85	0.43	1.66	1.03	0.99	1.08	246	176	52	294	180
	0.39	0.14	1.03	1.03	0.96	1.11	246	176	52	294	180
rs2583988	0.84	0.43	1.63	1.03	0.98	1.08	250	175	52	296	181
	0.39	0.15	1.03	1.04	0.96	1.11	250	175	52	296	181
rs2736994	0.81	0.41	1.57	0.99	0.95	1.04	291	160	25	296	180
	0.41	0.11	1.63	1.00	0.91	1.11	291	160	25	296	180

[15] All markers treated as categorical variables; no inherit mode was assumed; G0 as reference group. The patella lead levels were treated as continuous variable. Unconditional Maximum likelihood (UML) method was applied.

Table S 3.1 Tibia Lead Main Effects in the Final Dataset

All sites (n=531)[16]						All hospital sites (exclude HCPOA) (n=400)[17]					
Tibia Lead (µg/g)	Non-PD	PD	OR	LCI	UCI	Tibia Lead (µg/g)	Non-PD	PD	OR	LCI	UCI
≤1.74	53	84	-	-	-	≤1.74	26	84	-	-	-
1.74-7.83	54	83	1.32	0.73	2.40	1.74-7.83	18	83	1.32	0.62	2.81
7.83-13.92	48	89	1.51	0.83	2.77	7.83-13.05	15	77	1.50	0.69	3.28
>13.92	49	71	1.66	0.88	3.15	>13.05	14	83	1.73	0.77	3.87
p-Trend			0.10						0.16		

[16] The final dataset for gene-environment interaction models

[17] The final dataset for gene-environmental interaction models excluding the control subjects recruited from Harvard Cooperative Program on Aging (HCPOA)

Table S 3.2 Gene-Environment Independence Test Results

	Tibia Lead Models						Patella Lead Models					
	G1			G2			G1			G2		
Markers	θ_{GE}	*p* value	[18]	θ_{GE}	*p* value		θ_{GE}	*p* value		θ_{GE}	*p* value	
rs10005233	0.002	0.90		0.035	0.06	.	0.012	0.37		0.009	0.59	
rs11931074	-0.016	0.43		0.062	0.04	*	-0.004	0.82		0.032	0.20	
rs1812923	-0.019	0.34		-0.026	0.20		-0.006	0.71		-0.009	0.60	
rs2301134	-0.034	0.07	.	-0.037	0.05	.	0.004	0.78		-0.014	0.40	
rs2301135	0.002	0.91		0.051	0.01	*	0.018	0.21		0.029	0.07	.
rs2583988	-0.029	0.06	.	-0.016	0.45		-0.008	0.53		-0.048	0.02	*
rs2619364	-0.031	0.04	*	-0.020	0.33		-0.011	0.43		-0.044	0.03	*
rs2736994	-0.015	0.30		-0.021	0.41		0.015	0.25		-0.007	0.77	
rs2737029	-0.009	0.58		-0.003	0.89		-0.001	0.92		-0.014	0.42	
rs356168	-0.007	0.70		0.023	0.22		-0.002	0.89		0.016	0.33	
rs356186	0.002	0.95		0.016	0.55		-0.007	0.75		-0.021	0.34	
rs356188	-0.005	0.73		-0.001	0.98		0.013	0.32		0.028	0.17	
rs356219	-0.024	0.13		0.005	0.79		0.006	0.64		-0.009	0.59	
rs356221	-0.032	0.05	*	-0.019	0.32		-0.020	0.14		-0.014	0.38	

[18] * $p<0.05$, . $p<0.1$

Table S 3.3 Standardized Coefficients of Main Effects of Bone Lead from Gene-Environment Interaction Models

	Tibia Lead Models			Patella Lead Models		
Markers	Std. OR	Std. SE	*p* value	Std. OR	Std. SE	*p* value
rs356219	0.01	0.20	0.94	-0.02	0.20	0.91
rs11931074	0.17	0.12	0.17	0.04	0.13	0.77
rs356221	-0.03	0.19	0.86	0.00	0.17	0.98
rs356168	0.15	0.22	0.52	-0.07	0.23	0.78
rs356188	0.13	0.14	0.37	0.21	0.15	0.15
rs356186	0.13	0.55	0.81	0.00	0.51	1.00
rs2737029	0.09	0.20	0.64	-0.16	0.21	0.43
rs1812923	-0.43	0.30	0.16	-0.19	0.26	0.47
rs10005233	0.14	0.20	0.48	0.17	0.22	0.43
rs2301135	0.18	0.20	0.37	0.29	0.22	0.19
rs2301134	-0.58	0.31	0.06	-0.16	0.27	0.56
rs2619364	0.03	0.16	0.85	-0.15	0.16	0.33
rs2583988	0.07	0.15	0.63	-0.15	0.16	0.35
rs2736994	0.04	0.14	0.76	0.14	0.14	0.33

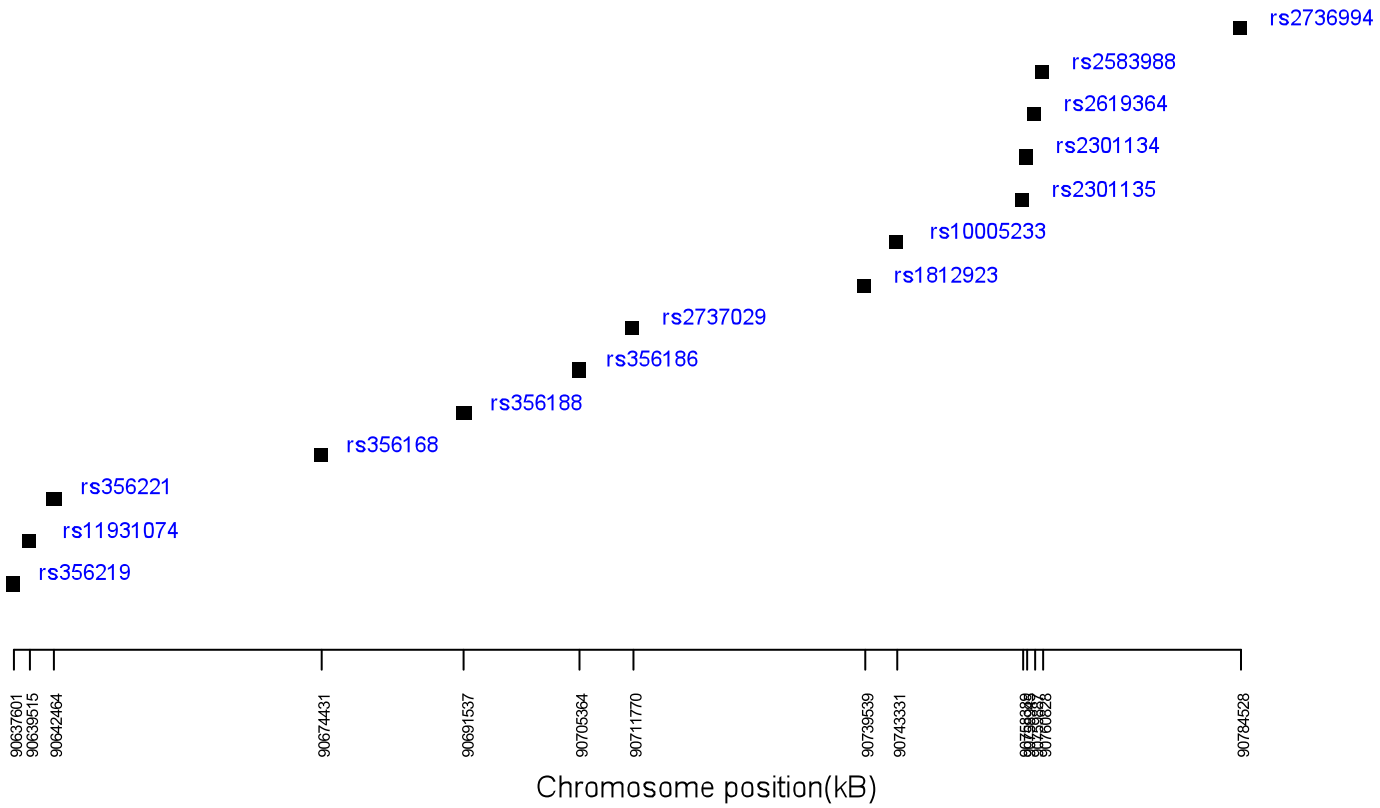
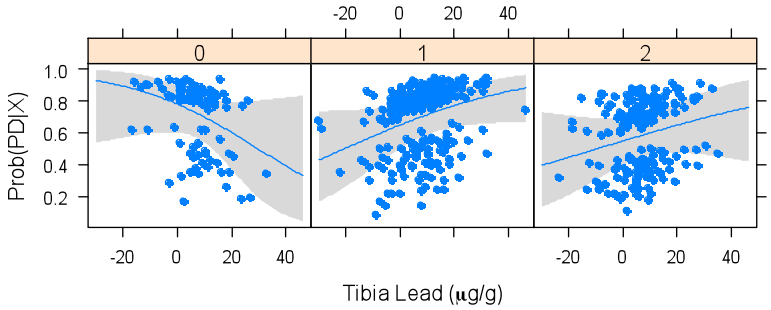


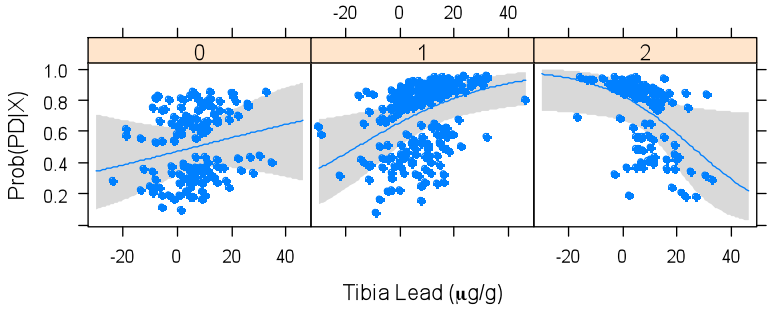
Figure 3.1 Physical Distance of 14 Markers in *SNCA* Gene

Tibia Lead Models

rs1812923

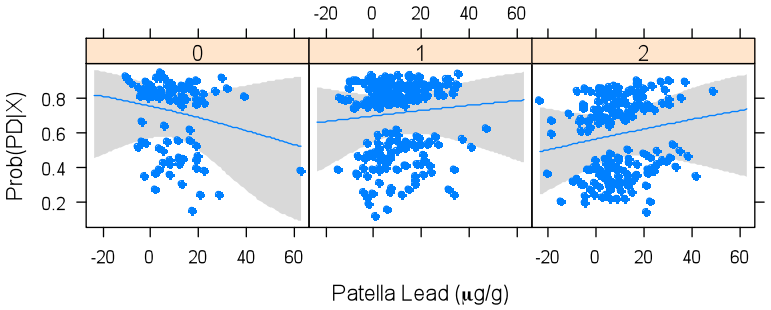


rs2301135



Patella Lead Models

rs1812923



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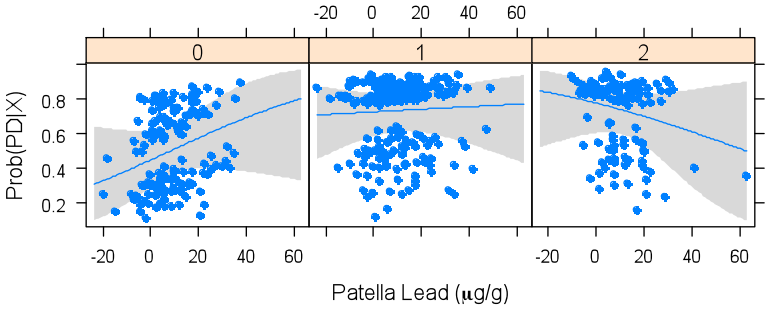

