**LEAD EXPOSURE, HOMOCYSTEINE, DNA METHYLATION AND
LATE-ONSET ALZHEIMER'S DISEASE**

by

Kelly M. Bakulski

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Environmental Health Sciences)
in The University of Michigan
2012

Doctoral Committee:

Professor Howard Hu, Co-Chair
Assistant Professor Laura S. Rozek, Co-Chair
Professor Henry L. Paulson
Assistant Professor Sung Kyun Park

ACKNOWLEDGMENTS

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8-oxo-dG	8-hydroxy-2'-deoxyguanosine
AD	Alzheimer's disease
ADRC	Alzheimer's disease research center
DOHad	Developmental origins of adult health and disease
EOAD	Early-onset Alzheimer's disease
EGCG	Epigallocatechin gallate
GAWS	Genome wide association study
HCY	Homocysteine
KXRF	Cd109 K-shell X-ray Fluorescence
LOAD	Late-onset Alzheimer's disease
Pb	Lead
MCI	Mild cognitive impairment
NGS	Next generation sequencing
PND	Post natal day
SAM	S-adenosyl methionine
SNP	Single nucleotide polymorphism
NAS	Veteran's Affairs Normative Aging Study
WBC	White blood cell

ABSTRACT

The causes of sporadic neurodegenerative disease in aging adults likely involve a complex interplay of genetics, epigenetics, and a lifetime of environmental exposures. The current dissertation uses two molecular epidemiology studies to investigate biomarkers of environmental exposures and neurodegenerative outcomes.

A major challenge of chronic disease environmental etiology research is the long latency between environmental exposures and the onset of disease. The Veteran's Affairs Normative Aging Study is an epidemiologic cohort designed to prospectively measure exposures and monitor early or subclinical disease. Repeated levels of recent exposure to lead were measured in blood and cumulative exposure to lead was measured by bone K-shell X-ray fluorescence. Homocysteine (Hcy), a risk factor for cardiovascular and neurodegenerative diseases when elevated, was measured concurrently with blood lead. Using repeated measures mixed effects models, this research demonstrated that an interquartile range higher blood Pb level (3 µg/dl) was associated with an 8.1% higher Hcy, compared to the percent change in Hcy with a 5-year increase in age (3.1%). We also demonstrated that individuals with diets rich in vitamins B6, B9, and B12, mitigated the effect of Pb on Hcy. This research suggests that interventions to reduce blood Pb and increase dietary B-vitamin intake would reduce circulating Hcy levels, potentially lowering risk for cardiovascular and neurodegenerative disease.

The second study investigated the role of epigenetic regulation, through DNA methylation, and its potential contribution to gene expression changes in late-onset Alzheimer's disease. In two separate thesis papers, the DNA methylomes and transcriptomes of frontal cortex tissues from deceased patients with Alzheimer's disease were mapped and compared to neuropathologically

normal controls using genome-wide approaches and bioinformatic analyses. In a proof-of-concept study, the top disease ranked DNA methylation site was validated for altered gene expression and protein levels. A novel biomarker and potential mechanism for LOAD pathogenicity with environmental implications was proposed.

This interdisciplinary thesis implemented laboratory biomarker studies, population exposure assessment and molecular epidemiology to approach the multi-faceted origins of neurological disease in aging populations.

CHAPTER I
Introduction

Alzheimer's Disease and Environmental Exposure to Lead: The Epidemiologic Evidence and Potential Role of Epigenetics

ABSTRACT

Several lines of evidence indicate that the etiology of late-onset Alzheimer's disease (LOAD) is complex, with significant contributions from both genes and environmental factors. Recent research suggests the importance of epigenetic mechanisms in defining the relationship between environmental exposures and LOAD. In epidemiologic studies of adults, cumulative lifetime lead (Pb) exposure has been associated with accelerated declines in cognition. In addition, research in animal models suggests a causal association between Pb exposure during early life, epigenetics, and LOAD. There are multiple challenges to human epidemiologic research evaluating the relationship between epigenetics, LOAD, and Pb exposure. Epidemiologic studies are not well-suited to accommodate the long latency period between exposures during early life and onset of Alzheimer's disease. There is also a lack of validated circulating epigenetics biomarkers and retrospective biomarkers of Pb exposure. Members of our research group have shown bone Pb is an accurate measurement of historical Pb exposure in adults, offering an avenue for future epidemiologic studies. However, this would not address the risk of LOAD attributable to early-life Pb exposures. Future studies

that use a cohort design to measure both Pb exposure and validated epigenetic biomarkers of LOAD will be useful to clarify this important relationship.

Keywords

DNA methylation; epigenetics; epidemiology, Late-onset Alzheimer's disease; lead exposure; Pb

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ALZHEIMER'S DISEASE

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General Alzheimer's Disease Epidemiology

Alzheimer's disease (AD) is a highly prevalent, progressive, and fatal neurodegenerative disease associated with aging. Clinical manifestation of AD includes progressive memory impairment and a gradual difficulty performing normal activities. A small percentage of cases, termed early-onset AD (EOAD), experience disease onset prior to age 60. EOAD cases are attributed to highly penetrant genetic mutations in amyloid pathway genes including amyloid precursor protein (*APP*) on chromosome 21, presenilin 1 (*PSEN1*) on chromosome 14, and presenilin 2 (*PSEN2*) on chromosome 1 (Bertram 2009; Hardy 1997).   These mutations lead to the accumulation of β-amyloid plaques, a pathological hallmark of AD.

Termed late-onset AD (LOAD), the majority of AD cases are sporadic and symptoms manifest after age 60. Numerous low-penetrant genetic risk factors conferring a modest increase in risk of disease have been identified for LOAD, the most studied of which is the apolipoprotein ε4 allele (*APOE-ε4*). The global population prevalence of *APOE-ε4* is 22%, while approximately 60% of LOAD cases carry at least one allele (Ashford 2004; Kim et al. 2009). Large, multi-center genome-wide association studies (GWAS) estimate the population attributable risk for *APOE* variants is 19-35% (Ertekin-Taner 2010).   GWAS have

identified additional polymorphisms associated with LOAD risk including genes for *ABCA7, BIN1, CD2AP, CD33, CLU, CR1, EPHA1, MS4A,* and *PICALM* (Harold et al. 2009; Hollingworth et al. 2011; Lambert et al. 2009; Naj et al. 2011), each associated with small increases in population attributable risk (PAR) ranging from 2-9.3% with a combined non-*APOE* PAR of 31-35%. Additional *APOE ε4* dose adjustment reveals 50% of the PAR for LOAD is accounted for by known single nucleotide polymorphisms (SNPs) (Naj et al. 2011). While these variants are important both for risk assessment and identification of novel mechanisms of pathogenesis, they are neither necessary nor sufficient for the development of LOAD.

Twin studies are an important epidemiologic tool for estimating the relative contribution of genetics and the environment in disease development. Incomplete twin concordance and variable age of onset supports a significant role for non-genetic factors in LOAD etiology. Among monozygotic twin (MZ) pairs, approximately 45-67% of twin pairs are concordant for LOAD (Gatz et al. 1997; Gatz et al. 2006; Nee and Lippa 1999). Heritability of liability based on twin studies is estimated to be 58-79% (Gatz et al. 1997; Gatz et al. 2006). Linkage analysis reveals age at LOAD onset is partially genetically linked to regions on chromosomes 4 (208 cM) and 10 (139 cM) (Li et al. 2002). However, among a group of MZ pairs in which both twins develop the disease, differences in age of onset range from 4 to 16 years (Gatz et al. 1997). Both genetic and environmental factors likely contribute to LOAD development.

Association studies have identified several non-genetic risk factors for LOAD, including depression (Saczynski et al. 2010), hypertension (Li et al. 2011; Sharp et al. 2011), stroke (Savva and Stephan 2010), diabetes (Li et al. 2011), hypercholesterolemia (Li et al. 2011), obesity (Anstey et al. 2011), head trauma (Plassman et al. 2000), smoking (Almeida et al. 2002; Li et al. 2011; Rusanen et al. 2011), and having greater than 6 siblings (Moceri et al. 2000). Protective factors, those that reduce the risk of developing LOAD or delay the onset of LOAD, include physical activity (Laurin et al. 2001; Lindsay et al. 2002), social

engagement (Fratiglioni and Wang 2007), mental activity (Fratiglioni and Wang 2007; Stern and Munn 2010), education (via the cognitive reserve hypothesis) (Fratiglioni and Wang 2007; Lindsay et al. 2002), statin use (G Li et al. 2010) , non-steroidal anti-inflammatory drug (NSAID) use (Lindsay et al. 2002), moderate alcohol consumption (Larrieu et al. 2004; Lindsay et al. 2002), coffee consumption (Lindsay et al. 2002), past vaccinations (Verreault et al. 2001), and childhood residence in the suburbs relative to the city (Moceri et al. 2000). In particular, nutrition may play a protective role in LOAD onset. Consumption of one meal/week of fish rich in omega-3 fatty acids reduced the risk of developing AD by 60% in the Chicago Health and Aging Project (Morris 2009). Individuals with plasma vitamin E less than or equal to 21.0 µmol/L had a higher risk of incident dementia than individuals with plasma levels greater than or equal to 25.5 µmol/L (Helmer et al. 2003). The natural plant polyphenols curcumin and green tea epigallocatechin gallate (EGCG) have anti-oxidant and neuroprotective properties that may be protective against LOAD (Mandel et al. 2007). EGCG reduces *APP* translation through modulation of the intracellular iron pool *in vitro* neuroblastoma cell culture (Reznichenko et al. 2006) and AD transgenic mice exposed *in vivo* to EGCG show reduced Aβ plaque density (Rezai-Zadeh et al. 2005). In an additional AD transgenic mouse model study, curcumin suppressed inflammation and oxidative damage in the brain and lowered levels of soluble Aβ and plaques (Yang et al. 2005).   Anthropometric measures of shorter adult knee height and arm span may reflect nutritional deficits in childhood (Jeong et al. 2005) and women in the lowest quartile of arm span in the Cardiovascular Health Cognition cohort study had 1.5 times elevated risk of dementia (Huang et al. 2008).

Proposed environmental exposures associated with LOAD include aluminum (Frisardi et al. 2010; Shcherbatykh and Carpenter 2007), copper (Brown 2009; Shcherbatykh and Carpenter 2007), zinc (Shcherbatykh and Carpenter 2007), mercury (Gerhardsson et al. 2008), lead (reviewed below), iron (Mandel et al. 2007), pesticides (Baldi et al. 2003; Santibanez et al. 2007), solvents (Kukull et al. 1995), electromagnetic field (Sobel et al. 1996), and

particulate matter in air pollution (Calderon-Garciduenas et al. 2004). Environmental exposure studies have been underrepresented in the AD literature, likely due to the challenges of retrospective exposure assessment in older adults.

LEAD (Pb) EXPOSURE

Overview of Pb Exposure as a Risk Factor

Zawia and colleagues have published a series of experimental studies on rodents and primates demonstrating that Pb exposure in early life results in late-life neuropathological changes similar to those of AD (reviewed elsewhere in this issue of *Current Alzheimer's Research*). This work, coupled with the recognition that exposure to Pb in the general population until recently has been high, has heightened interest in the epidemiology of Pb exposure and neurodegenerative disease. We discuss trends in Pb exposure and epidemiologic studies that provide evidence for the role of Pb as a risk factor for AD.

Pb Exposure Epidemiology

One of the greatest environmental health successes of our society was the regulatory action to reduce what had been decades of high Pb exposure in the US (Grosse et al. 2002). Between 1976 and 1991, the mean blood Pb levels for people in the US dropped 78% from 12.8 µg/dL to 2.8 (Pirkle et al. 1994). Since 1991 the standard elevated blood Pb level defining the need for action from Pb poisoning in children has been set to 10 µg/dL. The previously elevated mean blood Pb level of 12.8 µg/dL is a sobering testament to the high levels of Pb exposure endured by the general US population and other countries in the recent past.

However, hazardous public health impacts remain despite low mean population blood Pb levels. One issue is that the general reduction in Pb exposure is not universal. Pockets of high Pb exposure remain in certain sectors

of the US population where housing was constructed prior to 1950 and at which time leaded paint was used (Lanphear et al. 1998; Rabito et al. 2003), or where plumbing pipes and solder containing Pb have not yet been replaced (Edwards et al. 2009). Fly ash from municipal waste incineration contains high levels of heavy metals including Pb (Jung et al. 2004). In the high temperatures of the trash incineration process, Pb is converted to the volatile $PbCl_2$ compound, which can contaminate surrounding areas (Jung et al. 2004). It is estimated that the US has hundreds of defunct Pb battery recycling sites (Nedwed and Clifford 1997) and Pb/zinc mines and smelters. The surrounding soil and water of these industrial sites are often contaminated with high levels of Pb, leading to human exposure (Kaul et al. 1999), and many of these sites are designated Superfund sites on the National Priorities List by the US Environmental Protection Agency (EPA) (Lewin et al. 1999; von Lindern et al. 2003). In many developing countries the combustion of leaded gasoline continues and industrial emissions of Pb have been increasing (Meyer et al. 2008; Tong et al. 2000). Groups of people continue to experience high blood Pb levels based on their occupation or residential proximity to these hazards.

The pharmacodynamics of Pb in the human body makes past exposure to this heavy metal relevant to current and future health outcomes. Pb dust is inhaled or ingested and absorbed through the lung epithelia or gastrointestinal tract respectively. Pb is taken up by divalent metal transporters in the gut, binds tightly to heme molecules, and circulates throughout the body via blood. A small percentage of circulating Pb is highly toxic because it is free and bioavailable in the plasma (Hernandez-Avila et al. 1998). Plasma Pb contributes to both soft tissue Pb as well as bone Pb deposition (Rabinowitz et al. 1976). Pb can occupy both Ca^{2+} sites in the hydroxyapatite structure of bones (Barry and Mossman 1970) and greater than 95% of the adult body burden of Pb is stored in bones (Barry and Mossman 1970). Given that cortical bone turns over at a slow rate of approximately 2% per year in healthy adults, Pb can be stored for decades in bone (Barbosa et al. 2005; Hu et al. 1998; Rabinowitz 1991). Storage in bone is not a permanent Pb detoxification mechanism as Pb can have direct effects on

the cellular components of bone (Pounds et al. 1991), and bone Pb can be mobilized in times of higher bone turnover such as during pregnancy, lactation, and osteoporosis (Silbergeld et al. 1988). Individuals born in the US prior to the Pb phase out in the 1970s may have accumulated elevated bone Pb stores that become mobilized in later life.

Biomarkers of Pb Exposure

Whole blood Pb is the most common biomarker of Pb exposure. The half-life of Pb in blood is relatively short, approximately 35 days (Rabinowitz et al. 1976). This biomarker is best used for quantifying recent environmental exposures and mobilization of endogenous Pb (Silbergeld 1991). Similarly, soft tissues also have relatively high turnover of Pb with a mean half-life of approximately 40 days, but soft tissue Pb quantification is invasive and not typically used for epidemiologic studies (Rabinowitz et al. 1976).

An expert panel on adult Pb toxicity convened by the US Centers for Disease Control concluded that bone Pb levels were the best biomarker of cumulative Pb exposure (Hu et al. 2007). Spongy trabecular bone, such as that found in the patella, has an intermediate half-life of 5-15 years in adults (Hu et al. 1995). More dense cortical bone, as in the tibia, has a much longer half-life of 10-30 years (Chettle 2005). Thus, epidemiologic studies measuring Pb in bone can quantify a subject's life history of cumulative Pb exposure.

Bone Pb levels can be measured either *in vivo* using Cd^{109} K-shell X-ray Fluorescence (KXRF) (Hu et al. 2007) or by direct, chemical measurement of Pb in excised total joint replacement or post-mortem bone samples (Wittmers et al. 1988). Measurements with KXRF technology are painless and non-invasive, with minimal radiation exposure (Hu et al. 1995). The KXRF instrument uses low-level gamma radiation to provoke emission of fluorescent photons from a subject's tibia and patella (Hu et al. 1989). The photons are detected and quantified over a spectrum of wavelengths from which the characteristic emission profile of Pb can be extracted (Hu et al. 1995). Post-mortem bone samples can

be acid digested and quantified for Pb levels using inductively coupled plasma mass spectrometry (ICP-MS) (Garcia et al. 2001).

There are several approaches to predicting cumulative Pb exposure in the absence of direct bone Pb measures. The Park model incorporates blood Pb and information on subject demographics, medical history, and metabolic parameters to predict cumulative Pb exposure (Park et al. 2009). The Gorell system predicts cumulative Pb exposure based on blood Pb levels and physiologically based pharmacokinetic (PBPK) models incorporating industrial hygienist rated occupational Pb exposure for each job over the duration worked (Coon et al. 2006). Both strategies have been validated with bone Pb measures.

Pb as a Neurotoxicant and Risk Factor for LOAD

Pb is a well-known neurotoxicant in children. Even at relatively low (subclinical) levels, epidemiologic studies demonstrate that childhood Pb exposure affects IQ and behavior with major impacts on IQ and functioning (Fewtrell et al. 2004; Grosse et al. 2002).

A growing body of toxicological and population-based research indicates that cumulative environmental Pb exposure is neurotoxic in adults as well (Toscano and Guilarte 2005). Pb exposure is a significant risk factor for accelerated declines in cognition (Weisskopf et al. 2004; Wright et al. 2003), an effect that a recent CDC panel concluded was likely causal (Shih et al. 2007). The Veteran's Affairs Normative Aging Study (NAS) is a longitudinal cohort of men free of disease when recruited in 1963. Based on data from repeated measures of bone Pb, blood Pb, and cognitive tests in the NAS, there are significant associations between high Pb exposure and decreased cognition. The cognitive domains associated with increased Pb exposure differ depending on the time of exposure. In a cross-sectional analysis, higher blood Pb was associated with reduced ability to recall and define words, identify line-drawn objects, and difficulty with a perceptual comparison test (Payton et al. 1998). Both higher blood and bone Pb were associated with decreased spatial copying

skill (Payton et al. 1998). Higher bone Pb was associated with reduced pattern memory (Payton et al. 1998). Longitudinal analyses confirm that the Pb associated declines in cognitive function are greater than changes observed with normal aging alone (Schwartz et al. 2000). Also in the NAS, functional genetic polymorphisms in the δ-aminolevulinic acid dehydratase (*ALAD*) and hemochromatosis (*HFE*) genes modify the association between Pb and cognition measured by the Mini-Mental State Exam (MMSE), where variant carriers have more pronounced cognitive deficits associated with Pb exposure (Wang et al. 2007; Weuve et al. 2006). Future research may study the ecological association between geographic regions with elevated Pb exposure and prevalence of LOAD.

Pb is also a risk factor for increased hippocampal gliosis measured by magnetic resonance spectroscopy in the NAS (Weisskopf et al. 2007), an abnormality associated with LOAD development. Molecular epidemiology studies show cumulative Pb exposure is associated with an increased risk of amyotrophic lateral sclerosis (Kamel et al. 2005; Kamel et al. 2003; Kamel et al. 2002) and Parkinson's Disease (Weisskopf et al. 2010), suggesting that Pb exerts a significant neurodegenerative effect. This effect may have specificity through epigenetic change as a pathogenic mechanism.

Toxicological studies are consistent with the epidemiologic research. Early life Pb exposure in animal models is associated with latent *APP* pathway dysregulation. Rats exposed to Pb in early life showed increased expression of APP mRNA and elevated Aβ aggregation without changes in α-, β-, or γ-secretases at 20 months of age (Basha et al. 2005a; Basha et al. 2005b). Similarly, primates exposed to Pb during the first 2 months of life only had significant adverse brain changes at 23 years of age when compared to their unexposed counterparts (Wu et al. 2008a). Pb exposed primates had increased amyloidogenesis, senile plaque deposition, and up-regulation of key proteins in the amyloid processing pathway, such as *APP* and beta-site *APP*-cleaving enzyme 1 (*BACE1*) (Wu et al. 2008a).

OVERVIEW OF EPIGENETICS

Literally meaning "above the genome," the epigenome comprises the heritable changes in gene expression that occur in the absence of changes to the DNA sequence itself. Epigenetic mechanisms include chromatin folding and attachment to the nuclear matrix, packaging of DNA around nucleosomes, covalent modifications of histone tails, and DNA methylation. The influence of regulatory small RNAs and micro RNAs on gene transcription is also increasingly recognized as a key mechanism of epigenetic gene regulation (Morris 2011). Epigenetic mechanisms are important in growth and cellular differentiation (Jones and Taylor 1980). Epigenetic change can be stochastic (Feinberg and Irizarry 2010) or internally orchestrated as part of aging (Fraga and Esteller 2007). Longitudinal change in global and gene-specific DNA methylation clusters within families, suggesting that there is genetic control of methylation status (Bjornsson et al. 2008). Inappropriate epigenetic changes are associated with many diseases including cancers (Esteller 2008), Rett syndrome (Horike et al. 2005), Beckwith-Wiedemann syndrome (DeBaun et al. 2002) and other imprinting disorders. Environmental signals can trigger epigenetic responses and may be an important mechanism by which environmental exposures are associated with disease (Faulk and Dolinoy 2011). Furthermore, epigenetic mechanisms may play an important role in the developmental origins of adult health and disease (DOHaD) by providing a mechanism underlying the latent effects of adverse fetal, infant, and childhood environments on late-life chronic disease (Barker 2004; Hanson et al. 2011; Wadhwa et al. 2009).

Epigenetic Epidemiology and Alzheimer's Disease

Epigenetic epidemiology is the study of the effects of heritable epigenetic changes on the occurrence and distribution of diseases in populations (Jablonka 2004). This research includes both trans-generational and intra-individual cellular epigenetic inheritance systems. Epigenetic changes are associated with epidemiologic risk factors such as aging (Calvanese et al. 2009; Fraga 2009) and environmental exposures (Faulk and Dolinoy 2011), as well as psychiatric

outcomes (Sananbenesi and Fischer 2009) and neurodegeneration (Urdinguio et al. 2009).

Evidence for the role of epigenetics in AD pathogenesis is found in human studies of various tissues, animal models, and cell culture (2010; Mastroeni et al. 2011; Mill 2011). Global changes associated with AD have been observed in DNA methylation, miRNAs, and histone modifications. A human post-mortem case-control study identified global DNA hypomethylation in the entorhinal cortex of AD subjects by quantifying the percentage of positive 5-methylcytosine neuronal nuclear immunoreactivity (Mastroeni et al. 2008). Within a single MZ twin pair discordant for AD, DNA from the temporal neocortex neuronal nuclei was hypomethylated in the AD twin compared to their cognitively normal twin using similar methods to the previous study (Mastroeni et al. 2009). An AD case-control study in the post-mortem human parietal lobe cortex revealed differential regulation of miRNAs including miR-204, miR-211, and miR-44691 using a custom µParaflo array (Nunez-Iglesias et al. 2010). Age-matched AD cases have increased neuronal global phosphorylation of histone 3 relative to controls determined by immunolabeling in the hippocampus, a histone modification that suggests mitotic activation (Ogawa et al. 2003).

Given that epigenetics play an important regulatory role in gene expression, epigenetic dysregulation of important AD tau and amyloid processing pathway genes may point to a potential mechanism for AD disease progression. In experiments where neuroblastoma cells were cultured under low folate and vitamin B12 conditions, *PSEN1* and *BACE1* were hypomethylated, mRNA expression of *BACE1* and *PSEN1* was significantly induced, and Aβ production was increased (Fuso et al. 2005). Addition of S-adenosyl methionine (SAM) was able to restore *BACE1* and *PSEN1* expression to baseline levels, though DNA methylation reversal was incomplete (Fuso et al. 2005). An additional study using human neuroblastoma cells and male rat brain tissue shows *APP* mRNA expression is repressed by thyroid hormone (T3) sensitive histone modifications (Belakavadi et al. 2011). Treatment with T3 decreases H3K4 methylation and H3

acetylation at the *APP* promoter, leading to *APP* silencing that was reversed with histone deacetylase (HDAC) and histone lysine demethylase inhibitors (Belakavadi et al. 2011).

There have been several candidate-gene methylation studies in LOAD cases and controls. In a post-mortem brain study of 26 controls and 44 LOAD cases with varying degrees of disease severity, no differences were seen in DNA methylation in regions associated with Microtubule Associated Protein Tau (*MAPT*), *PSEN1*, and *APP*, nor were differences detected between frontal cortex and hippocampal DNA (Barrachina and Ferrer 2009). Investigation of 6 familial AD frontal cortex and cerebellum brain samples revealed no methylation at the *APP* promoter in any case in either brain region (Brohede et al. 2010). These studies were limited by a candidate-gene approach and highlight the need for genome-wide assessment of DNA methylation.

It is critical that epigenetic epidemiology studies of AD epigenetics consider age as an independent predictor of epigenetic change as age-specific epigenetic drift has been observed at AD related loci among healthy normal controls. In a set of control parietal cortex samples, the promoter of *APP* was hypomethylated in individuals greater than 70 years of age relative to younger subjects (Tohgi et al. 1999a). DNA methylation upstream of the *MAPT* gene also varied with age in the control parietal cortex and was associated with an age-related decline in *MAPT* gene expression (Tohgi et al. 1999b). Specifically, *MAPT* promoter CpG dinucleotides located in the *Sp1* transcriptional activator binding site were hypermethylated with age, while CpG dinucleotides located within the GCF transcriptional repressor binding region were hypomethylated with age (Tohgi et al. 1999b). Another study of post-mortem cerebral cortex in 125 subjects ranging from 17 weeks of gestation to 104 years of age measured methylation by MethyLight PCR at candidate tag loci for 50 genes selected for their relevance to LOAD, CNS differentiation, and cancer. CpG sites in the promoters of eight genes showed robust linear increases in DNA methylation across the lifespan (Siegmund et al. 2007). An additional study examined

prefrontal cortex samples across a 30 year age range and noted that the average DNA methylation in promoters of *MTHFR* and *APOE* increased by 6.8% across the age range, while control samples decreased by 10.6% with age (Wang et al. 2008). Given the likely epigenetic drift, clinical samples should be carefully matched on age.

Epigenetics and Heavy Metals, with a Focus on Pb

Epigenetic alterations have been observed following exposure to environmental metals (Salnikow and Zhitkovich 2008), including arsenic, nickel, chromium, cadmium, and Pb. Perhaps the heavy metal most studied in the field of cancer epigenetic epidemiology is arsenic. In a population-based study of 351 individuals with bladder cancer, elevated toenail arsenic measurements were associated with increased tumor sample promoter methylation of *RASSF1A* and *PRSS3* tumor suppressor genes (Marsit et al. 2006). Nickel, chromium, and cadmium epigenetics research has largely been in toxicologically based *in vitro* experiments. A cell line of human lung bronchoepithelial cells treated with nickel chloride show global histone modification changes including decreased H2A, H2B, H3, and H4 acetylation and increased H3K9 dimethylation (Ke et al. 2006). When the same cell line is treated with chromium, the cells exhibit increased H3K9 dimethylation at the *MLH1* gene promoter region, which correlates with decreased *MLH1* mRNA expression (Sun et al. 2009). Cadmium exposure in a rat liver cell line initially reduces DNA methyltransferase activity and global DNA methylation, but after 10 weeks of prolonged exposure, the cells show significant increases in DNA methyltransferase and global DNA methylation above the baseline (Takiguchi et al. 2003).

Evidence suggests that Pb, in particular, may play a role in epigenetics throughout the life course. In a study of 103 mother-infant pairs, maternal cumulative Pb exposure was inversely associated with offspring umbilical cord genomic DNA methylation of Alu retrotransposable elements (Pilsner et al. 2009). Similarly, bone Pb levels were inversely associated with peripheral blood genomic DNA methylation of LINE-1 retrotransposons in 517 elderly men from

the NAS (Wright et al. 2010). Individuals exposed to extremely high levels of Pb (51-100 µg/dL blood Pb) had higher methylation in the promoter of the *p16* tumor suppressor gene (Kovatsi et al. 2010). Research is needed to expand this early epidemiologic work on global and candidate gene DNA methylation to more comprehensively understand specific pathways influenced by Pb exposure in humans.