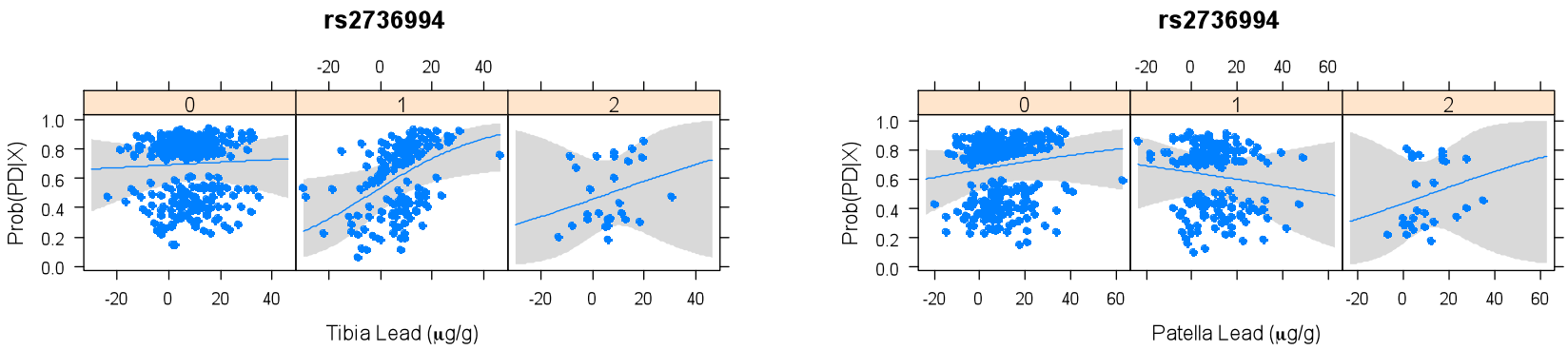

Figure 3.2 Bone Lead Levels and Probability of PD by *SNCA* Genotypes

Caption: X-axis: the bone lead levels in tibia and patella. Y-axis: the probability of being a Parkinson's disease case.