Animal studies have investigated the relationship between Pb exposure and epigenetics. Early life exposure to Pb in primates causes dysregulation of biological pathways important to LOAD pathogenesis in late life and is associated with reduced DNA methyltransferase 1 (*DNMT1*) activity (Wu et al. 2008a). Rat pheochromocytoma cells exposed to Pb show dose dependent decreases in global methylation and decreases in *APP* promoter methylation at 4 CpG sites (YY Li et al. 2010). These changes were associated with increases in *APP* mRNA and Aβ protein levels (YY Li et al. 2010). Toxicological and epidemiological studies suggest that Pb exposure may be associated with epigenetic change, but further research is needed.

DATA INTEGRATING ALZHEIMER'S DISEASE, EPIGENETICS, AND Pb EXPOSURE

Alzheimer's and Pb Exposure are Associated with Changes in One-Carbon Metabolism, the Substrate for DNA Methylation

De novo and maintenance DNA methylation is dependent on available methyl ($-CH_3$) groups. One-carbon metabolism reactions are reversible and deficiencies in methyl donors can cause DNA hypomethylation. For example, mice given diets deficient in the methyl donor choline showed lower global brain methylation (Niculescu et al. 2006) and elevated expression of *APP*, consistent with promoter hypomethylation (Niculescu et al. 2005). Epidemiologic studies indicate that AD patients have altered circulating levels of one-carbon metabolism members including homocysteine (HCY), SAM, folate, and vitamin B12. Elevated HCY is associated with increased risk of developing AD and

increased rate of disease progression among individuals with the disease. Prospective data from the Framingham Heart Study show that each standard deviation increase in log transformed plasma total HCY levels was associated with an adjusted relative risk of dementia of 1.8 (95% CI: 1.3-2.5) eight years after the HCY measurement (Seshadri et al. 2002). AD patients in the Oxford Project to Investigate Memory and Ageing have increased serum HCY relative to cognitively normal control subjects (n=164) and the individuals with the greatest disease progression over the subsequent three years had the highest original HCY levels (Clarke et al. 1998). SAM is a methyl-donor molecule that is hydrolyzed to form HCY, the substrate for DNA methylation. AD patients also have decreased cerebrospinal fluid SAM relative to cognitively normal controls (Bottiglieri et al. 1990).

Several proteins in the one-carbon metabolism cycle may be disturbed by Pb exposure because elemental Pb reacts with free sulfhydryl groups on proteins. HCY metabolism may be directly inhibited by Pb binding to the sulfhydryl group in HCY. Furthermore, HCY is transsulfurated into cysteine by cystathionine β-synthase (CBS) and CBS has two sulfhydryl groups with which Pb can react. There is also evidence for Pb's involvement in methionine processing. Rats developmentally treated with Pb have impaired long-term potentiation (LTP), memory, and synaptic plasticity. Co-treatment with SAM and Pb increases LTP relative to Pb treatment alone and reduces circulating blood Pb levels (Cao et al. 2008) . Similarly, neuroblastoma cells exposed to Pb experience viability loss, glutathione antioxidant depletion, membrane lipid peroxidation, DNA damage, and apoptosis; pretreatment with a methionine derivative reduces these harmful effects (Chen et al. 2011).

Pb exposure and HCY levels are linked in cross-sectional epidemiologic studies. In the Baltimore Memory and Aging Project involving greater than 1,000 adults, higher blood Pb was associated with higher HCY (Schafer et al. 2005). Analyses from the 1999-2002 National Health and Nutrition Examination Survey (NHANES) showed HCY was strongly associated (OR=1.92) with peripheral

arterial disease (PAD) (Guallar et al. 2006). Subsequent analysis showed the original association was actually due to confounding from smoking, blood Pb and cadmium levels, and impaired renal function (Guallar et al. 2006). This suggests that the association between HCY and chronic disease may be driven by environmental exposures.

Animal Model Studies Linking Pb Exposure, Epigenetics, and Amyloidogenesis

A series of rat and primate model studies conducted by the Zawia research group collectively demonstrate that early life Pb exposure reduces DNA methyltransferase activity and specifically alters the regulation of many AD pathway related genes including *APP* and *BACE1* that are known to be CpG rich. Rats exposed to Pb from post natal day (PND) 1 through PND 20 experienced a transient increase in *APP* mRNA expression in cortical brain tissue, which returned to basal levels at 1 year, and later resurged at 20 months of age in the absence of continued exposure (Basha et al. 2005b). The observed late-life rise in *APP* mRNA was accompanied by elevated Aβ, suggesting that early life Pb exposure may have long-term effects on amyloidogenesis in late life (Basha et al. 2005b). In a follow-up study on the same tissues, investigators noted the effects on Aβ formation and aggregations were not due to changes in protein levels of *APP* processing secretases (Basha et al. 2005a). In a third study using the early-life exposed rat brain tissues, elevated oxidative DNA damage measured by cerebral 8-hydroxy-2'-deoxyguanosine (8-oxo-dG) was observed in the exposed animals (Bolin et al. 2006). Local 8-oxo-dG is associated with hypomethylation at adjacent CpG sites (Cerda and Weitzman 1997). Direct oxidation of 5-methylcytosine to 5-hydroxymethylcytosine may be part of active DNA demethylation (Wu and Zhang 2010). Analogous primate experiments by Zawia *et al.* are consistent with these rodent findings. Primates exposed in early life to Pb had elevated levels of the Aβ peptide, 8-oxo-dG DNA, and mRNA from *APP* and *BACE1* on autopsy 23 years later relative to controls, suggesting Pb is involved in LOAD-like pathology (Wu et al. 2008a). Brain tissue from these

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exposed primates also had 20% reduced DNA methyltransferase 1 activity (Wu et al. 2008a) and lower methylation at the promoter of *APP* (Wu et al. 2008b). *In vivo* animal model studies spanning multiple organisms support an integrated role of Pb exposure and epigenetics in amyloidogenesis.

CHALLENGES TO LOAD EPIDEMIOLOGIC RESEARCH INTEGRATING EPIGENETICS AND Pb EXPOSURE

Human epidemiologic research integrating LOAD, environmental exposure to Pb, and epigenetics faces many challenges. Clinical criteria for AD include progressive impairment in memory in the absence of motor, sensory, or coordination deficits (McKhann et al. 1984). However, the standard of diagnosis for AD requires the pathologic post-mortem identification of Aβ plaques and tau neurofibrillary tangles. Epidemiologic studies can take advantage of predictive and diagnostic biomarkers, including a panel of plasma signaling proteins (Ray et al. 2007), cerebrospinal fluid protein analyses (De Meyer et al. 2010), magnetic resonance imaging (MRI) volumetric and structural measures (Jack et al. 1992), and positron emission tomography (PET) neuroimaging of metabolic rate and Aβ pathology (Klunk et al. 2004; Minoshima et al. 1995). However, these research methods require additional validation to become routine early detection methods (Dubois et al. 2007).

Another concern in environmental epidemiology is that the length of time between exposure and disease onset. Barker first introduced the hypothesis that early-life conditions could be linked to late life chronic disease, otherwise known as the developmental origins of health and disease (DOHaD) hypothesis (Barker and Osmond 1986). Fetal or childhood exposures have been associated with adverse health outcomes including impaired glucose tolerance (Ravelli et al. 1998) and hypertension (Barker et al. 1990; Bergvall et al. 2007). Indeed, several early life events related to growth, metabolism, and cognitive reserve have been associated with LOAD (Miller and O'Callaghan 2008). AD risk is

increased with limited education and income, and both factors are associated with poor early life environment and growth (Borenstein et al. 2006). Middle life risk factors including obesity (Whitmer et al. 2005), limited physical activity (Rovio et al. 2005), and diabetes (Luchsinger et al. 2001) are shared between AD and cardiovascular disease. Low birth weight and intrauterine growth restriction are related to metabolism, fat distribution, and insulin resistance at mid-life and it has been suggested that these early-life events may be associated with AD as well (Landrigan et al. 2005; Lester-Coll et al. 2006; Ross et al. 2007). However, LOAD is a chronic disease of old age and a prospective developmental exposure study could not feasibly follow an early life cohort for 75 years with our current late stage diagnostic measures. Additionally, retrospective exposure assessment is difficult. The human body has efficient detoxification and clearance mechanisms for many toxicants and many chemicals do not bioaccumulate in the human body. There is an acute need to develop biomarkers that correspond to prior toxicologic exposures.

Finally, an additional roadblock is that brain specific epigenetic measurements are only possible post-mortem. Molecular epidemiology research of toxicant induced disease is strengthened when performed with relevant tissue samples. Brain tissue collection is invasive and not possible longitudinally on live subjects. Model animal research and epidemiology studies of human pre-mortem available tissues such as skin, blood, colon, etc. are necessary to fill in stages of disease tissue not available through end of life epidemiologic brain banks.

POTENTIAL APPROACHES TO STUDY Pb EXPOSURE, EPIGENOMICS, AND ALZHEIMER'S DISEASE EPIDEMIOLOGY

To best understand the relationship between Pb exposure (both early-life and later life) and LOAD, studies should take advantage of available biomarkers of Pb and technologic advances in epigenetic measurements. Bone Pb levels are a strong predictor of negative health outcomes including elevated risks for hypertension (Cheng et al. 2001; Hu et al. 1996; Korrick et al. 1999), ischemic

heart disease (Jain et al. 2007), and mortality (Weisskopf et al. 2009), but the relationship between cumulative Pb exposure and LOAD has not been assessed. Cumulative Pb exposure of LOAD subjects can be measured either non-invasively *in vivo* using K-x-ray fluorescence (Hu et al. 2007) or by direct measurement of Pb in bone samples (Wittmers et al. 1988). At Alzheimer's Disease Research Centers (ADRCs) where LOAD subjects consent to brain tissue donation on autopsy, it would be most ideal to directly measure Pb in samples of cranial bone obtained at the time of brain harvesting. Measurement of Pb in the cranium is highly correlated with a weighted average of skeletal Pb levels, as well as the level of Pb in tibia bone (Hu et al. 1990), the latter being the bone most commonly measured in epidemiologic studies of chronic Pb toxicity (Hu et al. 2007). Sampling cranial bone Pb would make it possible to concurrently study LOAD epidemiology, brain tissue epigenetics and cumulative Pb exposure in post-mortem case-control studies.

Circulating epigenetic biomarkers would be useful to conduct case-control studies of Pb exposure (by *in vivo* KXRF) with live subjects. Post-mortem Alzheimer's disease brain tissue epigenetic studies are expanding, but use of this tissue collected at end of life is not feasible to track within individual changes over time as in longitudinal epidemiological aging cohort studies. Biologically-available biomarkers would allow for repeated epigenetic measures throughout the disease course. Epigenetic markers in white blood cells (WBC) have been used as biomarkers in other diseases. Global DNA methylation has been associated with several cancers, myelodysplastic syndrome, and schizophrenia and thus does not appear to be a disease specific biomarker. Gene-specific methylation data and risk factor methylation data are more limited and results are inconsistent (Terry et al. 2011). Larger, prospective cohort studies are needed to determine whether WBC gene-specific epigenetics will be informative with AD and with Pb exposure (**Figure 1.1**). Upon epigenetic biomarker development, cohort studies could integrate and target distinct age groups. Birth cohorts could investigate the role of *in utero* and postnatal Pb exposure on AD biomarkers to test the hypothesis suggested by animal research (Wu et al. 2008b) that early life

is a critical window for Pb's influence on developmental reprogramming. Mid-life cohorts could focus on later exposure periods and could incorporate traditional AD risk factors such as hypertension status and education achieved. Late-life cohorts would involve the best AD and mild cognitive impairment (MCI) diagnostic tools and study the role of cumulative lifetime Pb exposure.

Finally, epidemiologic data needs to be incorporated with epigenetic studies on ADRC brain bank tissues. Epigenetic changes are associated with age (Wang et al. 2008), sex (Anway et al. 2005), exposures (Dolinoy et al. 2007), and diseases (Waterland and Garza 1999). Alzheimer's disease specific epigenetic change may need to be extracted from a noisy background of age-specific epigenetic drift, sex-specific epigenetic marks, co-morbidity disease changes, and a lifetime of environmental exposures. The majority of existing studies of brain epigenetics focus on CpG islands and the application of array-based approaches that only cover a portion of the genome, largely in genic regions. Rapid advances in technology and reduction in costs have made new approaches using next-generation sequencing (NGS) feasible for larger sample sizes. These new approaches have been lauded as unbiased but criticized as relative (rather than quantitative) measures of DNA methylation. The depth of genome coverage will be able to provide the large amount of information needed to detect subtle changes from multiple sources. Integration of these data with ongoing studies of biomarkers in other neurodegenerative diseases and in non-diseased aging populations will help elucidate the specific epigenetic changes associated with LOAD, providing a foundation for prevention and treatment of this disease.
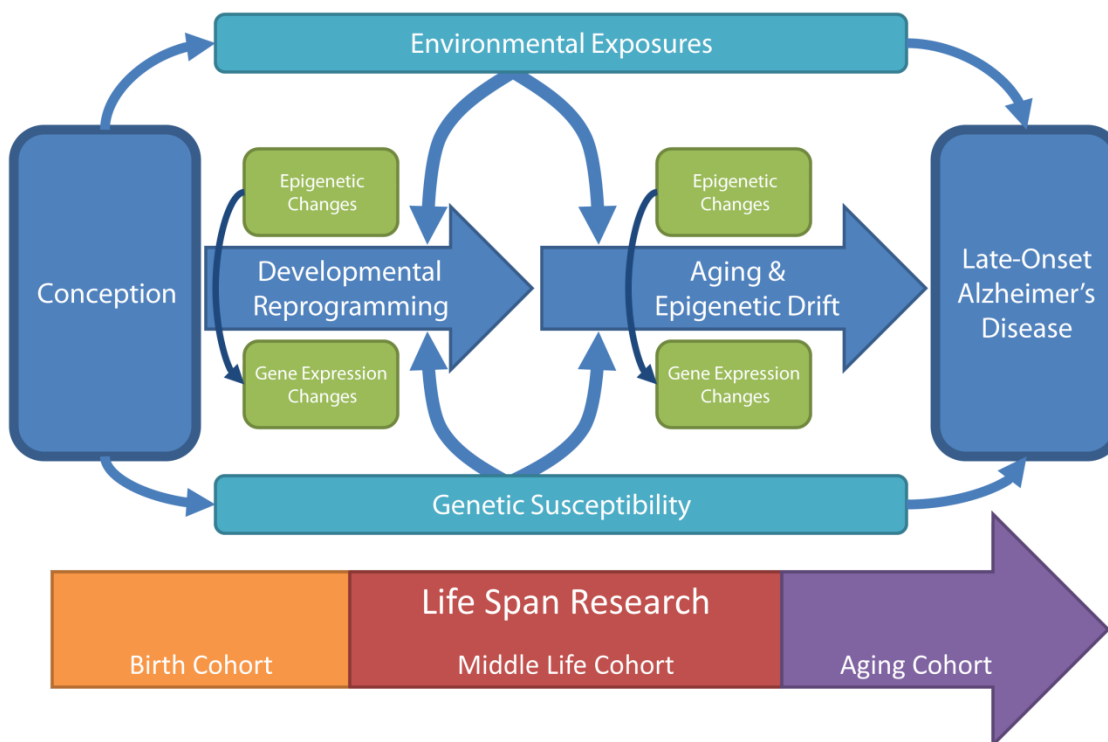
Figure 1.1. Conceptual diagram describing the relationship between environmental exposures, including to the heavy metal lead, with the development of late-onset Alzheimer's disease. There is a complex interplay of genetics and epigenetic programming. Epidemiologic cohort studies can be designed to study different stages in the life course leading to disease development.

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CHAPTER II
Research Chapter 1

Lead exposure, B-vitamins, and plasma homocysteine in the VA Normative Aging Study

ABSTRACT

Background: Epidemiologic studies suggest that elevated circulating level of homocysteine (Hcy), a one-carbon metabolite, is a risk factor for cardiovascular and neurodegenerative diseases. Although a few cross-sectional studies have evaluated the influence of environmental toxicant exposures on Hcy levels, longitudinal studies and studies of the interplay of environmental and dietary factors are lacking.

Objectives: We examined the association of recent and cumulative exposure to lead with Hcy levels cross-sectionally and longitudinally. We also determined whether lead exposure's association with Hcy varied by dietary intake of nutrients involved in one-carbon metabolism (folate and vitamins B6 and B12).

Methods: We followed 1,056 of the Normative Aging Study men (age 50-97 at visit 1) over 4 study visits (2,301 total observations) at which concurrent measures of blood lead and Hcy were collected. We determined baseline cumulative dose of lead via Cd^{109} K-shell X-ray fluorescence of the tibia and patella bones. We estimated cross-sectional differences in Hcy across levels of Pb exposure measures using generalized linear models. We also used mixed effects models to estimate differences in rate of change in Hcy over time associated with Pb exposure.

Results: Higher exposure to lead was associated with higher Hcy levels. An interquartile range (IQR) increment in tibia bone lead concentration (14µg/g) was associated with 3.64% higher Hcy at visit 1 (95% CI= 1.37-5.97). Similar results were found at other visits, but tibia Pb was not associated with trajectory in Hcy over time. An IQR increment in blood lead concentration (3 µg/dl) was associated with 8.14% higher Hcy (95% CI: 6.16-10.16) at visit 1, similar to the results at other visits. To put these findings in context, in our data, a 5-year increment in age corresponded to a 3.14% increase in Hcy (95% CI=2.88-7.87). The association between blood lead and Hcy was significantly larger among participants with lower dietary intakes of vitamins B6, B12, and folate.

Conclusion: Increasing levels of lead exposure were associated with elevated Hcy, and this relationship was stronger in individuals with low dietary folate, B6, and B12. Increased intake of folate, B6, and B12 may be an effective intervention on lead's effects on Hcy.

KEYWORDS: aging, folate, homocysteine, lead exposure

INTRODUCTION

The post-war baby boom and lengthening life spans are fueling an unprecedented growth in the population of older adults worldwide, with widespread implications for stress on public health and medical infrastructures. Thus, increasing numbers of older adults are at risk of chronic diseases that require care and monitoring over decades. Several chronic diseases, including cardiovascular disease (CVD) and neurodegenerative diseases, share a common risk factor, elevated homocysteine (Hcy), which is measured in the blood or cerebrospinal fluid. Relatively little is known about the causes of elevated Hcy; in particular, the potential influence of environmental toxicant exposures and their interplay with dietary factors. Research on the regulation of Hcy may result in opportunities for intervention prior to onset of chronic disease.

Homocysteine (Hcy)

Hcy is a thiol-containing amino acid that is highly reactive and thus short-lived in the body (Jocelyn 1972). It is an intermediate in the one-carbon metabolism cycle, formed in the production of methionine, an important methyl donor for epigenetic modifications to nucleic acids and proteins (**FIGURE 2.1**). Though physiologically normal cellular processes produce and require Hcy at low levels, elevated Hcy is associated with toxicity. Accessible cysteinyl residues in cellular proteins can react with free Hcy, forming Hcy-protein thiol-thiol interactions, altering native protein conformation and function. In addition, free Hcy can cleave accessible disulfide bridges, damaging native protein confirmations (Krumdieck and Prince 2000). Biochemical damage is based on the duration and concentration of exposure to Hcy. Long-lived proteins can accumulate irreversible Hcy-related damages, making these mechanisms especially relevant to the chronic diseases and morbidity of aging.

Elevated Hcy is a risk factor for both CVD and neurodegeneration. In epidemiologic studies, moderately elevated Hcy is associated with CVD. In a meta-analysis of 30 retrospective or prospective studies, reduced plasma Hcy

was protective against ischemic heart disease (IHD) (OR=0.89 for 25% lower Hcy), and stroke (OR=0.81) (Collaboration 2002). Similarly, a 5-µmol/L increase in Hcy corresponded to elevated odds of IHD (OR=1.23) and stroke (OR=1.42) in a meta-analysis of 20 prospective studies (Wald et al. 2002). The suggested mechanisms linking Hcy and cardiovascular outcomes include impaired endothelial elasticity and the production of reactive oxygen species (Perla-Kajan et al. 2007).

Elevated Hcy is also associated with decline in multiple cognitive domains. In the Veteran's Affairs Normative Aging Study (NAS) of older men, higher baseline Hcy was associated with reduced spatial copying and verbal recall over a three-year follow-up period (Tucker et al. 2005). Data from the Oxford Health Aging Project show high Hcy is associated with declines in general cognitive function as measured by the Mini-Mental Status Exam (MMSE) over a ten-year period (Clarke et al. 2007). In a two-year follow-up study in Korea, incidence of dementia increased across ascending quintile of Hcy at follow-up (Kim et al. 2008). In the Sacramento Area Latino Study on Aging, higher baseline Hcy was associated with increased risk of both incident dementia and cognitive impairment in the absence of dementia over the subsequent 4.5 years of follow-up (Haan et al. 2007). Hcy is also an important risk factor for Alzheimer's disease (Seshadri et al. 2002).

Circulating Hcy can be lowered with dietary interventions, primarily therapy with folic acid, vitamin B12, and vitamin B6 (Appel et al. 2000). Several randomized control trials have evaluated the effects of these therapies on cardio- and cerebrovascular events, their results have been mixed. A meta-analysis of seventeen trials of individuals with preexisting cardiovascular or renal disease showed no differences between the Hcy-lowering treatment group and control group with respect to coronary heart disease, stroke, cardiovascular events, or all-cause mortality (Mei et al. 2010). Dietary changes can reduce Hcy, but in individuals with pre-existing conditions, hypothesized improvements in health outcome do not seem to occur with the reduction (Mei et al. 2010).

Lead Exposure

The heavy metal lead (Pb), is a well-established and ubiquitous toxicant. The molecular mechanisms of lead's toxicity in the human body are numerous but incompletely characterized. Pb binds free sulfhydryl groups on proteins and can alter protein conformation and activity (Needleman 2004). Pb can form protein complexes in the kidney's proximal tube, which lead to poor blood pressure regulation and cardiovascular problems (Goyer 1989). Pb can also compete with or replace other divalent cations including calcium and iron. In the central nervous system, rodent model research indicates lead exposure reduces synaptic plasticity and hippocampal long-term potentiation (Toscano and Guilarte 2005). It also results in a cascade of molecular changes including reduced cAMP and protein kinase A activity, altered MAPK signaling, and disruption of CREB phosphorylation (Toscano and Guilarte 2005). Pb and Hcy both independently exert toxicity in part by binding to free sulfhydryl groups and damaging proteins.

Exposure to Pb is linked to numerous diseases and negative health outcomes throughout the lifespan. In late life, lead exposure, even at low levels experienced in the community, is related to CVD, as measured through hypertension (Cheng et al. 2001), heart rate variability (Park et al. 2006), and clinical CVD outcomes (Navas-Acien et al. 2007). Lead exposure is also associated with poor cognition and cognitive decline in aging populations (Shih et al. 2007; Weuve et al. 2009).

Lead Exposure and Homocysteine

Previous studies have demonstrated a relationship between Pb and Hcy at single time points. In the Baltimore Memory Study, blood Pb and plasma Hcy were significantly correlated with each other (Pearson's unadjusted r=0.27) (Schafer et al. 2005). Similarly, in Pakistan, a cross-sectional survey of persons aged 18-60 years, an increase of 1 µg/dL log blood Pb was associated with an increase of 0.09 µmol/L log Hcy after multivariable adjustment (Yakub and Iqbal

2010). In a third cross-sectional study, this time in Vietnam and Singapore, an increase of 1μg/dl log blood lead was associated with an increase of 0.04μmol/l log Hcy among occupationally exposed 449 workers (mean age=39; mean blood Pb= 22.7 μg/dl) (Chia et al. 2007).

One reason that lead exposure and elevated Hcy are associated with many of the same negative health outcomes is that lead exposure elevates Hcy. For example, in the 1999-2002 National Health and Nutrition Examination Survey (NHANES), Hcy was associated with peripheral arterial disease (PAD) (OR of PAD in the highest quintile of Hcy relative to the lowest was 1.92, p-trend=0.004), however the association disappeared following adjustment for blood lead and calcium levels as well as kidney function (OR=0.89, p-trend=0.87) (Guallar et al. 2006). This research suggests the Hcy may be a marker of exposure to other toxicants, rather than the toxic agent itself.

Pb and Hcy levels may be mechanistically linked. Several proteins in the homocysteine processing one-carbon metabolism cycle (**FIGURE 2.1**) contain sulfhydryl groups that may be potential reaction sites for lead (Schafer et al. 2005). Cystathionine β-synthase (CBS) catalyzes the breakdown of Hcy into cysteine. CBS has two sulfhydryl groups that may be subject to reaction with lead, potentially interfering with the enzyme's ability to transsulfurate Hcy into cysteine (Schafer et al. 2005). In addition, Hcy contains a sulfhydrl group and lead may directly inhibit its metabolism. There is also evidence for the protective effect of methionine on lead toxicity (Chen et al. 2011). It is hypothesized that Pb could directly influence the levels of Hcy in the body.

The association reported between lead and Hcy is plausible and supported by compelling data, but it requires further epidemiologic investigation in an additional population. Thus far, only one study has tested the Pb-Hcy relationship in a community-exposed population of older adults. There are currently no studies evaluating the relevant window of Pb exposure's effect on Hcy. Studies of cumulative Pb exposure and Hcy are needed to fill this gap. In

addition, there are no data indicating whether the association of Pb exposure with Hcy can be mitigated (or worsened) with intake of B vitamins.

Objectives

The goal of this study was to evaluate the relationship of lead exposure to plasma Hcy in a population of community-exposed older men. We tested the hypothesis that recent exposure to Pb (meausured by blood Pb) is associated with concurrent Hcy levels. Next, we evaluated whether people who have changes in their blood Pb over time also have corresponding changes in their Hcy levels. We also tested whether those with a diet low in methyl donors (folate, vitamin B6, and vitamin B12) are more susceptible to the effects of Pb exposure on Hcy. Finally we evaluated the relationship of cumulative Pb dose (tibia and patella bone Pb) and Hcy in comparison to acute/recent Pb exposure.

METHODS

Study Population

In 1963, 2,280 men in the greater Boston area between the ages of 21 and 80, and representing a range of educational and occupational backgrounds, were enrolled in the Veteran's Affairs Normative Aging Study (NAS) (Bell et al. 1972). All participants were free of disease at the onset of the study and participated in health assessments every three to five years that expanded in scope over time. Blood lead measurements began in 1977 and bone lead measurements began in 1991. Homocysteine was first measured in 1993. At each study visit, age, smoking status, medication use, physical activity, and dietary intake were assessed.

In the study of Hcy, up to six repeated measures of Hcy from 1,080 men for a total of 2,941 observations were available. Bone lead data was missing for 301 individuals and there were a total of 779 subjects across 2,252 observations that had bone lead data and Hcy measures. Approximately three years after the bone lead assessment, the first Hcy was measured. Concurrent blood lead data

was missing from 23 participants and there were 1,056 individuals with 2,301 observations of Hcy and blood Pb.   In an additional analysis of the changes in blood Pb and Hcy, a subset of the main dataset was used. At least two repeated measures of blood lead and concurrent Hcy were available from 747 men for a total of 1,830 repeated observations. The majority of blood lead measurements (>99%) were made within 30 days of Hcy measurements. The Human Subjects Institutional Review Boards at the Harvard School of Public Health, the Department of Veterans Affairs Outpatient Clinic in Boston, the Brigham and Women's Hospital, and the University of Michigan Medical School approved this study.