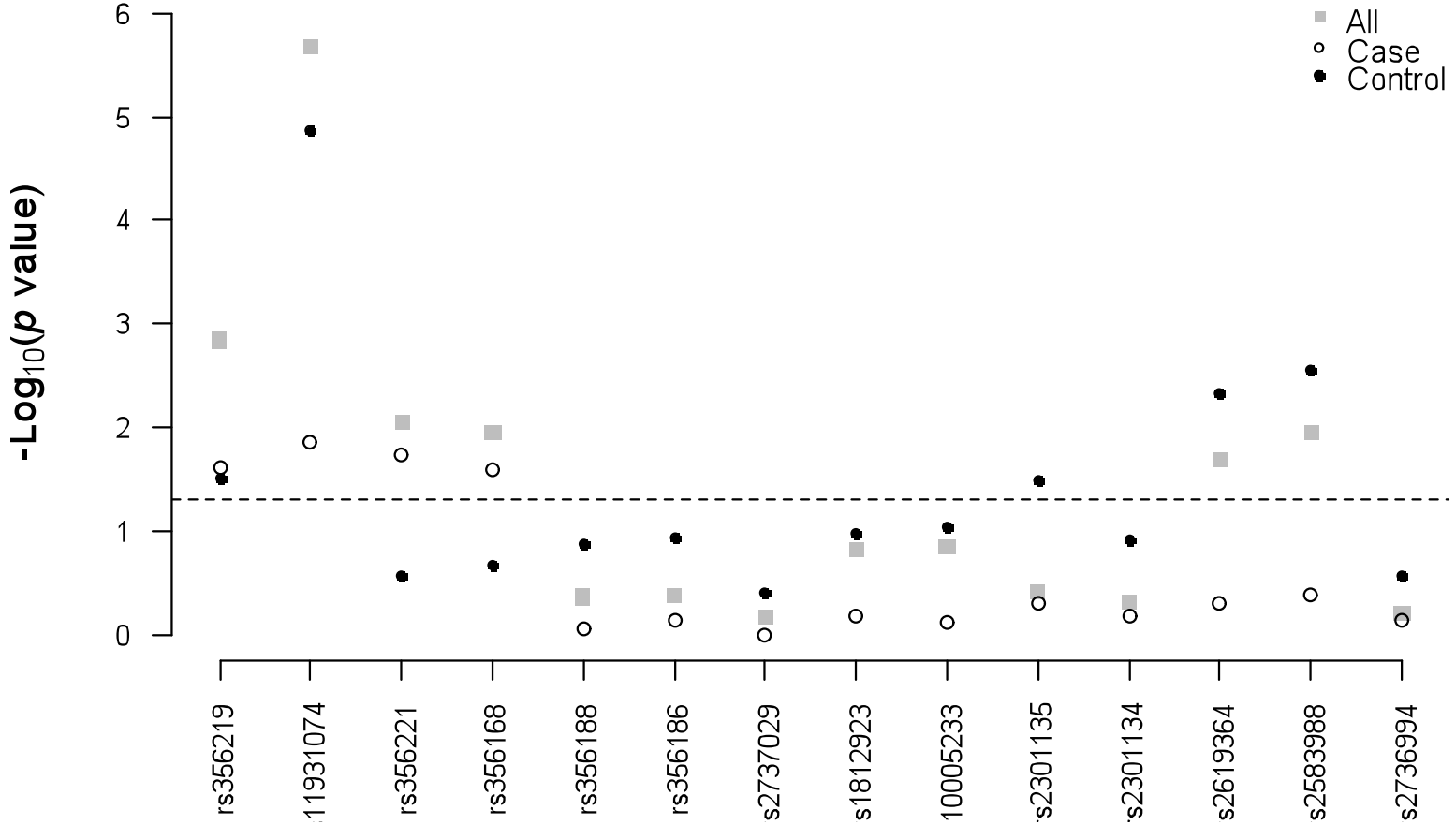

Figure S 3.1 –Log$_{10}$ (*p* value) from Hardy-Weinberg Tests

Caption: The dotted line corresponded to p =0.05

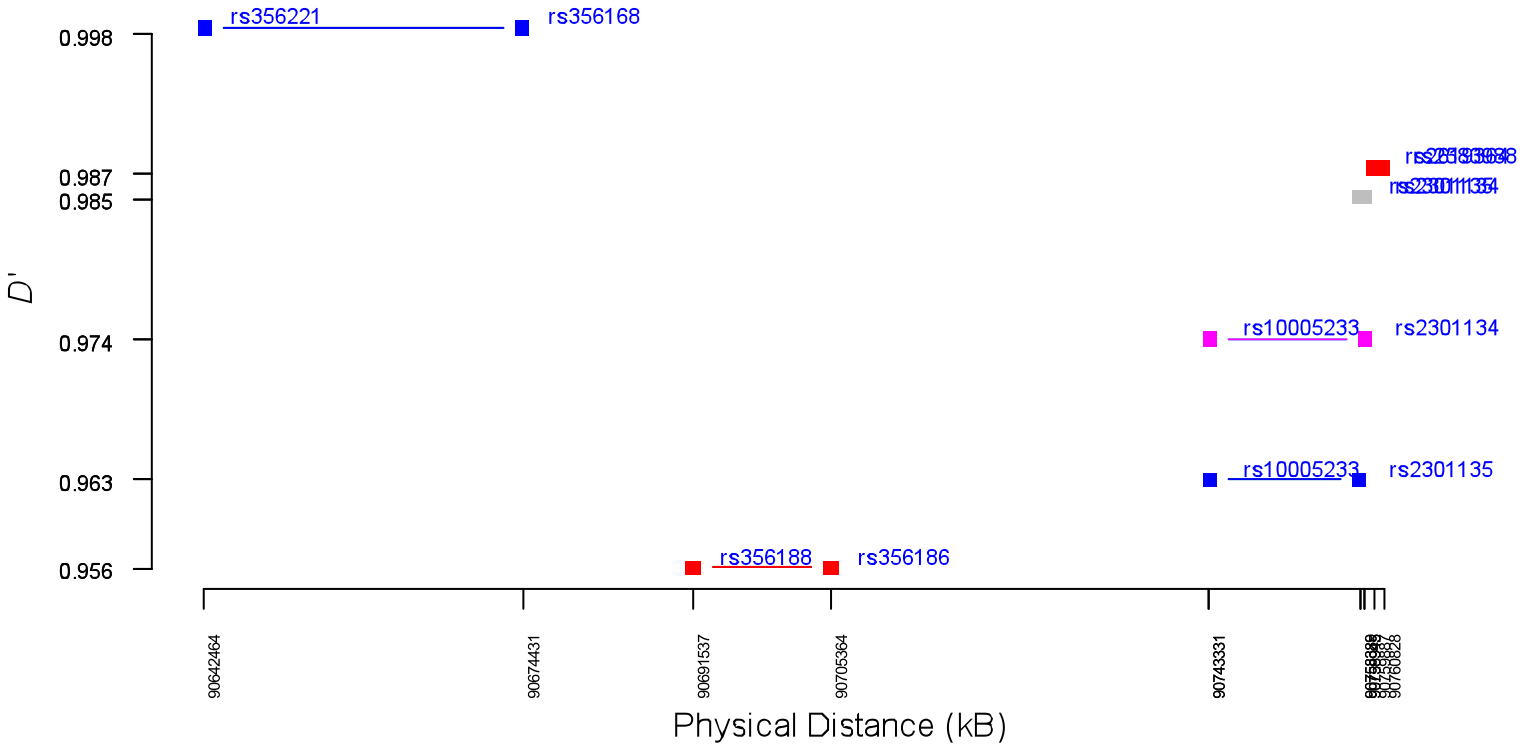

Figure S 3.2 High Linkage Disequilibrium Pairs

Caption: High Linkage Disequilibrium was defined as r^2 >0.8 and D' >0.9

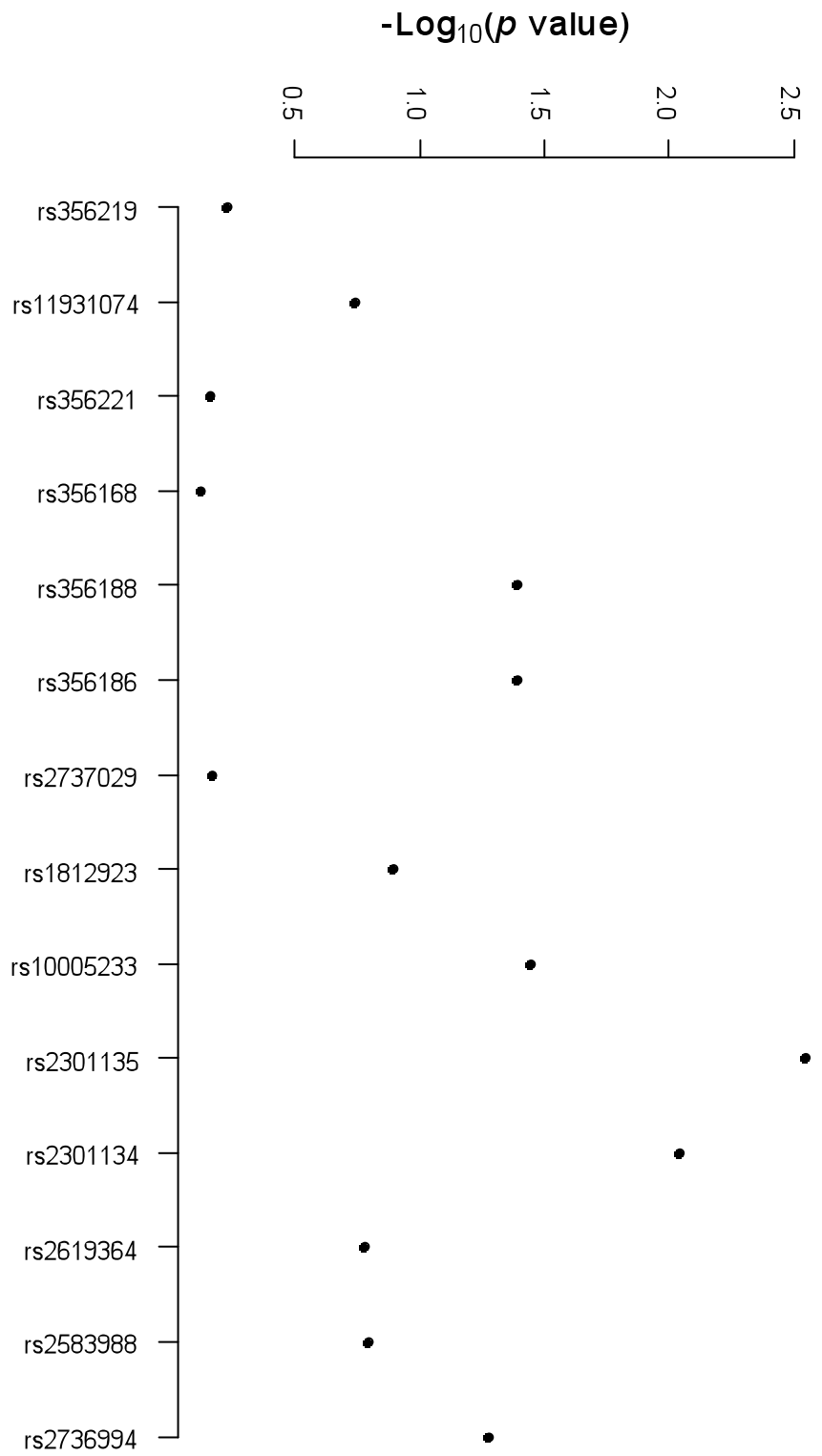

Figure S 3.3 –Log$_{10}$ (*p* Value) of Fisher Exact Tests at 14 Loci

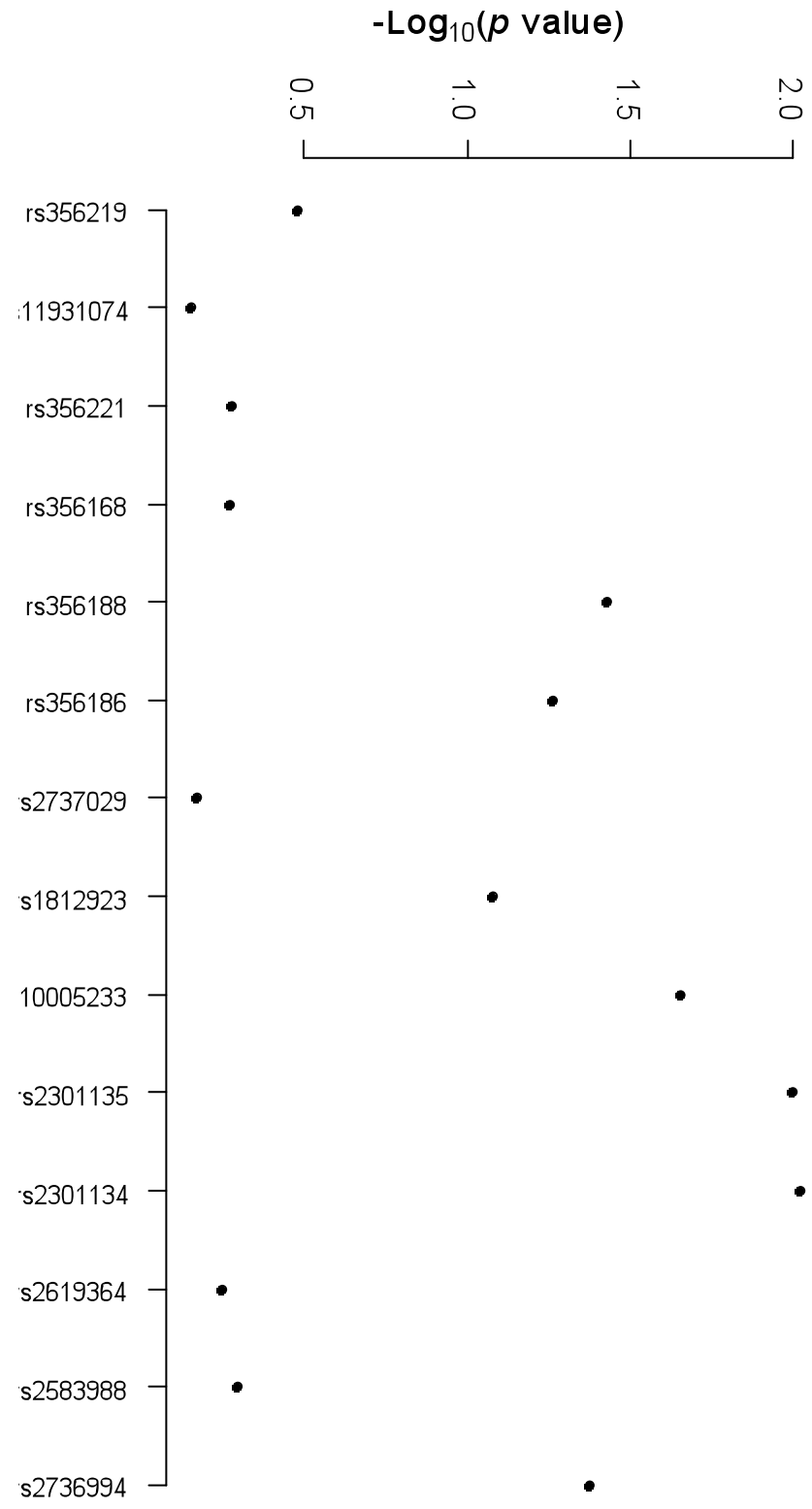

Figure S 3.4 –Log$_{10}$ (*p* Value) of Cochran-Armitage Tests at 14 Loci

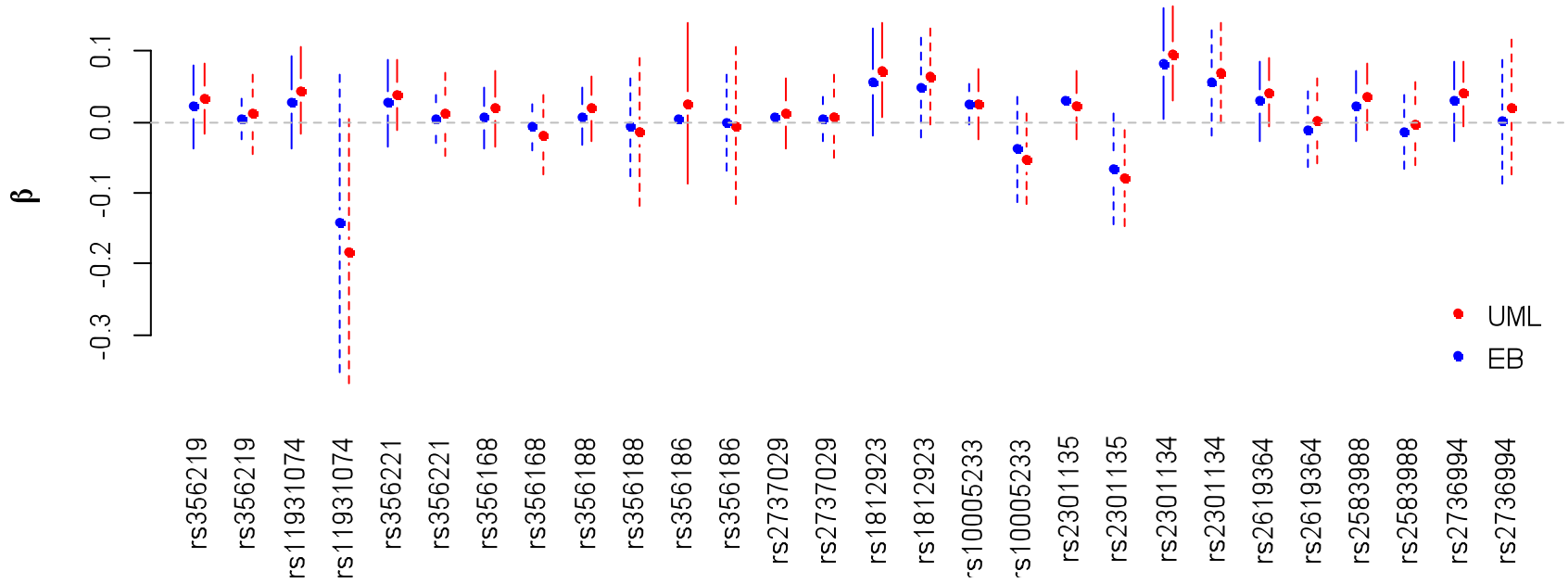

Figure S 3.5 Interaction Effect Estimates in Tibia Lead Models (Unconditional Maximum Likelihood vs. Empirical Bayes Likelihood Estimates)

Caption: Solid line - G1 group; dotted line - G2 group. Red - unconditional maximum likelihood (UML) estimates; blue - empirical bayes (EB) maximum likelihood estimates. The coefficients lack of 95%CI due to that the standard errors were unable to derive with the corresponding method.

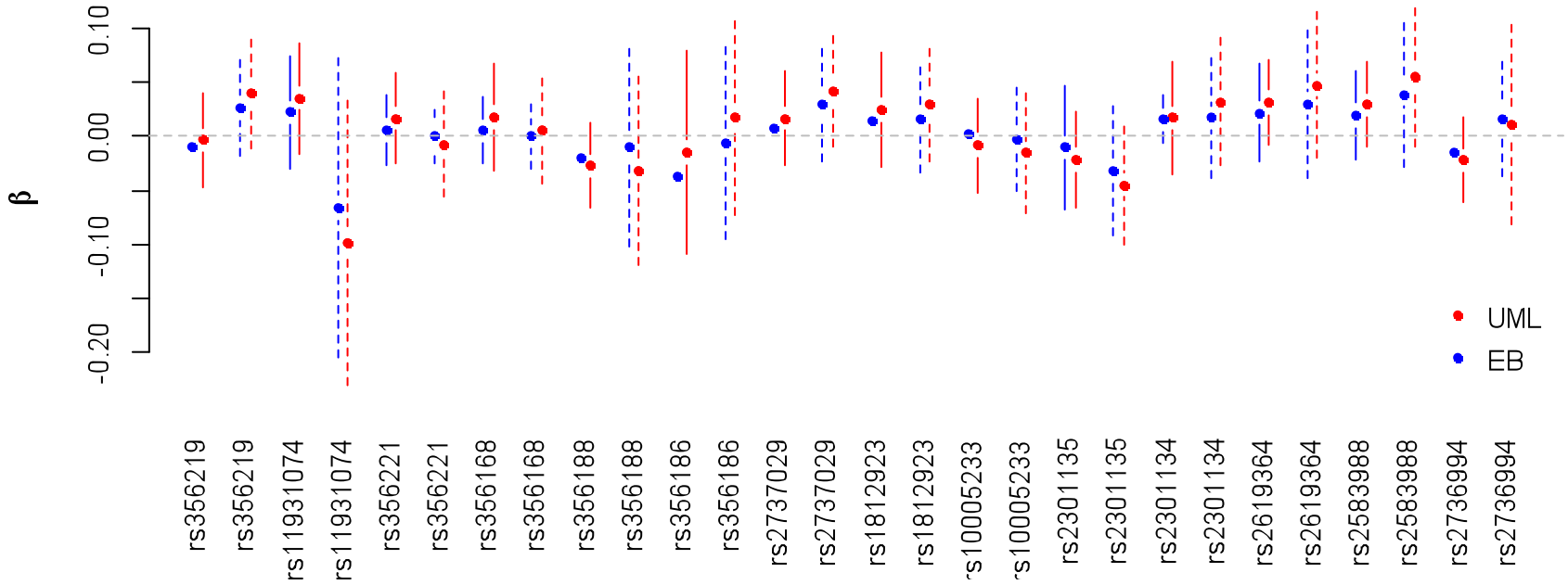

Figure S 3.6 Interaction Effect Estimates in Patella Models (Unconditional Maximum Likelihood vs. Empirical Bayes Likelihood Estimates)

Caption: Solid line - G1 group; dotted line - G2 group. Red - unconditional maximum likelihood (UML) estimates; blue - empirical bayes (EB) maximum likelihood estimates. The coefficients lack of 95%CI due to that the standard errors were unable to derive with the corresponding method.