Plasma Homocysteine Measures

Fasting blood plasma was collected during each clinic visit and frozen at negative 80°C. Samples were analyzed at the Jean Mayer US Department of Agriculture Human Nutrition Research Center on Aging. Total Hcy was measured via fluorescence detection with high-performance liquid chromatography (HPLC) (Araki and Sako 1987). Hcy detection methods have been previously described in detail (Tucker et al. 2005). Plasma folate levels below 3 ng/mL were considered low (LSRO 1984). Plasma B6 levels at 30nmol/L were considered adequate (US RDA, NIH Office of Dietary Supplements). Plasma B12 below 250 pg/mL was considered low (US RDA, NIH Office of Dietary Supplements). Participants with low nutrient status were included in the analysis.

Lead Exposure Measures

Lead levels can be measured in various tissue types across the body to reflect different exposure times. Blood lead levels indicate recent exposure (with a half-life of approximately 30 days) (Hu et al. 1998). Blood lead levels included in this analysis were measured up to six times between 1993 and 2011 via graphite furnace atomic absorption with Zeeman background correction.

Bone lead was measured in two bone types (tibia and patella) through Cd^{109} K-shell x-ray fluorescent (KXRF) spectroscopy using methods previously described (Hu et al. 1996). The half-life for lead in cortical tibia bone is estimated at 48.6 years in the NAS (Wilker et al. 2011). The trabecular patella bone has a shorter half-life for lead (between 10-15 years) (Hu et al. 1998). The primary statistical analyses were performed using tibia Pb, while patella Pb was used as a secondary sensitivity analysis.

Dietary Measures

Annual average diet was assessed with the semi-quantitative Willett Food Frequency Questionnaire using methods described previously (van de Rest et al. 2009). Before each study visit, participants were mailed the questionnaire. Their responses were checked for completeness at the study visit. The questionnaire assesses frequency of consumption of 126 items on a scale ranging from never to ≥2 times per day. Three one-carbon metabolism dietary factors were measured without supplements and were used in the current study (folate, vitamin B6, and vitamin B12). Nutrient quantification based on FFQs administered after August, 1997 were adjusted to consider folate fortification in the US. Total calorie intake-adjusted nutrient residuals (Willett et al. 1997) (Willett et al. 1997) were calculated for each individual and included in the models.

Statistical Analysis

All analyses were performed in R Statistical Software (version R 2.15.0). Univariate descriptive statistics were calculated for each variable. We also estimated bivariate associations between Hcy, and the three lead exposure measures and additional covariates were also calculated.

We assessed the associations between blood Pb and concurrent Hcy using multivariable-adjusted linear models. Because the distribution of Hcy levels is skewed, we natural log-transformed the variable Hcy. Thus, the exponentiated parameter estimates from these models are directly interpreted as

the percentage difference in Hcy per unit increment in predictor. We adjusted these analyses for several sets of covariates. All analyses were adjusted for the following "core covariates": age, education, smoking status, alcohol consumption, and body mass index (BMI) (Schafer et al. 2005). The following plasma levels of one-carbon metabolism related compounds were considered the "plasma sensitivity covariates": plasma PLP (a measure of vitamin B6), vitamin B12, and folate. Finally, dietary measures were categorized as the "dietary sensitivity covariates": intakes of total energy and calorie adjusted residuals for vitamin B6, vitamin B12, and folate. We tested for non-linear trends using generalized additive models with penalized splines in the mgcv R package.

Using the repeated measures of Hcy and linear mixed effects models with random intercepts, we compared rates of change in Hcy (as percentage change from baseline Hcy) by level of lead biomarker. We modeled interaction terms between time and baseline age to capture the trajectories of Hcy over the follow-up period. Several error covariance matrices were considered, but they did not improve model fit, so no within-subject correlation was used. All models were adjusted for Core Covariates, Plasma Sensitivity Covariates, and Dietary Sensitivity Covariates, similar to the concurrent exposure models. In addition, all models included a cross-product term between time and baseline age. The inclusion of other time-covariate cross-products did not change the results.

We used "change-change" models to assess whether change in Pb level over time was associated with concomitant changes in Hcy. In these models, we restricted the analyses to individuals with at least two measures of Pb and Hcy (747 individuals, 1,830 observations). We regressed change in blood Pb on change in Hcy, adjusting for the core covariates.

To assess the potential modification of the association between Pb and Hcy by key nutrients, we fit models stratified by median plasma or dietary levels of folate and vitamins B6 and B12. We used methods by Payton et al. (Payton et al. 2003) to test for differences in the effect of lead on Hcy between the high and low nutrient strata. This permits other covariate-Hcy associations to vary across

the strata, associations that may influence the stratum-specific Pb association. In addition, however, we fit models, incorporating all observations that included a cross-product term between each Pb marker measure and nutrient measure.

Additional analyses were performed to determine whether the effects of cumulative lead dose were mediated through current blood lead levels. Our secondary aim was to determine whether lead-associated changes in Hcy were associated with recent exposure measured by blood lead or cumulative dose measured by tibia lead. We used mixed effects core covariate models containing tibia Pb, both tibia Pb and blood Pb, and blood Pb alone as a basic mediation analysis.

RESULTS

The univariate descriptive statistics for subjects with blood Pb levels are presented in Table 1. These values are similar to those for the population with tibia bone lead measures. The geometric mean Hcy level in this population was 10.2 nmol/ml (GSD= 1.3) (**TABLE 2.1**). Mean (SD) blood, tibia bone, and patella bone lead levels were 4.4(2.5) µg/dl, 21(13) µg/g, and 29.9(19.5) µg/g, respectively. Similar to the general US population at this time, blood lead levels decreased across study visits. In general, this population was elderly (age 70.9 (7.3)), overweight (BMI 28(4)), educated (64.2% with some post-high school education), moderate-heavy drinkers (20.6% with more than 2 drinks per day), and former smokers (66.2%).

At visit one, log(Hcy) was positively correlated with blood Pb, tibia bone Pb, and patella bone Pb (respectively Pearson's r=0.28 p=2×10^{-16}; r=0.15 p=2×10^{-5}; r=0.16, p=3×10^{-5}) (**Supplemental Figure 2.1**). The bivariate associations between Hcy and other covariates before and after age-adjustment at study visit 1 are listed in **Table 2.2**. Hcy was positively associated with increasing linear trend in categories of blood Pb, tibia bone Pb, patella Pb, age, alcohol consumption, and smoking status. Hcy was inversely associated with plasma PLP (B6), B12, folate and dietary B6 levels. Similar associations were

observed at the other study visits (data not shown). A notable exception is that smoking was only associated with Hcy at study visit one.   Similar results are also observed in the population with tibia bone Pb levels (n=774) (data not shown). The bivariate associations between covariates and blood Pb as well as tibia bone Pb are also presented in **Table 2.2**. Blood Pb was positively associated with age and alcohol intake. Blood Pb was negatively associated with increased education, plasma B6, plasma B12, plasma folate, dietary B6, and dietary B12. Tibia bone Pb was positively associated with smoking status (current smokers with higher exposure) and tibia bone Pb was negatively associated with increased education, plasma B6, plasma folate, and dietary B6.

After adjustment for age, education, alcohol, smoking, and BMI (Core Covariates) an IQR increment in blood Pb (3 ug/dl) at visit 1 was associated with an 8.0 percent increase in Hcy (95% CI: 6.0-10.0). By comparison, a 5-year increment in age was associated with a 3.2 percent increase in Hcy (95% CI=2.0-4.4) (**FIGURE 2.2A**). The percent difference in Hcy at visit 1 between never smokers and current smokers was 14.0 (95% CI: 5.6-23.1). The difference in Hcy between never smokers and former smokers was not statistically significant. Next we added to the model plasma factors that influence Hcy metabolism. After further adjustment for the plasma sensitivity covariates (plasma PLP, B12 and folate), an IQR increment in blood Pb was associated with 6.4% increase in Hcy (95% CI: 4.5-8.3), while after further adjustment for the dietary sensitivity covariates (core covariates plus total energy consumption, dietary B6, B12, and folate), an IQR increment in blood Pb was associated with 5.6 in Hcy (95% CI: 1.4-9.9). These results were consistent across the 4 study visits (**FIGURE 2.2A**).

Results from the mixed effects models of the repeated blood Pb and Hcy measures were consistent with those from the models based on single visits (**TABLE 2.3**).   Plasma B6, plasma B12, plasma folate, and dietary B6 were significant negative predictors of log(Hcy). In the core model, a 3 ug/dl (IQR) increase in blood Pb is associated with 6.0% increase in Hcy (95% CI: 4.6-7.5).

The adverse association between blood Pb and Hcy was stronger among men whose dietary intake of vitamin plasma B6, B12, or folate fell below the median B6: 2.24 mg/day, B12: 6.185 mcg/day, folate: 343 mcg/day) (**FIGURE 2.4**). Results were similar albeit weaker for the plasma vitamin measures. None of the blood lead estimates for men with the higher vitamin levels was significantly different from the corresponding estimates for men with the lower vitamin levels. Data was stratified by median plasma level (B6: 64.1 nmol/L, B12: 447.5 pg/mL, folate: 11.4 ng/mL) or median dietary levels (B6: 2.24 mg, B12: 6.185 mcg, folate: 343 mcg). However, when we modeled the interaction between blood Pb and the nutrients using cross-product terms, the interactions corresponding to dietary B6, dietary folate, and plasma folate were statistically significant (p-values for interaction: 9.6×10^{-5}, 0.0016, and 0.024 respectively), and the interaction corresponding to dietary B12 was borderline significant (p-value for interaction: 0.051).

Among the 747 men with two or more measures of blood Pb and Hcy, blood Pb levels generally declined over time (mean) but this decline was variable (SD or range). Changes in Hcy generally "tracked" changes in blood Pb, with a 1-µg/dL drop in blood Pb corresponding to a 5% drop in Hcy, but this association was not statistically significant (**Supplemental Tables 2.1 and 2.2**).

Higher cumulative exposure to Pb, as measured by tibia bone lead concentration, was also significantly associated with increased plasma Hcy. Using all four study visits, tibia bone Pb was a significant predictor of log(Hcy) (p-value =0.0012) (**TABLE 2.4**). Similarly, at visit 1 alone, tibia Pb predicted log(Hcy) (p-value=0.016). Next we looked at each visit individually using linear regression with three sets of covariates. Similar to the blood lead analysis, after adjusting for the Core Covariates (age, education, alcohol, smoking, and BMI) an IQR change in tibia bone Pb (14 ug/g) at visit 1 was associated with a 3.6 percent increase in Hcy (95% CI: 1.4-6.0) (**FIGURE 2.2B**). After further adjustment for plasma PLP, B12 and folate, an IQR change in tibia Pb was associated with 2.4 percent increase in Hcy (95% CI: 0.2-4.6). Finally in the

dietary sensitivity model, an IQR change in tibia Pb was associated with 2.5% increase in Hcy (95% CI: -0.3-5.4). Similar results were observed across the four study visits.

While cumulative exposure to lead was associated with consistently higher plasma Hcy over the course of follow-up, it was not associated with the degree to which plasma Hcy *changed* over time (**TABLE 2.5)**. On average, Hcy levels increased by about 1.2% per year of follow-up. An IQR increment in tibia Pb (14 µg/g) corresponded to a negligible 0.02% slower percentage change in Hcy over time (95% CI: XX).

There were no significant differences in the concurrent effect of bone Pb on Hcy between persons with low and high plasma or dietary B vitamins or folate. In a subset of individuals (n=221) with at least three repeated visits with dietary information (n=691 observations), we tested for a long term dietary influence on Hcy. We averaged dietary intake of folate and vitamins B6 and B12 over three-four visits and used mixed effects models to test for dietary associations with Hcy. In this smaller set of individuals, tibia Pb was no longer a significant predictor of Hcy and we did not observe a long term dietary trend (**Supplemental Table 2.3**).

To determine whether the association between cumulative exposure to Pb and Hcy was mediated by current exposure to Pb, we further adjusted the analyses of tibia Pb for blood Pb as a predictor (**TABLE 2.6**). Adding blood Pb to the model removed the association between tibia Pb and Hcy. We see similar results when we restrict the dataset to just visit one and compare the linear multivariate models containing (1) core covariates and tibia Pb, (2) core covariates, tibia and blood Pb, and (3) core covariates and blood Pb (**Supplemental Table 2.4**). Tibia Pb is an significant predictor of Hcy at visit one, but it drops out of the model with the addition of blood Pb. This suggests that the association between tibia Pb and Hcy may be mediated by blood Pb.

DISCUSSION

This cohort study examined the association between Pb exposure and circulating Hcy levels in a population of elderly community-dwelling men in the greater Boston area. Increased current blood Pb exposure was associated with greater Hcy levels. These effects were stronger in individuals with low dietary vitamins B6, B12, and folate as well as plasma folate. Greater cumulative exposure to Pb, measured in the tibia and patella bones, was associated with higher levels of Hcy. Further analyses revealed that these results may be mediated through current blood Pb and that Hcy responds to acute Pb dose.

The present finding that Pb exposure is associated with Hcy levels is consistent with prior epidemiological studies. The Baltimore Memory Study examined the cross-sectional association between Pb and Hcy in a population of 1,037 adults (mean age= 59) with similar blood Pb and Hcy to our study. The analysis observed a 1.0 ug/dL increase in blood lead was associated with a 0.43 µmol/L increase in Hcy in males, but no association was observed with tibia Pb (Schafer et al. 2005). Among 872 adults in Pakistan age 18-60, with mean blood Pb 11.65± 5.5 µg/dl, an increase of 1 µg/dl log blood Pb was cross-sectionally associated with an increase of 0.09 µmol/l log Hcy (Yakub and Iqbal 2010). In a group of 276 workers occupationally exposed to Pb in Vietnam, blood lead and Hcy were associated (Pearson's correlation=0.255, $p<0.01$) (Chia et al. 2007). Our research has confirmed the association between lead exposure and Hcy demonstrated in the above studies. However, these studies did not examine longitudinal change in Hcy and they did not assess how the association might be influenced by intake of folate and B-vitamins.

The results of this study are plausible given that Pb and Hcy share toxic mechanisms. Both form stable disulfide bonds with protein cysteine residues, potentially altering protein function. In particular, albumin (the dominant protein in blood) Cys34 has a low pKa that readily reacts with metals and Hcy (Carter and Ho 1994). In addition, both are associated with inflammation (Hcy through NF-kB activation of IL-8 and MCP-1 (Perla-Kajan et al. 2007); and Pb through cytokine production (Heo et al. 1996)). Given the independent cell culture and

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animal model evidence on each toxicant (Pb and Hcy), our epidemiological observations on the associations between Pb and Hcy, with interactions with B-vitamins, are plausible. Studies are needed to examine the health effects of combined exposure to Pb and these vitamins, and the degree to which they are mediated by Hcy.

The current analyses are influenced by a few limitations of the data. First, there may be error in the measurement of Hcy. The time of day of blood draw for Hcy measures was not standardized. In healthy individuals over a 24-hour period, Hcy levels showed a daily rhythm characterized by an evening peak and nighttime low (Bonsch et al. 2007). Whole blood genomic DNA methylation varies throughout the day and is inversely correlated with Hcy levels (Bonsch et al. 2007). Unstandardized Hcy collection times in our study would have biased our results towards the null hypothesis of no association between Hcy and Pb. In addition, plasma measures of Hcy assess the pool of Hcy released after reduction of all disulfide bonds in the sample. Total Hcy does not include homocysteine thiolacetone (a product of misincorporation of Hcy into proteins and subsequent error-editing) or Hcy bound to protein by an amide bond (Perla-Kajan et al. 2007). These Hcy groups are potentially toxic and may not be correlated with plasma concentrations of Hcy (Perla-Kajan et al. 2007).

Another potential weakness of this study is the limited power to detect interactions. This problem is exacerbated in our study due to missing nutrient data and smaller sample sizes in dietary models. The change-change analyses also have limited power as participants were lost to follow-up over time.

Epidemiologic studies that identify mediators linking exposure and outcome strengthen the model's biological plausibility and potential causal relationship (Hafeman 2011). In this study, we measured blood Pb as a mediator of tibia Pb. Our results are based on the assumption that there are no unmeasured confounders of the causal effect of the mediator (blood Pb) on the outcome (Hcy) (Cole and Hernan 2002). With these caveats in mind, our analysis incorporating blood Pb and tibia Pb suggest that the influence of Pb exposure on

Hcy is fairly immediate and thus that recent exposure to Pb (measured in blood) may be driving the association between Pb and Hcy. Despite cumulative exposure, interventions that lower acute blood Pb levels may be an effective strategy to lower Hcy.

The current study is strengthened by the use of repeated measures of Hcy and blood Pb as well as repeated measures in Hcy with baseline bone Pb. This allows us to look at longitudinal changes in Hcy with Pb exposure, not simply cross-sectional associations. This study is the first to examine the Pb-Hcy relationship while examining plausible dietary interactions, namely folate and vitamins B6 and B12.

Since Pb exposure is related to elevated Hcy levels, toxicology studies are needed to determine the mechanism and potential reversibility of Pb and disturbed Hcy metabolism. Based on the current study, dietary intervention with folate and vitamins B6 and B12 may be a potential option to remediate elevated Hcy high Pb exposed individuals.

In the 1999-2002 US National Health and Nutritional Examination Study (NHANES), Hcy was cross-sectionally associated with peripheral arterial disease (PAD), but adjustment with blood Pb, Cd, smoking, and glomerular filtration rate removed the Hcy association (Guallar et al. 2006). This suggests that Pb and Hcy levels are related and are associated with cardiovascular effects. Future research may test this association and the association with cognitive decline in cohort studies.

In conclusion, we report a significant association between blood, patella, and tibia Pb levels with higher levels of plasma Hcy in a group of older men. The association corresponding to blood Pb was strongest, suggesting that circulating lead may influence circulating Hcy through its metabolism, even at very low levels of exposure. The effects of chronic lead exposure are also supported by these results.

Diet may modify the association between blood Pb and Hcy. The adverse effect of blood lead on Hcy may be worse in the presence of low folate, vitamin B6, and vitamin B12 intake (equivalently: the adverse effect of low folate intake on Hcy may be worse in the presence of lead, even for very modest levels of lead exposure). Altered Hcy levels, such as those observed in the range here, may increase risk for cardiovascular and neurodegenerative disease and measures should be taken to reduce blood lead levels and improve dietary methyl donor diet status.

TABLES

Table 2.1. Univariate Statistics: Characteristics of individuals with complete Hcy and blood Pb (2234 obs, 1048 individuals). Mean (S.D.), except where noted.

	All Visits		Visit 1		Visit 2		Visit 3		Visit 4	
N	1056 individuals (2301 observations)		1056		747		400		98	
Parameter	**Mean (SD)**	**N Missing (%)**	**Mean (SD)**	**N Miss(%)**	**Mean (SD)**	**N Miss(%)**	**Mean (SD)**	**N Miss(%)**	**Mean (SD)**	**N Miss(%)**
Homocysteine (µmol/l)[*]	10.2(1.3)	0(0)	10.1(1.3)	0(0)	10.2(1.3)	0(0)	10.5(1.3)	0(0)	10.8(1.3)	0(0)
Blood Pb (µg/dl)	4.4(2.5)	0(0)	4.9(2.7)	0(0)	4.2(2.3)	0(0)	3.6(2.2)	0(0)	3.4(2.2)	0(0)
Age (years)	70.9(7.3)	0(0)	69(7.4)	0(0)	71.3(6.9)	0(0)	74(6.5)	0(0)	76.4(5.9)	0(0)
Education < HS [n(%)] HS [n(%)] Some college [n(%)] College [n(%)] > College [n(%)]	157(6.8) 668(29) 630(27.4) 449(19.5) 397(17.3)	0(0)	80(7.6) 303(28.7) 288(27.3) 202(19.1) 183(17.3)	0(0)	48(6.4) 219(29.3) 204(27.3) 151(20.2) 125(16.9)	0(0)	24(6.0) 118(29.5) 107(26.8) 79(19.8) 72(18.0)	0(0)	5(5.1) 28(28.6) 31(31.6) 17(17.3) 17(17.3)	0(0)
Smoking Status Never [n(%)] Former [n(%)] Current [n(%)]	663(28.8) 1524(66.2) 114(5)	0(0)	296(28.0) 698(66.1) 62(5.9)	0(0)	211(28.2) 502(67.2) 34(4.6)	0(0)	114(28.5) 272(68.0) 14(3.5)	0(0)	42(42.9) 52(53.1) 4(4.1)	0(0)
Alcohol Consumption ≤ 2 drinks/day [n(%)] > 2 drinks/day [n(%)]	1827(79.4) 474(20.6)	0(0)	834(79.0) 222(21.0)	0(0)	594(49.5) 153(20.5)	0(0)	320(80.0) 80(20.0)	0(0)	79(80.6) 19(19.4)	0(0)
BMI	28(4)	0(0)	28(3.9)	0(0)	28.2(4)	0(0)	27.9(4.2)	0(0)	27.5(3.7)	0(0)
Tibia Pb (µg/g)			21.4(13.5)	285(27)						
Patella Pb (µg/ml)			30.6(20.1)	289(27.4)						
Plasma B6 (nmol/l)	93.8(92.5)	27(1.2)	87.5(87.2)	12(1.1)	97(93.7)	9(1.2)	99.2(93.5)	5(1.2)	114.9(125.5)	1(1)
Plasma B12 (pg/ml)	490(235.8)	26(1.1)	466.4(222)	21(2)	493.4(244.1)	2(0.3)	519.9(230.5)	3(0.8)	593.4(289.4)	0(0)
Plasma Folate (ng/ml)	14.2(11.7)	35(1.5)	11(7)	30(2.8)	14.8(12.9)	2(0.3)	19.4(15.2)	3(0.8)	22.2(14.7)	0(0)
Total Energy Consumption (kcal)	1978.3(643.1)	150(6.5)	1967.4(618.4)	70(6.6)	1985.3(629.6)	43(5.8)	2012.7(730.8)	28(7)	1898.9(626.3)	9(9.2)
Dietary B6 (w/o supp) (mg)	2.4(1)	104(4.5)	2.3(0.9)	49(4.6)	2.4(0.9)	25(3.3)	2.4(1.1)	22(5.5)	2.4(1.1)	8(8.2)
Dietary B12 (w/o supp) (mcg)	7.5(4.8)	104(4.5)	7.9(5.5)	49(4.6)	6.9(3.8)	25(3.3)	7.4(4.4)	22(5.5)	8.1(5)	8(8.2)
Dietary Folate (w/o supp) (mcg)	379.1(179)	1034(44.9)	344(150.5)	794(75.2)	356.5(164)	210(28.1)	418.5(195)	22(5.5)	451.2(218.4)	8(8.2)

[*] Geometric Mean and Geometric Standard Deviation are reported.

Table 2.2. Bivariate statistics based on visit 1. Characteristics of individuals with complete Hcy and blood Pb (1056 individuals).

Variable	Category	N (%)	Geometric Mean Hcy (GSD)	P-trend	Age-Adjusted Least Squares Mean Hcy	Age-Adjusted P-trend	Mean Blood Pb (SD)	P-trend	Mean Tibia Pb (SD)	P-trend
Blood Pb (ug/dl)	≤ 3	367(34.8)	9.3(1.3)		9.7		2.4(0.8)		16(8.5)	
	3<x≤5	347(32.9)	10.1(1.3)	7E-16	10.4	5E-15	4.4(0.5)	4E-270	21.1(12.3)	6E-23
	> 5	342(32.4)	11.1(1.4)		11.6		8(2.4)		27.4(16.4)	
Tibia Pb (ug/g)	≤ 15	267(25.3)	9.5(1.3)		10.0		3.7(2.1)		9.8(4.1)	
	15<x≤23	248(23.5)	10.1(1.3)	3E-06	10.5	2E-04	4.6(2.3)	4E-26	19.2(2.3)	4E-150
	> 23	256(24.2)	10.7(1.3)		11.1		6.2(3.2)		35.7(13.3)	
	Missing	285(27)	10.2(1.3)		10.6		4.8(2.6)		NaN(NA)	
Patella Pb (ug/g)	≤ 20	255(24.1)	9.7(1.3)		10.1		3.8(2.1)		13.4(7.1)	
	20<x≤34	258(24.4)	9.8(1.3)	6E-07	10.2	4E-05	4.5(2.4)	4E-24	18.6(8)	9E-58
	>34	253(24)	11(1.3)		11.3		6.3(3.2)		32.5(15.6)	
	Missing	290(27.5)	10.1(1.3)		10.6		4.8(2.6)		13.6(12.5)	
Age Tertile	50-65	355(33.6)	9.8(1.4)		10.6		4.6(2.6)		16.5(9.3)	
	66-71	327(31)	9.9(1.3)	2E-05	10.3	0.8	4.9(2.8)	0.03	21.8(12.3)	0.4
	72-97	374(35.4)	10.7(1.3)		10.8		5(2.8)		26(16.3)	
Education	< HS	80(7.6)	9.8(1.3)		10.1		5.8(3)		30.2(18.6)	
	HS	303(28.7)	10.2(1.4)		10.7		5.2(2.9)		24.7(15.9)	
	Some College	288(27.3)	10.1(1.3)	0.9	10.6	0.8	4.8(2.7)	8E-06	20.3(11)	3E-15
	College	202(19.1)	9.9(1.3)		10.3		4.5(2.3)		18.9(11.3)	
	> College	183(17.3)	10.2(1.3)		10.7		4.4(2.8)		17(9.4)	
Smoking Status	Never	296(28)	9.8(1.3)		10.2		4.8(3)		20(13.7)	
	Former	698(66.1)	10.1(1.3)	0.005	10.6	0.0002	4.8(2.6)	0.1	22(13.6)	0.002
	Current	62(5.9)	11.2(1.3)		12.0		5.8(3.2)		22.1(11.5)	
Two Drinks/Day	No	834(79)	9.9(1.3)	0.00007	10.3	0.000006	4.6(2.7)	2E-07	21.4(13.9)	0.5
	Yes	222(21)	10.8(1.4)		11.5		5.7(2.9)		21.2(12.1)	
BMI	< 25	216(20.5)	10.1(1.3)		10.4		4.9(2.8)		21.4(11.5)	
	25≤x<30	574(54.4)	10.1(1.3)	0.9	10.6	0.5	4.8(2.7)	0.5	21.8(14.3)	0.3
	≥30	266(25.2)	10.1(1.3)		10.6		4.8(2.8)		20.4(13.3)	
Plasma B6 (nmol/L)	<30	108(10.2)	11.3(1.4)		12.0		5.7(3.4)		27.2(14.6)	
	≥30	936(88.6)	10(1.3)	1E-05	10.4	7E-06	4.7(2.6)	5E-04	20.6(13.2)	2E-05
	Missing	12(1.1)	9.5(1.2)		9.7		5.3(2.8)		30.7(12.4)	
Plasma B12 (pg/mL)	<250	82(7.8)	12.3(1.4)		13.0		5.8(3.2)		22.6(12.6)	
	≥ 250	953(90.2)	9.9(1.3)	1E-10	10.3	2E-11	4.8(2.7)	6E-04	21.2(13.5)	2E-01
	Missing	21(2)	10.5(1.4)		11.2		4.9(2.4)		28.2(24.1)	

Plasma Folate (ng/mL)	< 3	24(2.3)	13.7(1.5)		15.0		7.2(4.2)		29.1(15.2)	
	≥ 3	1002(94.9)	10(1.3)	9E-08	10.4	1E-08	4.8(2.7)	3E-05	21.1(13.2)	4E-03
	Missing	30(2.8)	10.3(1.4)		10.8		4.7(2.2)		28.6(21.5)	
Total Energy Consumption	<2300	739(70)	10.1(1.3)		10.5		4.9(2.8)		21.6(13.7)	
	≥2300	247(23.4)	10.2(1.3)	0.8	10.7	0.7	4.7(2.8)	0.5	20.8(13.6)	0.6
	Missing	70(6.6)	10.1(1.3)		10.4		4.8(2.4)		21.4(10.5)	
Dietary B6 (mg)	<1.7	774(73.3)	10(1.3)		10.4		4.7(2.7)		21.1(13.2)	
	≥1.7	233(22.1)	10.6(1.3)	0.006	11.2	0.001	5.3(2.8)	0.007	22.7(15)	0.05
	Missing	49(4.6)	10.1(1.4)		10.4		4.8(2.5)		20.2(10.9)	
Dietary B12 (mcg)	<2.4	960(90.9)	10.1(1.3)		10.5		4.8(2.7)		21.3(13.6)	
	≥2.4	47(4.5)	10.6(1.4)	0.3	11.3	0.2	5.6(2.9)	0.04	25(14.7)	0.06
	Missing	49(4.6)	10.1(1.4)		10.4		4.8(2.5)		20.2(10.9)	
Dietary Folate (mcg)	<400	73(6.9)	9.9(1.3)		10.1		4.1(2.2)		19.8(19.6)	
	≥400	189(17.9)	9.9(1.3)	0.9	10.2	0.8	4.6(2.5)	0.1	21.1(14.7)	0.2
	Missing	794(75.2)	10.2(1.3)		10.7		5(2.8)		21.6(12.5)	

Table 2.3. Concurrent Pb exposure is associated with plasma homocysteine: Mixed effects model, random intercept only. Equivalent to cross-sectional model taking into account correlated nature of observations from the same individual. Linear mixed effects model of $\log(Hcy)_t = b0 + b1[\text{blood Pb}]_t + \text{covariates}$. Continuous variables have been centered so the intercept is interpretable.