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CHAPTER 4 CUMULATIVE EXPOSURE TO LEAD AND INCIDENCE OF TYPE-2 DIABETES: THE VA NORMATIVE AGING STUDY

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by insulin deficiency from loss of pancreatic beta cell or insulin resistance in peripheral tissues. Type 2 diabetes mellitus (T2DM) is a dominant subtype of DM. Oxidative stress induced endoplasmic reticulum (ER) distress and systemic inflammation are the major pathogenic processes involved in the development of T2DM [1 2 3 4]. Insulin deficiency or resistance can cause hyperglycemia. If untreated, hyperglycemia can lead to ketoacidosis or endothelial infraction, which can cause cardiac and renal function impairments[5, 6]. The incipient stage of T2DM is characterized by hyperglycemia, polyuria, polydipsia, polyphagia and weight loss. Early stage T2DM patients typically are overweight, with BMI greater than 25, or have excessive body fat around abdominal region[7, 8].

According to the Centers for Disease Control and Prevention, there were 1.9 million incident cases of T2DM in people aged 20 or older in 2010[8]. 18.8 million prevalent T2DM cases were recorded, which approximately 8.3% of the US population in year 2010. Furthermore, 7 million additional people were suspected to be undiagnosed and an estimated 79 million were pre-diabetic. Specifically 11.3% of population in age 20 and older and 26.9% of population in age 65 and older were suspected diabetic[8]. The T2DM incidence increased from 1.1 million in 2000 to 1.7

million in 2010[9]. Even with increasing attention and interventions directed towards diabetes prevention, the incidence rate is still increasing. In 2001, CDC predicted that by the year 2050 the national DM prevalence would be 7.9% of the population, an estimated 29 million DM cases[10]. However the growth of DM in recent years has exceeded the predictions. Along with this rapid increase in incidence, the financial burden associated with diabetes healthcare and treatment for related complications also increased concurrently[11].Therefore, in order to understand and control the epidemic of T2DM, more knowledge on the etiology of T2DM are needed.

Genetic inheritance plays a role in the development of T2DM[12]. However, genetics alone cannot fully explain the emerging epidemic of T2DM observed in the recent decades, as genetic drift would need a long period to manifest phenotypes at the population level. Unhealthy diet, physical inactivity, obesity, aging, race, cigarette smoking and poor nutrition during pregnancy are well-known non-genetic risk factors for T2DM [13, 14]. Growing evidence has shown that environmental chemical exposure is another non-negligible aspect in the etiology of T2DM. Heavy metals, such as arsenic and cadmium, can exacerbate oxidative stress and trigger inflammation processes creating an environment poorly suited for the survival of pancreas cells (examples: Arsenic [15-17], cadmium [18, 19] nickel and mercury[20]).

Despite the established link between exposure to heavy metals and T2DM, few studies have been conducted to explore the role of environmental lead exposure in the etiology of T2DM. Lead is a pro-oxidant divalent metal[21-24] that can induce oxidative stress through direct impact on cell membranes[23], interactions with δ-aminolevulinic acid dehydratase[24] and inhibition of reductase functions [25] and mitochondria [26, 27]. Lead

is known to disrupt endocrine functions, especially estrogen metabolism[28] . Therefore, it is biologically plausible to hypothesize that lead exposure may contribute to the T2DM risk. We examined the association between cumulative exposure to lead, as measured in bones, and incident T2DM in a community-based cohort of men.

Methods

Study Design

This analysis is nested in the Normative Aging Study (NAS), which was initiated by a multidisciplinary investigation team in early 1960's. The study was established in order to study the process of healthy aging. Each individual in the study undergoes a series of physical examinations in the following domains: biochemistry, clinical medicine, oral medicine, neurology, anthropometry, psychology and sociology. Clinical examinations were performed every five years in subjects younger than 50 and every three years in subjects 50 and older. Bone lead measurements were conducted from 1991 to 2002, with up to four consecutive measurements at tibia and patella bone sites. A graphical representation of exposure assessment and health outcome measurements is presented in Figure 4.1.

Study Population

The study was initially constituted of veterans from the Spanish-American war and later shifted to veterans who served in World War II. Subjects were enrolled through Veteran Administration outpatient clinics in Boston, Massachusetts. In 1972, an active cohort of 2032 subjects was retained in the study. Subjects were followed from enrollment until either death or withdrawal. Attrition rate did not exceed more than one

percent a year in the early study stage. Subjects in the study were selected to represent a wide range of socioeconomic classes and were ensured to be geographically stable. In the early stage of the study, the participants were mainly middle aged, with 35 percent of subjects aged less than 40, and 60 percent aged between 40 and 59. Additionally, this study excluded participants with pre-existing health conditions with stringent criteria. For example, subjects with blood pressure higher than 140/90 mmHg were excluded from the study, which led to 50% of initial elderly subject exclusions. As a consequence, the remainder in the study are relatively healthier compared to the group being filtered out [29]. This selection approach can lead to a 'healthy subject' bias, which was foreseen by the researchers[30]. The initial recruitment included a wide range of social economic classes. Among the recruited participants, 14 percent had less than high school education, 25 percent were high school graduates, 35 percent were educated beyond high school and 26 percent were college graduates. This population structure shifted upwards with additional recruitment along with the upgrading of the educational system in recent decades. Occupations in the original study were evenly spread among managers, professionals, clerks, craftsmen, as well as service workers and with minor representation of operatives and laborers. However, at the initial stage, only 2% of the study participants were African-Americans.

The current analyses were based on this existing cohort, targeting the subpopulation with bone lead measurement available. A total of 878 subjects with valid bone lead measurements were included in the current data set. The majority of the subjects were born in the middle1920's.

Bone Lead Assessment

Bone lead levels were measured using K shell X-ray fluorescence (K-XRF) approach at the Harvard University/Channing lab. Subjects who consented to lead measurements were asked to undergo a series of bone lead measurements at each clinic visit. The two bone sites were selected to represent the cortical and trabecular bone types. The tibia midpoint was selected to reflect lead burden in cortical bones; whereas patella was selected to reflect lead burden in trabecular bones. Due to low blood exchange rate, cortical bones have lower lead decay rate compared to trabecular bones therefore lead measured from cortical bones more accurately reflect the past history of lead exposures. Tibia lead reflects the retrospective lead exposures of the previous 14 to 16 years [31]. Trabecular bones have higher exchange rate with blood compared to cortical bones and are the main contributors to endogenous lead exposure. In our analysis, some T2DM incidental cases occurred before the lead measurement. However as these two bone sites have good retention of lead and can refer to the exposure at retrospective time points, we decided to use the first bone lead measurement as a proxy of past lead exposure. To further clarify the temporality of lead effects with the information from two bone types, we obtained information about 24 hour urinary g-crosslinked N-telopeptides of type I collagen (NTx) as indicator of bone turnover.

Type 2 Diabetes Identification

The determination of T2DM cases in this study population was based on the American Diabetes Association standard published in 2002[7]. Here, the incident cases of

T2DM met any of the following criteria: Single observation of plasma fasting glucose level greater than or equal to 126 mg/dL; current or ever anti-diabetic medication use indicated in the questionnaire; medical record of the diagnosis indicated in the questionnaire; or a 2 hour standardized glucose tolerance test value ≥ 200 mg/dL. All the identified cases were classified as T2DM with cases evaluated at each clinical visit.

Other Information

Factors with regard to gaining effect estimates precisions or to controlling for confounding effect were body mass index (BMI), current smoking status, physical activity, educational level, and birthdates. This information was obtained via a series of questionnaires that were applied at the recruitment and during the follow-up clinical visits. BMI were derived from weight in kilogram divided by height in meter squared measured at each clinical visit.

Urinary g crosslinked N-telopeptides of type I collagen (NTx) levels were measured using archived 24-hour urine samples collected in 1987. NTx concentrations were measured via commercially available competitive-inhibition enzyme-linked immunosorbent assay[32]. Urinary NTx concentration was normalized to urinary creatinine secretion and is expressed as of nanomoles of bone collagen equivalents per mmol creatinnine (nM BCE/mM creatinine).

Statistical Analyses

Univariate and Bivariate Analyses

All statistical analysis was performed in R version 2.15 [33]. The R *survival* package was applied for survival analysis [34]. Univariate analyses were performed to examine the distribution of variables of interest. Each bone lead marker was first used as a continuous variable to examine a potential dose-response relationship or deviation from linearity. For the purpose of significance testing, bone lead levels were categorized at the 25th and the 75th percentile. Covariates were determined based on biological relevance of confounding effects, and includes educational level, calorie consumption from physical activity (kcal/wk), current smoking status, birth date and BMI. Physical activity was log-transformed due to right skewed distribution. Educational level was categorized into less than high school, high school graduates, and higher than high school groups. Subjects were grouped into three cohorts based on birthdates information: born before 1920, between 1920 and 1930, and after 1930.

Bivariate analyses were conducted to compare the distributions of covariates in the exposure groups or by the T2DM status. Additionally, to examine bias introduced by potential differential attrition in the subset with bone lead information, comparisons between baseline health status and health behaviors were made between subjects with and without bone lead measures by diabetes status observed later in the study. Comparisons of T2DM biomarkers, blood pressure and health behaviors were conducted among lead exposure groups and among the three birth cohorts.