	Core Model*		Plasma Model**		Diet Model***	
	2301 obs from 1056 indiv R2=0.10		2240 obs from 1033 R2=0.16		1241 obs from 779 indiv R2=0.13	
	Beta(SE)	p-value	Beta(SE)	p-value	Beta(SE)	p-value
(Intercept)	2.30(0.015)	0	2.30(0.014)	0	2.29(0.017)	0
Blood Pb (per µg/dl)	0.0195(0.0023)	4E-17	0.0159(0.0023)	6E-12	0.0197(0.0035)	2E-08
Age	0.0091(0.00084)	2E-26	0.0105(0.00085)	2E-33	0.0118(0.0012)	3E-22
Education (reference>hs)	-0.0127(0.013)	0.3	-0.0157(0.013)	0.2	0.00255(0.017)	0.9
Alcohol Consumption	0.0576(0.015)	2E-04	0.0572(0.015)	0.0002	0.0713(0.02)	0.0005
Former Smoker	0.0176(0.017)	0.3	0.0148(0.016)	0.4	0.0123(0.019)	0.5
Never Smoker	0.0690(0.032)	0.03	0.0646(0.031)	0.04	-0.0077(0.042)	0.9
BMI	0.00191(0.0018)	0.3	0.00129(0.0017)	0.5	0.00326(0.0021)	0.1
Plasma B6			-0.000256(6e-05)	2E-05		
Plasma B12			-0.000186(2.4e-05)	5E-15		
Plasma Folate			-0.00115(0.00043)	0.008		
Dietary B6 Residual					-0.0310(0.014)	0.03
Dietary B12 Residual					-0.00308(0.0021)	0.1
Dietary Folate Residual					3.03e-05(6.4e-05)	0.6
Total Energy Intake					-1.64e-05(1.1e-05)	0.1
*Core model adjusts for blood Pb, age, bmi, education, smoking status, and alcohol consumption.						
**Plasma model adjusts for core model covariates and plasma B6 (PLP), B12, and folate.						
***Diet model adjusts for core model covariates and total energy adjusted dietary FFQ vitamin B6, vitamin B12, and folate.						

Table 2.4. Cumulative exposure is associated with plasma homocysteine: Mixed effects model, random intercept only. Equivalent to cross-sectional model taking into account correlated nature of observations from the same individual.

	Core Model			
	Visits 1-4		**Visit 1**	
	2158 observations from 777 individuals R2=0.6		777 obs from 777 individuals R2=0.061	
	β(SE)	**p-value**	**β(SE)**	**p-value**
(Intercept)	2.32(0.016)	0	2.29(0.02)	0
Tibia Pb	0.00215(0.00066)	0.001	0.00256(0.00081)	0.002
Age	0.00921(0.00094)	4E-22	0.00526(0.0015)	0.0007
High School	-0.0148(0.015)	0.3	-0.036(0.022)	0.09
Two Drinks/Day	0.0675(0.017)	8 E-05	0.0817(0.025)	0.001
Former Smoker	0.00934(0.019)	0.6	0.0291(0.022)	0.2
Current Smoker	0.0422(0.035)	0.2	0.143(0.047)	0.002
BMI	0.00113(0.002)	0.6	0.00278(0.0026)	0.3

Table 2.5. Longitudinal mixed effects models: Log(hcy) is outcome and tibia pb is main predictor. Continuous covariates have been mean adjusted so that the intercept can be interpreted as the log(hcy) at the mean of those covariates and when dummy variables =0. Four visits of tibia Pb and Hcy have been used. Random intercept and slope.

	Core Model*		Plasma Sensitivity Model**		Diet Sensitivity Model***	
	n= 2158, R2=0.0811		n= 2106, R2=0.114		n= 1328, R2=0.098	
	β (SE)	p-value	β (SE)	p-value	β (SE)	p-value
Intercept	2.28(0.017)	0	2.25(0.019)	0	2.24(0.022)	0
Tibia Pb	0.00255(0.00077)	0.0009	0.00254(0.00076)	0.0009	0.00214(0.001)	0.04
Time Since Baseline	0.0123(0.0014)	2E-18	0.0149(0.0015)	1E-23	0.0178(0.0022)	6E-15
Baseline Age	0.0049(0.0015)	0.0009	0.005(0.0014)	0.0006	0.00665(0.0021)	0.001
Education	-0.0186(0.014)	0.2	-0.0211(0.014)	0.1	-0.00374(0.018)	0.8
Alcohol Consumption	0.0701(0.017)	4E-05	0.0684(0.017)	6E-05	0.0748(0.022)	0.0008
Former Smoker	0.00509(0.019)	0.8	0.00736(0.018)	0.7	0.0034(0.021)	0.9
Current Smoker	0.0301(0.036)	0.4	0.031(0.035)	0.4	-0.00147(0.045)	0.9
BMI	0.00131(0.002)	0.5	0.000636(0.002)	0.8	0.00288(0.0024)	0.2
Plasma B6			-0.000165(5.8e-05)	0.004		
Plasma B12			-6.4e-05(1.8e-05)	0.0005		
Plasma Folate			-0.00159(4e-04)	7E-05		
Dietary B6					-0.0284(0.014)	0.04
Dietary B12					-0.000564(0.0018)	0.8
Dietary Folate					7.84e-05(6.4e-05)	0.2
Total Energy Intake					-3.03e-05(1.1e-05)	0.005
Baseline Age * Time	0.000881(0.00022)	8E-05	0.000901(0.00022)	5E-05	0.000675(0.00031)	0.03
Tibia Pb * Time	-1.72e-05(0.00011)	0.9	-3.24e-05(0.00011)	0.8	3.77e-05(0.00015)	0.8

*Core model adjusts for tibia Pb, baseline age, bmi, education, smoking status, alcohol consumption, time since baseline, baseline age*time since baseline, and tibia Pb*time since baseline.

**Plasma model adjusts for core model covariates and plasma B6 (PLP), B12, and folate.

***Diet model adjusts for core model covariates and total energy adjusted dietary FFQ vitamin B6, vitamin B12, and folate.

Table 2.6. Basic mediation analysis. All visits main effect of tibia Pb with and without current blood Pb adjustment. Note: Tibia Pb is no longer significant after adjusting for blood Pb.   **Research Question: Does cumulative exposure to Pb influence Hcy levels, independent of current exposure to Pb?**

	Tibia Only		Blood and Tibia		Blood Only	
	n=1766 obs, n=771 indiv		n=1766 obs, n=771 indiv		n=1766 obs, n=771 indiv	
	R2=0.0711		R2=0.104		R2=0.104	
	Beta(SE)	p-value	Beta(SE)	p-value	Beta(SE)	p-value
(Intercept)	2.3(0.017)	0	2.3(0.017)	0	2.3(0.016)	0
Tibia Pb	0.0025(0.00069)	0.0003	0.000832(0.00072)	0.3		
Blood Pb			0.0183(0.0028)	4E-11	0.0194(0.0026)	8E-14
Age	0.00715(0.001)	2E-12	0.00886(0.001)	2E-17	0.00922(0.00098)	2E-20
Education (>hs reference)	-0.0148(0.015)	0.3	-0.0171(0.015)	0.3	-0.014(0.015)	0.4
Alcohol Consumption	0.0794(0.018)	1E-05	0.0682(0.018)	0.0001	0.0676(0.018)	0.0001
Former Smoker	0.00525(0.019)	0.8	0.0101(0.019)	0.6	0.0119(0.019)	0.5
Current Smoker	0.0606(0.037)	0.1	0.0505(0.036)	0.2	0.0521(0.036)	0.2
BMI	0.000694(0.0021)	0.7	0.00162(0.002)	0.4	0.00172(0.002)	0.4

FIGURES
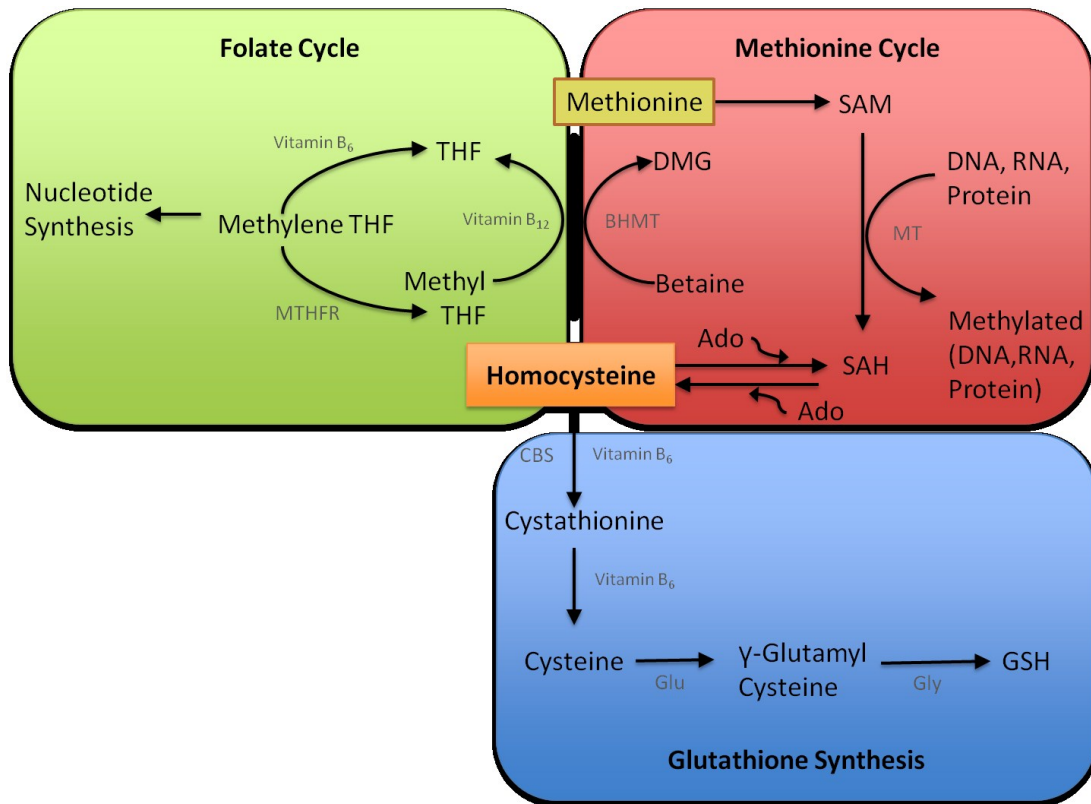
FIGURE 2.1. One-Carbon Metabolism Pathway. Homocysteine can be elevated in conditions of low folate, low B6, or low B12. The sulfhydryl groups on several proteins, including Cystathionine β-synthase (CBS), in the one-carbon metabolism pathway are potential sites for lead's interferences. Abbreviations Used: Adenosine (Ado), S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), Glutathione (GSH), Glutamate (Glu), Glycine (Gly), Tetrahydrofolate (THF), Methionine Transferase (MT), Betaine-Homocysteine S-Methyltransferase (BHMT), Dimethylglycine (DMG), Methylenetetrahydrofolate Reductase (MTHFR)
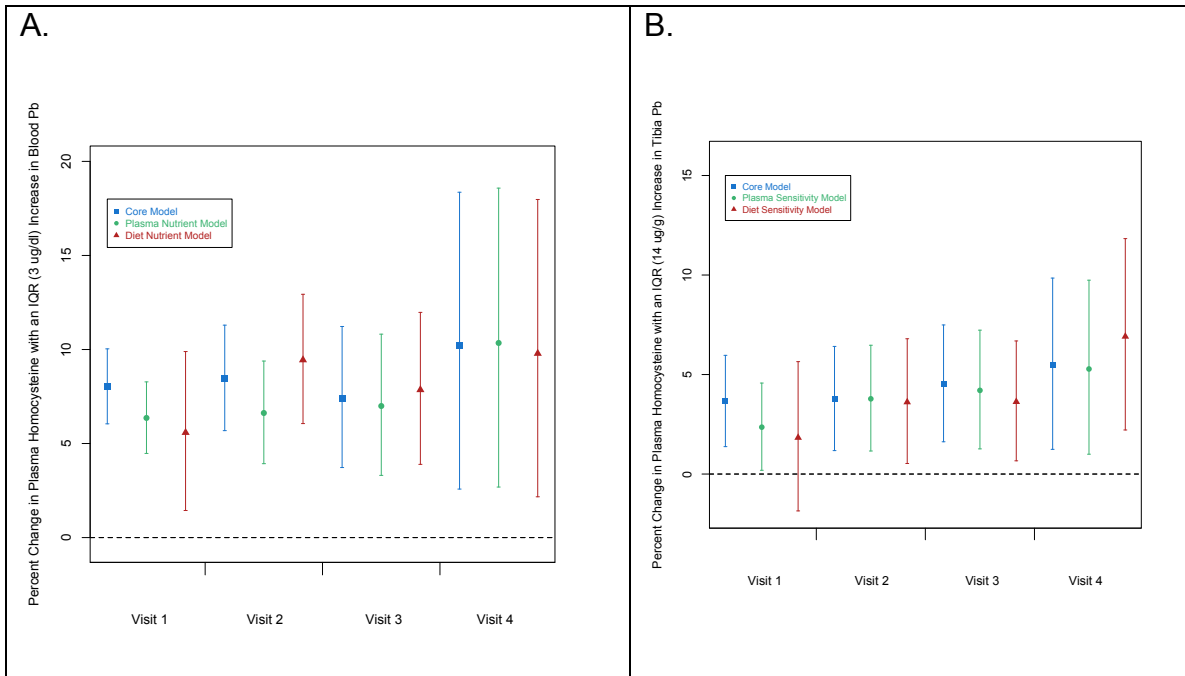
Figure 2.2. The adjusted cross-sectional association between Pb exposure and homocysteine. Y-axis: With an IQR increase in Pb exposure (blood: 3 ug/dl; tibia Pb: 14 ug/g), corresponding percent increase in plasma Hcy. X-axis: Visit number. Results from cross-sectional multivariate linear regression models. Core covariates are age, blood Pb, education, smoking status, alcohol status, and BMI. Plasma sensitivity covariates include the core model plus plasma PLP, B12, and folate. Diet sensitivity model includes the core covariates and the total energy consumed and the dietary adjusted residuals for B6, B12, and folate. (A) Blood Pb. (B) Tibia Pb.