Survival Analyses

Nelson-Aalen estimators were constructed and graphed for crude comparisons among exposure groups as well as the covariates of interest. Cox's proportional hazard (PH) model using the semi-parametric maximum likelihood estimation method was

applied for model construction. Using age as the time scale, we tested the lead effect on incident T2DM with two choices of modeling with regard to handling 'healthy subject' effect- hereon referred to survivor effects. First we used stratified analysis by birth cohort as recommended by Korn et al [35]. The lead effects were estimated conditional on arbitrary survival functions of three birth cohorts (Model 4.1). This method does not assume any type of probability distribution of birth cohort variable. Alternatively, we applied a gamma frailty model (Model 4.2) for effect estimates. This model assumes the survival function as independent among three birth cohorts while being correlated within the cohorts. b_j denotes the frailty parameter, which is the random effect of birth cohort variable with three levels and follows the gamma distribution. Coefficients β in model 4.2 estimates conditional hazard ratios of variable Z in a given birth cohort. Maximum likelihood of the variance of random effect was derived using Expectation–Maximization algorithm[36]. In addition, the test for clustering b_j is given by a score test, which is robust to distribution assumptions of the frailty term. All of the survival analyses were performed in a longitudinal data format. The time scale was defined as age at clinical visit during follow-up period. BMI and smoking status were treated as time varying variables whereas the rest of covariates were time invariant. All test results were considered as significantly different from the null hypothesis at $p< 0.05$.

$$\lambda_{ij}(a_{start}, a_{stop} | Z, b_0 \in B_j) = \lambda_{0ij}(a)\exp(\sum_k \beta_k^{\mathrm{T}} Z_{ij} + \sum_p \beta_p^{\mathrm{T}} Z_{ij}(a)) \quad (4.1)$$

$$\lambda_{ij}(a_{start}, a_{stop} | Z, b_0 \in B_j) = b_j \lambda_{0i}(a)\exp(\sum_k \beta_k^{\mathrm{T}} Z_{ij} + \sum_p \beta_p^{\mathrm{T}} Z_{ij}(a)) \quad (4.2)$$

Caption: *i*- individual 1, 2…n; *j*- 1, 2, 3- levels in *b*; *b*- birth cohorts; *Z*, exposure and/or covariates of interest; *a*- age at clinic checkup; *p*, number of parameters of time-varying covariates; *k*, number of parameters of time invariant covariates.
Model 4.1: stratified survival model, b_i as strata term.
Model 4.2: gamma frailty model, with b_i as frailty term, which follows a gamma distribution.

Without prior knowledge on dose response relationship, decisions have been made to categorize tibia and patella lead levels at 25th and 75th percentile for the significance tests. Penalized spline smoothing methods were applied to visually examine the dose-response relationships.

Model fit was diagnosed by examinations of Martingale residuals and Schoenfeld residuals methods[36].The PH assumptions were tested by visual examination and by the test of constancy provided in the R *survival* package, which is based on scaled Schoenfeld residuals and indicates violation of PH assumption at $p< 0.05$. Influential data points were detected by identifying delta beta greater than $2/\sqrt{n}$, which equaled to 0.33 in our analyses. Test of significance of frailty parameter θ (variance) was performed using score test against null hypothesis that θ=0.

Sensitivity Analyses

We examined the effects of lead stratified by urinary NTx level analyses. Urinary NTx level was dichotomized at the median. Two sets of models were fitted independently in low (less or equals to the median) and high (higher than the median) urinary NTx groups. Covariates were chosen as the same in main hypothesis models.

Results

During a median of 15.58 follow-up years, 230 out of 878 subjects who had bone lead measurement developed T2DM. Table 4.1 shows baseline comparisons of characteristics between subjects with and without bone lead measurement by T2DM status. In general, compared to subjects without bone lead measurements, subjects who underwent bone lead measurements were younger. There were fewer smokers in

the bone lead measurement group. Further comparisons in T2DM subjects with regard to bone lead measurement statuses showed that subjects with bone lead measurement in general had late onset of T2DM, but tested worse on 2-hour glucose tolerance tests, and exhibited higher BMI with a corresponding higher prevalence of chronic diseases (data not shown).

For subjects with bone lead measurements, we compared T2DM biomarkers and health behaviors among the three birth cohorts (Table 4.2). BMI gradually decreased from the young to the old cohorts. The younger cohorts had earlier T2DM onset: the averages of the onset age were 57.9 years (SD: 8.89) in the young, 64.89 years (SD: 10.37) in the middle, and 68.3 years (SD 9.4) in the old cohorts, respectively. The proportions of current or ever smokers were higher in younger groups. The oldest cohort showed lower values on lipid and glucose metabolism tests.

Table 4.3 shows the distributions of diabetic markers and health behaviors by patella bone lead levels among diabetic subjects. Patella bone lead levels were categorized at the 25^{th} and 75^{th} percentiles, which corresponded to 18 μg/g and 39 μg/g, respectively. Compared to the lower 25^{th} percentile group, subjects in the higher patella lead groups were older, yet had lower fasting glucose and cholesterol levels. Late onset of T2DM was observed in the higher patella lead level groups. Heavier smokers were more frequently observed in higher patella lead groups. Similar results were found in tibia lead groups (data not shown).

Table 4.4 shows the comparisons on effect estimates of bone lead with different choices of adjustments in the models. The effect estimates reached statistical

significance in the most of the patella lead models. Patella lead manifested a ceiling effect with respect to the risk of T2DM. It showed that the medium patella lead group had higher risk than the high patella lead group. The directions of effect estimates from these modeling approaches were consistent. Compared to the birth cohort stratified analysis, gamma frailty modeling did not improve statistical efficiency drastically. However, the result from score test confirmed significance of dependence in the frailty term. The magnitude of patella lead effects was increased in the models with adjustment on birth cohort. In the tibia lead models, the effect estimates oscillated around null and none of the models found a statistically significant effect of tibia lead.

Table 4.5 showed the result from the final models using gamma frailty modeling method. Compared to the lower 25^{th} percentile bone lead group, the group in 25^{th} to 75^{th} percentile bone lead group had hazard ratios (HRs) of 1.57 (95%CI: 1.26, 1.94) in the patella lead model and 1.02 (95%CI: 0.83,1.25) in the tibia lead model. The HRs estimated from both patella and tibia lead models showed non-linear patterns in penalized spline smoothing plots (Figure 4.3, 4.4). In the patella lead model, the lead effect appeared to plateau at 30 μg/g, whereas the effect of tibia lead did not exhibit drastic increase at low levels and slowly declined after around 30 μg/g.

Current smoking status was positively associated with risk of T2DM. Compared to current nonsmokers, the HR for a current smoker was 1.58, (95% CI: 1.17, 2.13) in patella lead model and 1.64, (95% CI: 1.22, 2.21) in tibia lead model. Physical activities showed a protective effect of T2DM in both models. Increase in BMI was positively associated with T2DM with HR of 1.11, (95% CI: 1.09, 1.13) in patella lead model and

1.10, (95% CI; 1.08, 1.12) in tibia lead model. Educational level did not show significant impacts on T2DM risk in any of the models.

After stratifying by urinary NTx levels, lead effect in the low NTx group showed an increased dose-response relationship, while in the higher NTx group lead effect estimates showed a reverse U-shape relationship (Table S 4.1). None of the lead effect estimates reached to statistical significance.

Discussion

In this longitudinal analysis with data on cumulative lead exposures, we found a significant association of incident T2DM with patella lead but not with tibia lead. Our analyses adjusted for smoking status, educational level, BMI, physical activity; thus the association with patella lead appears to be independent of these factors. We further examined lead effects stratified by urinary NTx levels. However, the results did not support the endogenous exposure suspicion. We also observed survivor effects in this dataset as the oldest cohort, who had the relatively higher bone lead concentrations, exhibited later onset of T2DM. Overall, the data supports the hypothesis that cumulative lead exposure increases the risk of T2DM.

Justification of the Decisions in Survival Analyses

Age at onset instead of time-on-study was chosen as the time scale in this survival analysis. We chose this because age is an important risk factor for the chronic diseases in longitudinal studies. Furthermore, in the case of using biomarkers to reflect cumulative exposure, the observed exposure level is a monotonic function of age. This situation has two implications.

First, age behaves as a confounder that is associated with both exposures and health outcomes. When applying the Cox's regression with time-on-study scale, age should be adjusted in the model as in model 4.3. However, a simulation study showed that even with the adjustment considered, bias still cannot be eliminated [37]. Alternatively, when using age as timescale, the effect estimate is conditional on the age effect; therefore the confounding effect of age can be eliminated (Model 4.4).

Second, because the risk of T2DM showed age-dependent penetrance, age scale can capture the increasing trend of the disease's hazard based on biological relevance therefore reflecting the true effects[37]. Hence the distribution of the hazard function on the age scale determines deviations of estimated effects from the true effects if other time scales were used. For instance, when the hazard function distribution follows exponential distribution family on the age scale, due to the memoryless property of exponential distributions, the effect estimates from other time scales behave in the same pattern as on the age scale. However, if the hazard function follows distributions other than exponential distribution, the effect estimates would behave differently between two scales (age scale and the other time scale). As a result, bias is introduced. The direction of the bias depends on the magnitude of the true effect and on the strength of correlations between the covariate (*Z*) and age as well as on if the adjustment of age was made[37].

$$\lambda_T(t|Z,A) = \lambda_0(t)\exp(\beta^{\mathrm{T}}Z + \gamma A) \qquad (4.3)$$

$$\lambda_A(a|Z) = \lambda_0(a)\exp(\beta^{\mathrm{T}}Z) \qquad (4.4)$$

$$\lambda_A(a|Z,b_0 \in B_j) = \lambda_{0jA}(a)\exp(\beta^{\mathrm{T}}Z) \qquad (4.5)$$

Caption: *t*, time-on-study; *a*, biological age at the beginning of study; *A*, age at baseline; *Z*, variable of interest; *b*, B_j, birth year (B_j as random variables).

Model 4.3: time-on-study as time scale, adjusted for age at baseline
Model 4.4: biological age as time scale without adjustment for birth cohorts
Model 4.5: biological age as time scale stratified by birth cohorts. This model is recommended by Korn et al[35].

Furthermore, the PH assumption requires hazard ratio (β) to be independent of time. This can hardly be achieved in a longitudinal study, especially when the disease occurrence depending on age or when a biomarker is a function of time. In this study, bone lead monotonically increases with age. Similarly, BMI and smoking status varied along with age and were related to disease occurrence. Therefore, the PH assumption could barely hold if we used time-on-study as the time scale. In addition, the true influence of these risk factors on T2DM development varies at different stages of life. Therefore proportional hazard assumption could only be valid when conditioning on the age by treating age as timescale. Misspecifying timescale using time-on-study can lead to the departure from the PH assumption and introduce bias in the effect estimates. The direction of bias is unpredictable, and the magnitude of the bias is proportional to the true effect [37].

In addition, due to the original inclusion criteria of the study, the subjects entered into the study were relatively healthier than the general population. This issue was especially pronounced in the subjects who were born before1920. Figure 4.2 shows that in the study subjects with bone lead measurements, those who borned earlier exhibited late onset of T2DM compared to subjects whom borned later. We here refer this survivor effect to the phenomenon that the old cohort that was selected in this study exhibited some extent of resistance to T2DM or to lead intoxication compared to the younger cohorts. We created a birth cohort variable that categorized the study population into three groups: born before 1920, born between 1920 and 1920 and born

after 1930. In order to control this survivor effect, stratification on birth cohort approach is recommended [35] as in Model 4.5. We tested our hypotheses in a set of models with different choice of adjustment for this survivor effect. Our result showed that with adjustment of birth cohort variable in the models generally reduced the survivor effect. However, the HR estimates at the higher tail of lead exposure were inevitably biased beyond the null.

Lead exposure in the Etiology of Type 2 Diabetes

Oxidative stress induced cellular dysfunction is the central pathophysiological mechanism involved in the genesis of metabolic disease[1]. Oxidative stress affects functions of intracellular organelles such as mitochondria and endoplasmic reticulum (ER). Mitochondria distress results in insufficient energy supply and deregulation of insulin pathway signaling [3]. ER distress is the major pathological observation in diabetes[38], which involves the induction of unfolded protein response[39]. As a consequence, ER stress response results in leptin/insulin resistance in hypothamus; inflammatory process in fat tissue induces, insulin secretion impairment and apoptosis of beta cells in pancreas [39].

Lead induced oxidative stress can be observed in the many tissues [21, 40]. For instances, lead binds to δ-ALAD[24] in erythrocytes, which leads to accumulation of δ-aminolevulinic acid (δ-ALA) in the cell cytoplasm. δ-ALA is auto-oxidative at pH 7.0-8.0 and generates free radicals [41]. Lead inhibits the functions of a variety of reductases[22, 25]. Reduction in these bioactive antioxidants weakens defenses against oxidative stress. Moreover, lead imposes stress on mitochondria by impeding calcium reuptake and

stimulating calcium efflux from mitochondria [27, 42]. Lead depletes the calcium from ER and triggers the ER stress responses [4, 43].

On the other hand, recent studies showed that osteocalcin plays an important role in insulin production and in improving insulin resistance [44, 45]. Under the physical conditions, osteocalcin requires calcium binding to become bioactive. Lead ion (Pb^{2+}) can displace calcium ion (Ca^{2+}) at sulfhydryl ligand [42, 43] and causes osteocalcin more adsorptive to hydroxyapatite at much lower concentration. This leaves decreased proportion of bioactive osteocalcin in the circulation[46]. Experimental evidence also showed that lead suppresses the mRNA expression of osteocalcin[47, 48].

Our results supported the contention that lead increases the risk of T2DM by showing the increased HRs in elevated patella lead groups. However, we did not observe the significant impact in tibia lead models. To elucidate the temporality of lead effect, we explored the bone lead effect on T2DM with regard to bone turnover activity. Urinary NTx is a reliable biomarker for bone resorption activity and was shown to help identifying the endogenous exposure to lead [49]. Under the high bone turnover condition, lead in trabecular bones mobilizes into blood and creates secondary endogenous lead exposure. Therefore the prominent effect in patella lead could be explained by effect of endogenous exposure. If this were the case, the patella lead effect would be stronger in high urinary NTx group. However, the results from the stratified analyses did not support this contention. Further investigation is needed to explain the discrepancies observed between these two types of bones.

Limitations

The findings from this analysis should be interpreted with cautions. The main concerns were raised from the structure of study population and the biomarker for cumulative lead exposure. First, as we illustrated in the discussion session, the study subjects recruited were relatively healthier than the general population. Loss of participation to bone lead assessment created a secondary sample attrition, which left the subjects remained in the subset slightly healthier. This issue becomes noteworthy in the oldest birth cohort since the most of susceptible subjects had been filtered out from the final dataset. Subjects in this birth cohort were less likely to be affected by the environmental lead exposures and were more resistant to T2DM. Secondly, due to the summary feature of bone lead, it was difficult to distinguish the effect of lead apart from the aging effect. It also created paradoxical relationship when the old cohort exhibited less susceptibility to lead intoxication and more resistance to the disease. In order to resolve this issue, we created birth cohort groups to quantify the frailties in the subjects that were born at different calendar periods. This method can reduce the bias in the effect estimates to some extent but could not completely remove the survivor bias. A better method should be developed to overcome this limitation in the population structure and to derive unbiased estimates.

Other issues arisen from the lead exposure assessment and T2DM case identifications could also potentially affect the observed HRs. For example, all the DM cases identified in this study were classified as T2DM with a slim possibility that a few cases could belong to other types of DM. Additionally, the accuracy of bone lead concentration using K-XRF is affected by the thickness of skin. The measurement

uncertainties can be inflated with thicker skins, which is commonly seen in subject with high BMI. However the deviations of coefficient estimates from true effects with regard to these two issues were not well studied. Additionally, statistically significant associations were only observed in the patella lead models. Yet the NTx stratified test results did not support the endogenous exposure contention. This requires cautions when interpreting the lead effect with respect to the timing of lead exposure.

Despite the limitations in our observations, our findings provided a valuable piece of evidence to establish the association between cumulative lead exposures and T2DM with a few strengths. First, this is the first study with long-term follow-ups and repeated measurements in health outcomes and confounding factors. It also adopted the biomarkers for cumulative exposure to lead, which renders higher power to detect biological associations. In addition, the observation of the survivor effect provided a piece of evidence showing the heterogeneities in the risk population with regard to the mechanism of lead exposure on the development of T2DM. This provokes further investigations on the interactions between genetic predispositions and environmental lead exposures in the development of T2DM.

In conclusion, this analysis showed that cumulative exposure to lead increases the risk of T2DM with a ceiling effect. The observation of heterogeneities of susceptibility to T2DM in sub-study populations requires further investigations to elucidate the mechanisms of lead exposure on T2DM development. Animal studies are desired to clarify the biological pathways of lead involved in the pathogenesis of T2DM.

Tables

Table 4.1 Characteristics of Study Population at Baseline in T2DM and Non-T2DM Groups [19]

	T2DM Group					Non-T2DM Group				
	Without Pb measure		With Pb measure			Without Pb measure		With Pb measure		
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
Age	44.6	8.6	40.06	6.95	**	43.6	10.58	39.89	7.32	**
Birth Year	1921.19	8.53	1925.89	6.88	**	1922.14	10.51	1926.25	7.41	**
Education										
Less than high school [N (%)]	27(0.07)	-	24(0.06)	0.57		153(0.09)	-	63(0.04)	-	*
High school graduates [N (%)]	98(0.25)	-	144(0.37)			651(0.38)	-	367(0.21)	-	
More than high school [N (%)]	42(0.11)	-	50(0.13)			308(0.18)	-	194(0.11)	-	
Smoking Pack-years	19.65	18.99	16.36	18.16		19.63	19.35	15.03	17.78	**
Ever Smoking										
Never smoker [N (%)]	28(20.89)	-	49(28.32)	-		212(23.32)	-	161(30.61)	-	
Regular smoker [N (%)]	56(41.79)	-	66(38.15)	-		411(45.21)	-	179(34.03)	-	
Quit smoking [N (%)]	50(37.31)	-	58(33.52)	-		286(31.46)	-	186(35.36)	-	
Total Cholesterol (mg/dL)	208.93	47.79	209.79	45.47		204.39	44.69	199.69	43.8	*
Serum Triglycerides (mg/dL)	160.19	94.64	150.79	75.98		132.11	51.37	130.19	50.56	
Fasting Glucose (mg/dL)	101.7	11.75	101.27	9.55		97.66	9.68	97.52	9.85	
2-Hour Glucose Test (mg/dL)	113.94	25.47	112.02	20.23		103.52	18.98	103.14	18.35	
Body Mass Index	26.65	3.24	26.83	3.03		25.52	2.85	25.47	2.54	
Systolic Blood Pressure (mmHg)	125.89	11.93	124.82	11.68		123.77	11.97	122.84	11.14	
Diastolic Blood Pressure (mmHg)	77.98	7.4	78.37	7.56		76.93	7.78	76.73	7.91	

[19] Comparisons were made in T2DM and non-T2DM groups separately by ANOVA or Fisher's exact tests. *p< 0.05; **p<0.01

Table 4.2 Comparison of Characteristics in T2DM Subjects in the Final Dataset by Birth Cohorts

	Old			Middle			Young [20]			
	N	Mean (or %)	SD	N	Mean (or %)	SD	N	Mean (or %)	SD	
Birth Year	46	1915.8	2.88	119	1925.31	2.7	65	1934.01	3.11	**
Education										
Less than high school [N (%)]	4	0.09	-	113	2.12	-	5	0.08	-	
High school graduate [N (%)]	25	0.58	-	15	0.13	-	49	0.79	-	
More than high school [N (%)]	14	0.33	-	70	0.62	-	8	0.13	-	
Smoking Pack-years	29	14.99	18.98	28	0.25	23.27	46	21.12	19.05	
Current Smoker [N (%)]	1	0.03	-	8	0.08	-	5	0.09	-	
Former Smoker [N (%)]	17	0.49	-	67	0.67	-	38	0.68	-	
Total HDL (mg/dL)	30	46.67	11.57	87	44.76	11.67	51	42.92	11.34	
Total Cholesterol (mg/dL)	46	229.07	52.14	119	235.51	51.33	65	226.74	47.61	
Serum Triglycerides (mg/dL)	46	147.24	58.48	116	198.17	111.59	63	185.68	81.87	**
Fasting Glucose (mg/dL)	46	132	48.67	118	131.84	36.2	65	133.52	38.77	
2-Hour Glucose Test (mg/dL)	40	197.1	47.62	103	198.07	70.59	59	224.51	87.25	
Body Mass Index	35	26.49	3.81	101	29.41	4	65	30.95	4.59	**
Systolic Blood Pressure (mmHg)	46	138.67	17.82	119	135.27	17.65	65	135.74	19.3	
Diastolic Blood Pressure (mmHg)	46	80.62	10.01	119	80.43	9.28	65	83.18	9.91	
Physical Activity (Fast walk adjusted, kcal/wk)	22	1806.32	1160.07	67	1290.29	1231.95	42	1285.13	1137.74	
Physical Activity (No fast walk, kal/wk)	22	1251.89	827.05	67	1200.41	1181.74	42	1193.86	1072.4	
Diabetes Diagnosis [N (%)]	46	0.22	-	119	0.17	-	65	0.25	-	
Diabetes Medication (Current) [N (%)]	45	0.02	-	119	0.09	-	65	0.05	-	
Diabetes Onset Age	23	68.3	9.44	58	64.89	10.37	38	57.9	8.89	**

[20] The old cohorts were born before 1920; the middle cohort was born between 1920 and 1930; the young cohort was born later than 1930. *p* values are derived from either Fisher's exact test or ANOVA tests. *, $p<0.05$; ** $p<0.01$

Table 4.3 Comparison of Characteristics in T2DM Subjects by Patella Lead Groups [21]

	≤25 percentile (≤18 µg/g)			25-75 percentile (18 -39 µg/g)			>75 percentile (>39 µg/g)			
	N	Mean	SD	N	Mean	SD	N	Mean	SD	
Birth Year	46	1928.51	7.32	117	1926.68	6.09	67	1922.63	6.79	**
Education										
Less than high school [N (%)]	4	0.09	-	15	0.13	-	5	0.08	-	**
High school graduate [N (%)]	25	0.58	-	70	0.62	-	49	0.79	-	
More than high school [N (%)]	14	0.33	-	28	0.25	-	8	0.13	-	
Smoking Pack-years	27	20.96	18.3	85	22.06	22.26	40	23.73	22.45	
Current Smoker [N (%)]	37	0.11	-	100	0.08	-	54	0.04	-	
Former Smoker [N (%)]	37	0.62	-	100	0.62	-	54	0.69	-	
Total HDL (mg/dL)	32	44.94	10.53	89	44.2	11.74	47	44.91	12.07	
Total Cholesterol (mg/dL)	46	222.13	48.18	117	231.81	49.91	67	238.22	52.44	
Serum Triglycerides (mg/dL)	45	154.91	62.38	114	193.82	105.27	66	187.77	97.17	
Fasting Glucose (mg/dL)	46	134.65	44.72	117	132.26	43.77	66	130.91	25.92	
2-Hour Glucose Test (mg/dL)	43	215.7	73.65	100	200.19	73.32	59	207.41	71.97	
Body Mass Index	41	30.89	5.03	106	29.11	4.24	54	28.83	4.06	*
Systolic Blood Pressure (mmHg)	46	141.5	17.57	117	132.21	18	67	139.12	17.45	**
Diastolic Blood Pressure (mmHg)	46	83.92	8.18	117	80.24	10.64	67	81.17	8.43	
Physical Activity (No fast walk, kal/wk)	26	1511.92	1041.61	65	1046.14	1049.84	40	1270.05	1152.68	
Diabetes Diagnosis [N (%)]	10	0.22	-	24	0.21	-	12	0.18	-	
Diabetes Medication (Current) [N (%)]	4	0.09	-	8	0.07	-	3	0.05	-	
Diabetes Onset Age (Self-Reported)	23	60.74	11.39	63	62.52	10.55	33	66.64	8.9	
Onset Age of T2DM (Calculated)	46	61.66	11.58	117	62.41	10.29	67	65.57	10.4	

[21] *p* values are derived from either Fisher's exact tests or ANOVA F-statistic tests. *, $p<0.05$; ** $p<0.01$

Table 4.4 Comparison of Coefficients in Tibia and Patella Lead Models[22]

Patella Lead Models						
	Q1 (≤18μg/g)	Q2-Q3 (18 –39μg/g)		Q4 (>39 μg/g)		*p* for trend
N	221	437		214		
Events	46	117		67		0.01
Model 1	-	0.17 (0.08)	*	0.06(0.09)		0.74
Model 2	-	0.40(0.11)	**	0.28(0.12)	*	0.05
Model 3	-	0.45(0.11)	**	0.39(0.12)	**	<0.01
Model 4	-	0.45(0.11)	**	0.39 (0.12)	**	0.11
Tibia Lead Models						
	Q1 (≤13 μg/g)	Q2-Q3 (13 –28μg/g)		Q4 (>28 μg/g)		*p* for trend
N	230	443		204		
Events	51	118		61		0.07
Model 1	-	0.02(0.08)		0.042(0.09)		0.64
Model 2	-	-0.02 (0.10)		-0.051(0.12)		0.67
Model 3	-	0.01 (0.10)		0.051(0.12)		0.67
Model 4	-	0.02(0.10)		0.05(0.12)		0.81

[22] Major covariates: current smoking status, physical activity, educational level, and BMI. Model 1: crude lead effect estimate; Model2: adjusted for major covariates; Model3: stratified on birth cohort; Model4: gamma frailty model, birth cohort as frailty term; *p* values (not presented in the table) are derived from score tests against H_0 β=0. * p<0.05; ** p<0.01

Table 4.5 Hazard Ratios and 95% Confidence Intervals in the Final Models[23]

	Patella Lead Model [24]			Tibia Lead Model[25]		
	HR	LCI	UCI	HR	LCI	UCI
Lead (25^{th} -75^{th} percentile)	1.57	1.26	1.94	1.02	0.83	1.25
Lead (>75^{th} percentile)	1.48	1.16	1.89	1.05	0.83	1.34
Current Smoker	1.58	1.17	2.13	1.64	1.22	2.21
Physical Activity Expended (kcal/wk)	0.81	0.74	0.87	0.79	0.73	0.86
Body Mass Index	1.11	1.09	1.13	1.10	1.08	1.12
Education: High school graduate	1.11	0.86	1.44	1.10	0.85	1.42
Education: More than high school	0.87	0.64	1.17	0.81	0.60	1.10

[23] The frailty terms in both models were significant

[24] Patella lead at 25^{th} and 75^{th} percentiles corresponded to 18 μg/g and 39 μg/g

[25] Tibia lead at 25^{th} and 75^{th} percentiles corresponded to 13 μg/g and 28 μg/g

Table S 4.1 Lead Effects Stratified by Urinary NTx Levels[26]

		Tibia lead Models[27]						Patella lead Models[28]					
	Lead Levels	Low NTx (≤50%)[29]			High NTx (>50%)			Low NTx (≤50%)			High NTx (>50%)		
		β	Se(*β*)	*p* value	*β*	Se(*β*)	*p* value	*β*	Se(*β*)	*p* value	*β*	Se(*β*)	*p* value
Model 1	25th -75th percentile	0.04	0.16	0.81	0.13	0.19	0.47	0.31	0.18	0.08	0.16	0.18	0.38
	> 75th percentile	0.23	0.19	0.22	0.07	0.22	0.74	0.47	0.20	0.02	0.02	0.21	0.93
Model 2	25th -75th percentile	0.04	0.16	0.83	0.11	0.19	0.55	0.32	0.18	0.08	0.16	0.19	0.40
	> 75th percentile	0.23	0.19	0.21	0.06	0.22	0.77	0.47	0.20	0.02	0.02	0.21	0.92
Model 3	25th -75th percentile	0.03	0.16	0.86	0.17	0.18	0.34	0.23	0.18	0.18	-0.01	0.17	0.96
	> 75th percentile	0.22	0.18	0.22	0.04	0.21	0.86	0.41	0.20	0.04	-0.02	0.20	0.91

[26] Major covariates: current smoking status, physical activity, educational level, and BMI. Model1: Cox's model, birth cohort as covariate; Model 2: Cox's model, stratified on birth cohort; Model 3: gamma frailty model, birth cohort as frailty term.

[27] Tibia lead at 25th and 75th percentiles corresponded to 13 μg/g and 28 μg/g

[28] Patella lead at 25th and 75th percentiles corresponded to 18 μg/g and 39 μg/g

[29] The 50th percentile of NTx level corresponded to 41.44 nM BCE/mM creatinine

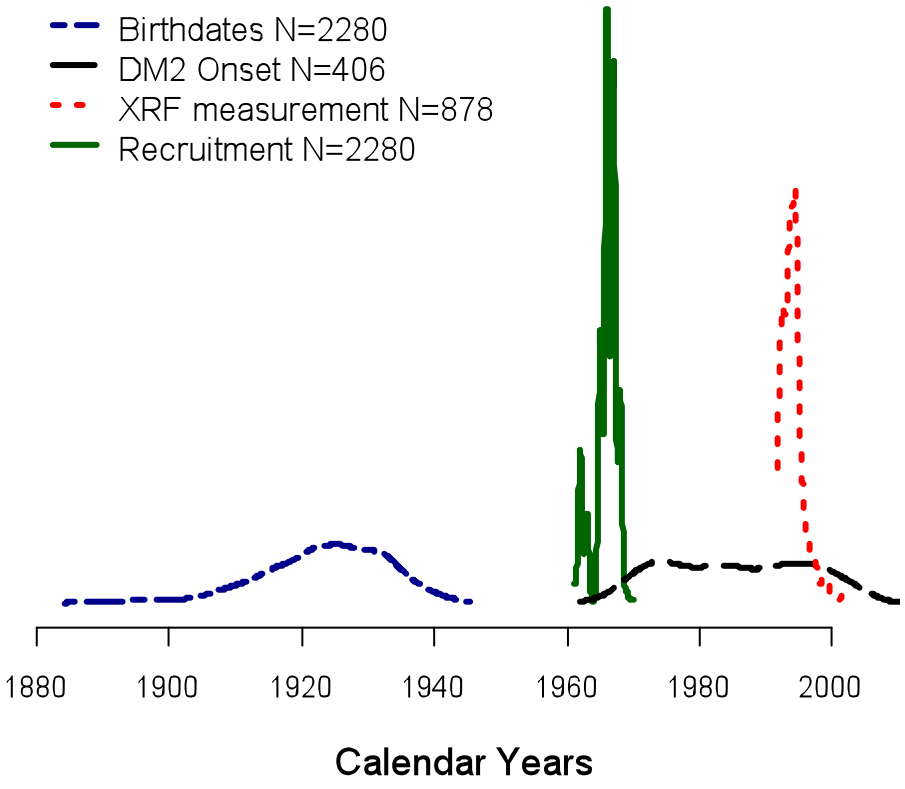

Figure 4.1 Event Timeline in NAS (Frequency Density Plot)

Caption: The majority of the study subjects were born in early 1900's (blue dashed line). They were recruited beginning in the early 1960's with continuous enrollment until early 1970's (solid line). Physical checkups were performed every three years for each subject 50 or older and every five years for subjects younger than 50. Bone lead measurements were added to the study in 1991 and were continued until 2002 (red dotted line). Diabetes incidence, represented by the black dash line, rose from early 1960's till the end of study period. For purpose of this analysis, the density plot presented in this graph is limited to the subjects in the current study. The total number of subjects in this figure does not represent entire NAS study population.

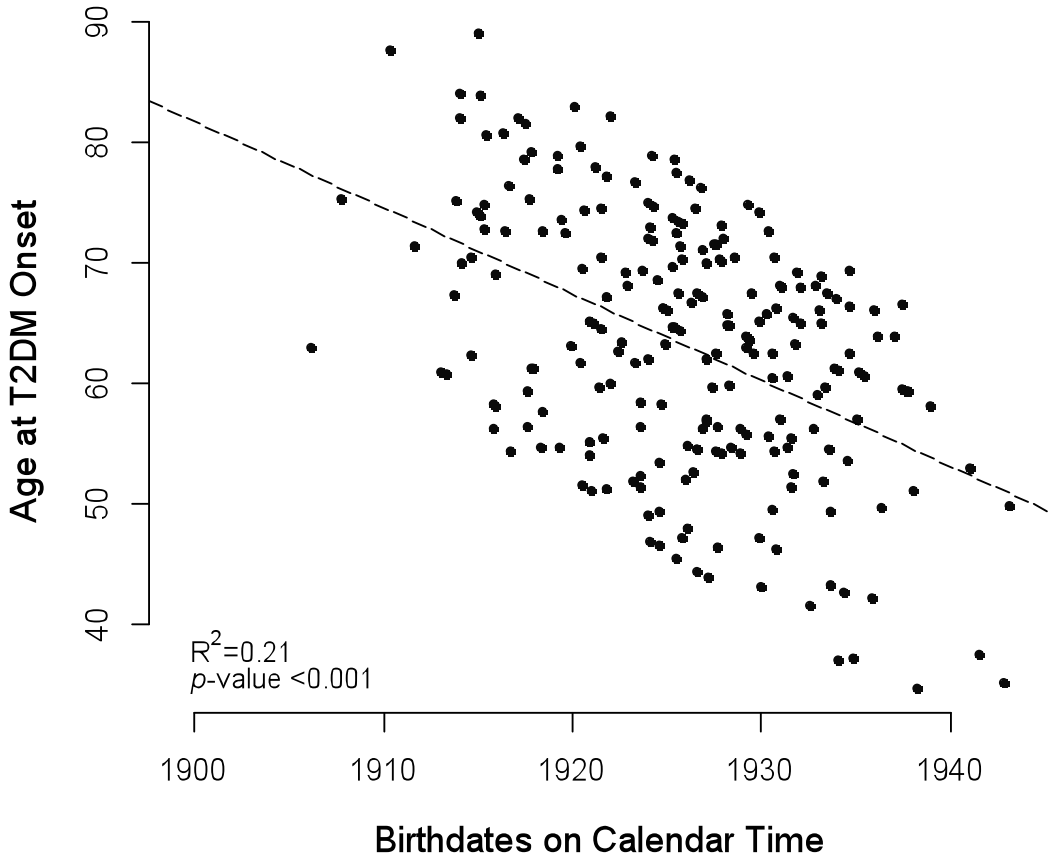

Figure 4.2 Survivor Effect (T2DM Onset vs. Birth Year)

Caption: X-axis: the birthdates of study subjects in calendar years. Y-axis: the age at T2DM onset.

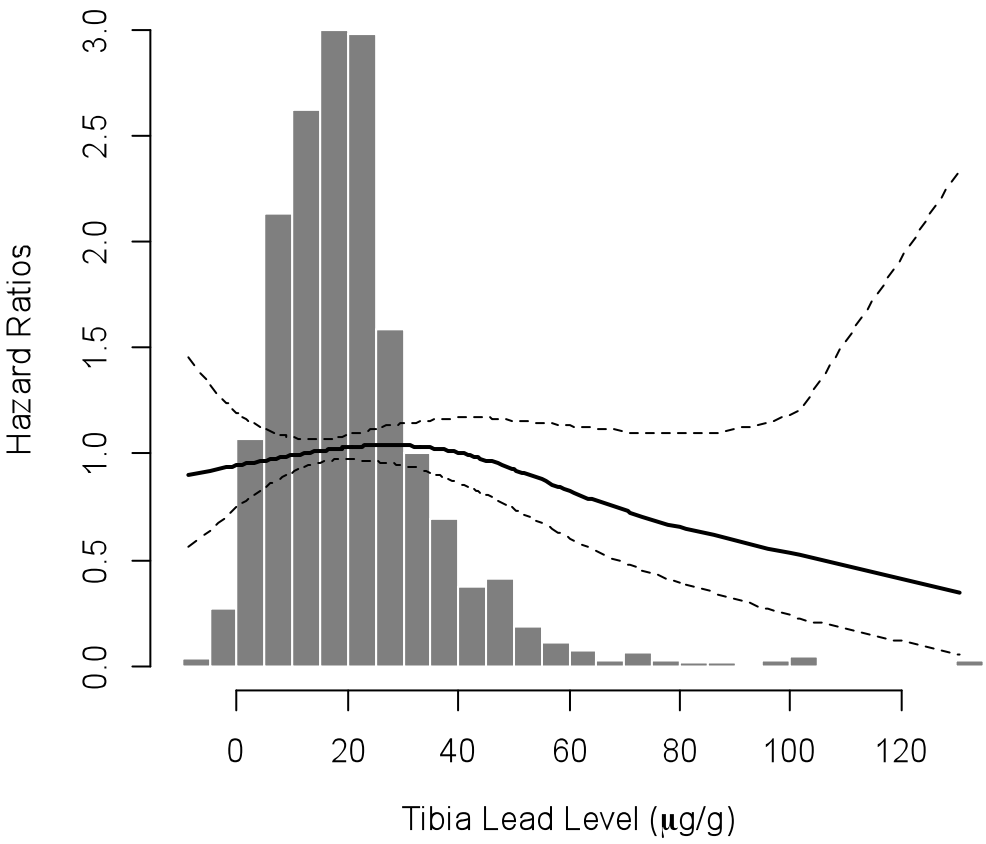

Figure 4.3 Hazard Ratios vs. Tibia Lead Levels

Caption: X-axis: the tibia lead level in µg/g. Y-axis: the hazard ratios estimated from the gamma frailty model treating the tibia lead as a continuous variable.

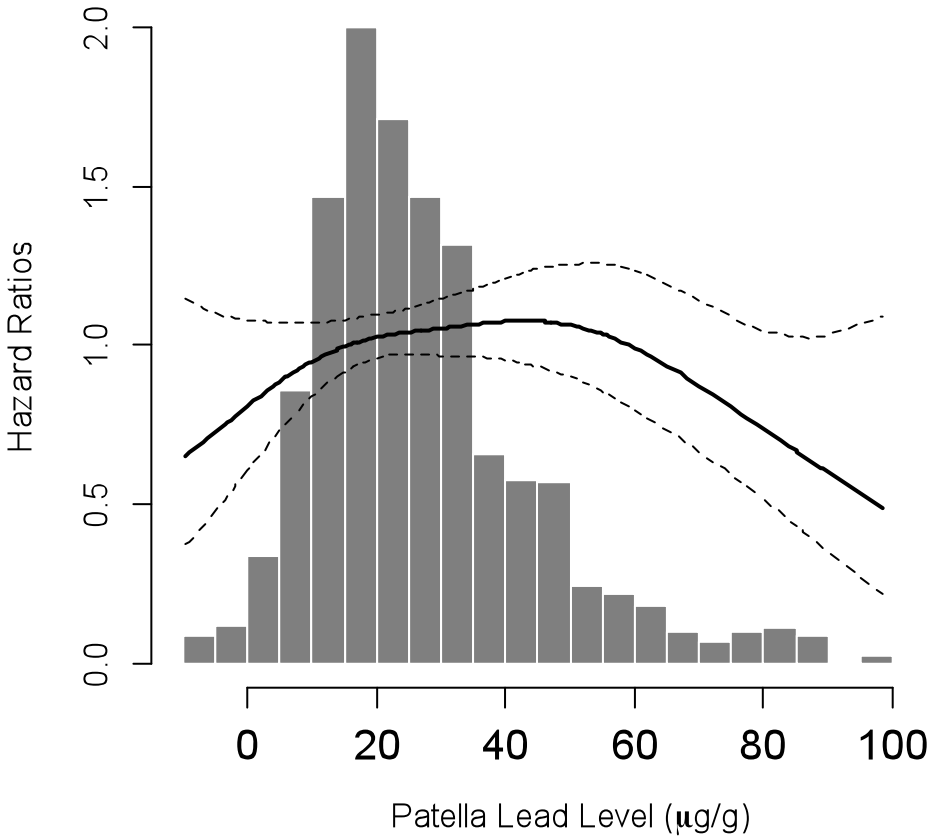

Figure 4.4 Hazard Ratios vs. Patella Lead Levels

Caption: X-axis: the patella lead level in μg/g. Y-axis: the hazard ratios estimated from the gamma frailty model treating the patella lead as a continuous variable.

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CHAPTER 5 CONCLUSIONS

Lead exposure remains an enormous public health problem worldwide. Evidence from basic science, clinical and epidemiological studies has provided fundamental knowledge on the toxic effects of lead exposure on human health. A majority of the epidemiologic research has focused on the cross-sectional links between lead exposures and health outcomes. In the handful of longitudinal studies, researchers have found that the effects of lead can be long lasting[1, 2]. Yet limited research has addressed the issue of timing of exposure to lead with respect to health outcomes in the long term. In this dissertation, I investigated the effect of exposure to lead in terms of windows of exposures. With the advantage of longitudinal study designs and the use of biomarkers for cumulative as well as short-term lead exposures, I aimed to establish the relationship between exposure to lead and a variety of health outcomes regarding the timing of lead exposure. The scope of health outcomes in this thesis covered psychobehavioral development, degenerative neurological disease (Parkinson's disease) and type 2 diabetes. Specifically, I investigated the timing of exposure to lead in the perinatal period and in early life in relation to the psychobehavioral development in children aged from 6 to 13 in Chapter 2. Chapter 3 and Chapter 4 focused on the potential influence of lifetime exposures to lead on risk of diseases that typically occur in late life. The goal of Chapter 3 was to understand the potential impact of lifetime exposure to lead on Parkinson's disease occurrence in subjects with genetic predisposition to Parkinson's disease in relation to polymorphisms of the SNCA gene.

Chapter 4 aimed to discover the relationship between lifetime lead exposure and risk of type 2 diabetes. All three chapters together attempted to assess the temporal dynamics of the influence of lead exposures across the lifespan.

In Chapter 2, the results did not show statistically significant associations between lead exposures in early life and behavioral outcomes measured in childhood/ early adolescence. However, the data suggested that exposure to lead during the prenatal period and the first two years after birth have strong impacts on inattention and hyperactivity behaviors. It appeared to affect emotion controls and somatic perceptions in children. Compared to effects of exposures during the perinatal period, the effect of recent lead exposure did not show strong impact on children's behaviors. These findings can be explained in relation to two respects. First, during early life, the immature brain does not have the protective mechanism to prevent against lead poisoning [3-5]. Previous studies have shown that the immature brain is a particularly sensitive target for lead poisoning. Second, the limbic system, of which functions are involved in emotional memory and socialization, develops and matures quickly in the first three years of life [6, 7]

However the limitations in these observations are that, first, the exposure levels in perinatal period was relatively higher than the recent lead exposure levels and second, these observations were limited to the subjects who had complete exposure measurements over the years of observation, which rendered a significant drop in sample size. This decreased the study's power. Third, the psychobehavioral assessments were only performed at one time point. We do not have observations of behavioral change overtime as a function of early life lead exposure. Future research

may need to clarify the effects of the temporal exposure patterns on a larger scale, as well as to provide new understanding on effect of timing of lead exposures on psychobehavioral development as a dynamic growing process. Nevertheless, this study provides an importance piece of evidence demonstrating the critical windows of lead exposures on the neurodevelopment in a long term.

In Chapter 3, I focused on the effect of lead from cumulative exposure on Parkinson's disease under different levels of genetic susceptibility. I investigated the lead effect among SNCA genetic variants on the disease occurrence. After counting for linkage disequilibrium, I found three loci that strongly predicted Parkinson's disease. Lead increases the odds of PD only among subjects who were genetically relatively resistant to the disease. Subjects with higher genetic susceptibility were less affected by lead exposure. This result implies that lead exposure and genetic predisposition in SNCA gene does not have a synergistic effect on PD development. In addition, I did not observe that genotypes of SNCA were strongly associated with bone lead levels. This indicates that SNCA gene does not modify the toxicodynamic of lead. These findings on the main effects of genetic markers were consistent with current knowledge on genetic etiology to PD[8-17]. However the main effects of lead were attenuated compared to our previous findings from a bigger sample[18]. In addition, this result showed that tibia lead appeared to be associated with PD when combined with genetic information more frequently than patella lead. This may be due to the low decay rate of cortical bone lead [19]. This underscores the importance of cumulative exposure to lead exposure on PD development, a topic that has been discussed in detail in our previous publications[18].

The mechanism of how lead exposures may cause Parkinson's disease is not clearly understood. The current conceptual framework involves oxidative stress [20-22]and alpha synuclein aggregation [23-27]in dopamine neurons. The result however was not in support of the idea of lead aggravating the effects of deleterious genetic variants of the SNCA gene. While these results are clearly preliminary, and are in need of replication, along with other work to better understand the pathogenesis of PD.

Chapter 4 provided novel evidence that exposure to lead could affect energy metabolism and result in type 2 diabetes. I found a significant increase of T2DM risk with an incremental increase in patella lead levels. I observed a discrepancy between the tibia lead and the patella lead effects on T2DM development. The NTx stratified analysis results did not support the contention that secondary endogenous lead exposure from increased resorption of bone stores of lead plays a significant role in modifying the effects of bone lead. These findings suggested that cumulative exposure to lead increases the risk of T2DM with a ceiling effect. The biological plausibility of the findings relates to two potential mechanisms involving inflammation and osteocalcin pathways. Lead imposes great oxidative stress on cells[28-31]and affects osteocalcin through calcium interference [32, 33]. Lead exposure results in impaired beta cell function and deranged osteocalcin hemostatsis, which may result in the perturbations of lipid and glucose metabolism.

The use of cumulative exposure markers posed some particular methodological challenges. This use carries the inherent difficulty of distinguishing the effect of the long-term exposure apart from the aging effect. It also created paradoxical relationships when an older cohort exhibits decreasing susceptibility to either the exposure or the

disease (“survivor effect”). On the other hand, the survivor effect reflects the heterogeneities in risk associated with the pool of subjects with late stage T2DM. This will require additional investigations on the interactions between genetic factors and environmental lead exposures in the etiology of T2DM. In addition, animal studies may, in parallel, help clarify the biological pathways of lead effects in relation to T2DM.

The studies in this thesis provide a landscape of lead effects on human health across the life span with consideration of the timing of the exposures, and interactions with genetic vulnerabilities. It explored the associations with diseases of which etiologies were not clearly understood and paves the way for future research.

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