Baseline Tibia Pb Predicts Hcy Over Time
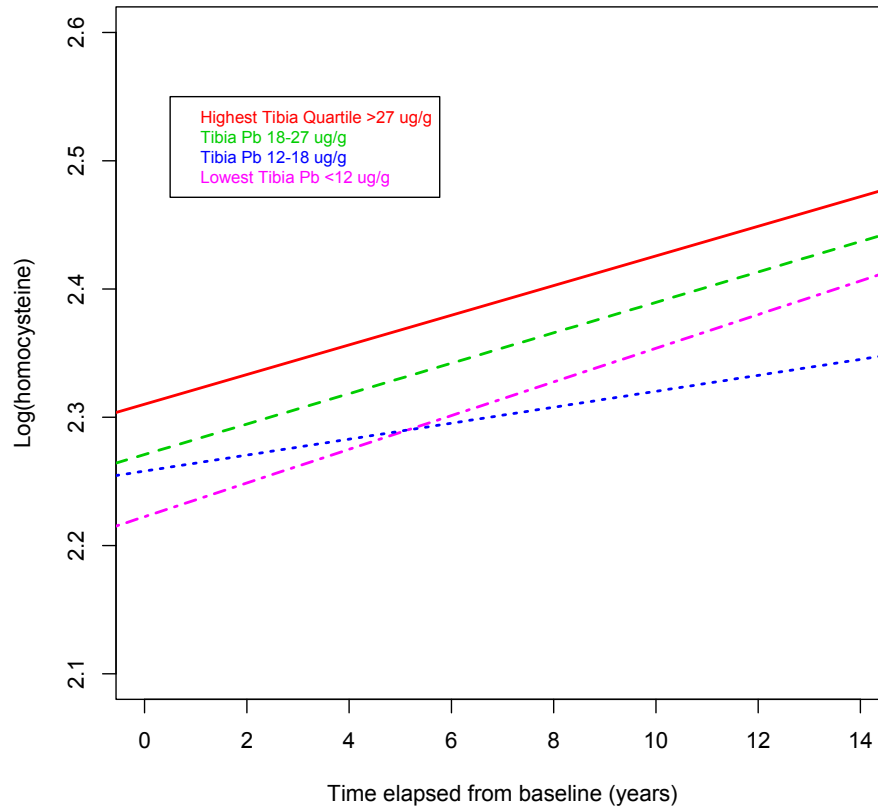
Figure 2.3. Longitudinal core model. Tibia Pb. Data has been centered on the mean continuous variables so intercept can be interpretable. Tibia Pb exposure quartiles are defined at baseline:
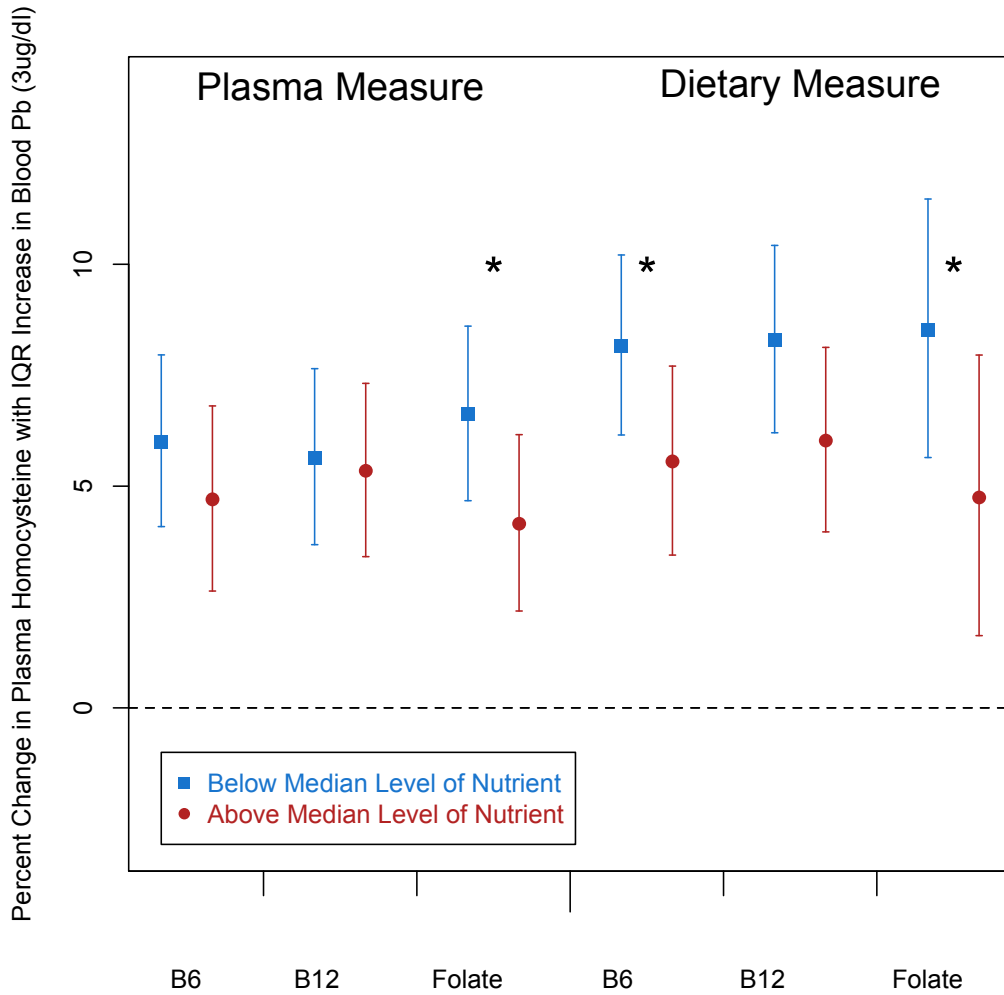
Figure 2.4. Adjusted association between Pb and homocysteine stratified by nutrient status. Y-axis: Percent change in Hcy with an IQR increase in Pb biomarker. Analyses stratified by level of B6, B12 or folate measured in either plasma or dietary FFQ. Longitudinal mixed effects regression of Pb exposure (either blood or tibia bone) on log(homocysteine). *High group is significantly different from low group (p for interaction <0.05).

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SUPPLEMENTAL TABLES

Supplemental Table 2.1. Visits 1-2 all samples, linear regression, split by change in blood Pb n=747

	No Change Blood Pb		Increase in Blood Pb		Decrease in Blood Pb		All Observations	
	n=171		n=177		n=399		n=747	
	Beta(SE)	p-value	Beta(SE)	p-value	Beta(SE)	p-value	Beta(SE)	p-value
(Intercept)	-1.29(0.9)	0.15	-0.42(0.68)	0.83	0.449(0.55)	0.41	-0.0266(0.36)	0.94
delta			0.0578(0.15)	0.7	0.0995(0.11)	0.34	0.0401(0.053)	0.45
bage.c	0.172(0.13)	0.18	0.0326(0.084)	0.7	0.0287(0.065)	0.66	0.0598(0.046)	0.2
as.factor(hs)1	0.0937(0.45)	0.83	0.378(0.41)	0.34	-0.515(0.33)	0.12	-0.138 (0.22)	0.54
as.factor(twodrink)1	-0.312(0.58)	0.59	0.762(0.5)	0.13	-0.147(0.38)	0.7	0.072(0.27)	0.79
as.factor(smkcat)2	-0.628(0.49)	0.2	-0.294(0.47)	0.53	-0.285(0.36)	0.42	-0.341(0.24)	0.16
as.factor(smkcat)3	-1.18(1.1)	0.27	-1.62(1)	0.11	-1.39(0.83)	0.095	-1.31(0.55)	0.017
bmi.c	-0.0405(0.047)	0.39	0.0123(0.053)	0.82	0.0385(0.042)	0.36	0.00542(0.027)	0.84
timebase	0.0543(0.27)	0.047	0.0844(0.15)	0.56	0.135(0.12)	0.26	0.168(0.086)	0.051
bage.c:timebase	-0.025(0.037)	0.51	0.00439(0.023)	0.85	-0.0015(0.017)	0.93	-0.00505(0.013)	0.69

Supplemental Table 2.2: All visits, all samples, mixed effects. n=747 with 1245 observations The average change in Hcy over the average time interval (3.9 years).

	Delta Change from Baseline Model			Adjacent Change Model	
	N=747 indiv, n=1245 obs			N=747 indiv, n=1245 obs	
	R2=0.0482			R2=0.022	
	Beta(SE)	p-value		Beta(SE)	p-value
(Intercept)	0.263(0.23)	0.26	(Intercept)	0.337(0.26)	0.2
delta	0.133(0.042)	0.0015	d.adj.bpb	0.0475(0.045)	0.29
bage.c	0.0125(0.023)	0.59	age.c	0.0469(0.013)	0.00043
as.factor(hs)1	0.0286(0.19)	0.88	as.factor(hs)1	-0.0231(0.18)	0.9
as.factor(twodrink)1	-0.163(0.24)	0.49	as.factor(twodrink)1	0.104(0.21)	0.63
as.factor(smkcat)2	-0.557(0.23)	0.013	as.factor(smkcat)2	-0.435(0.19)	0.024
as.factor(smkcat)3	-1.53(0.49)	0.0018	as.factor(smkcat)3	-1.35(0.45)	0.003
bmi.c	0.00321(0.024)	0.89	bmi.c	0.0117(0.021)	0.59
timebase.c	0.134(0.026)	1.9e-7	d.adj.time	0.0722(0.054)	0.18
bage.c*timebase.c	0.00909(0.0036)	0.012			

Supplemental Table 2.3: Secondary analysis, long-term dietary trend in tibia model

Variable	Core Model: Just Individuals with At Lead 3 Waves of Dietary Information n=691 obs, n=221 indiv R2=0.81		Dietary Model on Invididuals With At Least 3 Waves of Dietary Data n=691 obs, n=221 indiv R2=0.088	
	Beta(SE)	p-value	Beta(SE)	p-value
(Intercept)	2.31(0.032)	0	2.31(0.032)	0
tib.c	0.00163(0.0012)	0.16	0.00167(0.0012)	0.15
age.c	0.0131(0.0019)	4.4E-12	0.0135(0.0019)	1.6E-12
as.factor(hs)1	-0.0302(0.028)	0.28	-0.0278(0.028)	0.32
as.factor(twodrink)1	0.0364(0.033)	0.27	0.038(0.033)	0.25
as.factor(smkcat)2	0.00264(0.035)	0.94	0.00674(0.036)	0.85
as.factor(smkcat)3	0.0946(0.071)	0.18	0.0869(0.071)	0.22
bmi.c	0.00527(0.0036)	0.14	0.0056(0.0037)	0.13
b6res.lt			0.0304(0.045)	0.5
b12res.lt			-0.00972(0.0067)	0.15
folres.lt			-0.000158(0.00023)	0.49
calor.c			-3.2e-05(1.9e-05)	0.092

***note, when we subset only the people with 3 or 4 dietary measures (n=221 individuals across 691 observations), tibia lead is no longer a significant predictor of log(hcy). None of the long term dietary measures are significant either.

Supplemental Table 2.4: Mediation analysis, visit 1.

	Tibia Only		Blood and Tibia		Blood Only	
	n=771		n=771		n=771	
	R2=0.066		R2=0.11		R2=0.11	
	β(SE)	p-value	β(SE)	p-value	β(SE)	p-value
(Intercept)	2.27(0.02)	0	2.27(0.02)	0	2.26(0.019)	0
Tibia Pb	0.00262(0.00081)	0.001	0.000655(0.00085)	0.4		
Blood Pb			0.0241(0.0039)	1E-09	0.0252(0.0036)	9E-12
Age	0.00551(0.0016)	0.0005	0.00587(0.0015)	0.0001	0.00625(0.0014)	2E-05
Education (>hs reference)	-0.0408(0.022)	0.06	-0.0495(0.021)	0.02	-0.0463(0.021)	0.03
Alcohol Consumption	0.0881(0.025)	0.0004	0.0598(0.025)	0.02	0.0588(0.025)	0.02
Former Smoker	0.0326(0.023)	0.2	0.0403(0.022)	0.07	0.0419(0.022)	0.06
Current Smoker	0.144(0.047)	0.002	0.134(0.046)	0.004	0.136(0.046)	0.003
BMI	0.00282(0.0027)	0.3	0.00267(0.0026)	0.3	0.00276(0.0026)	0.3

SUPPLEMENTAL FIGURES

Supplemental Figure 2.1. Bivariate scatterplots for the unadjusted associations between lead measurement and Hcy at visit 1. Red line is the linear regression line of best fit and the blue lines are the 95% confidence intervals. (A) Tibia lead (n=774). (B) Patella lead (n=774). (C) Blood lead (n=1048).
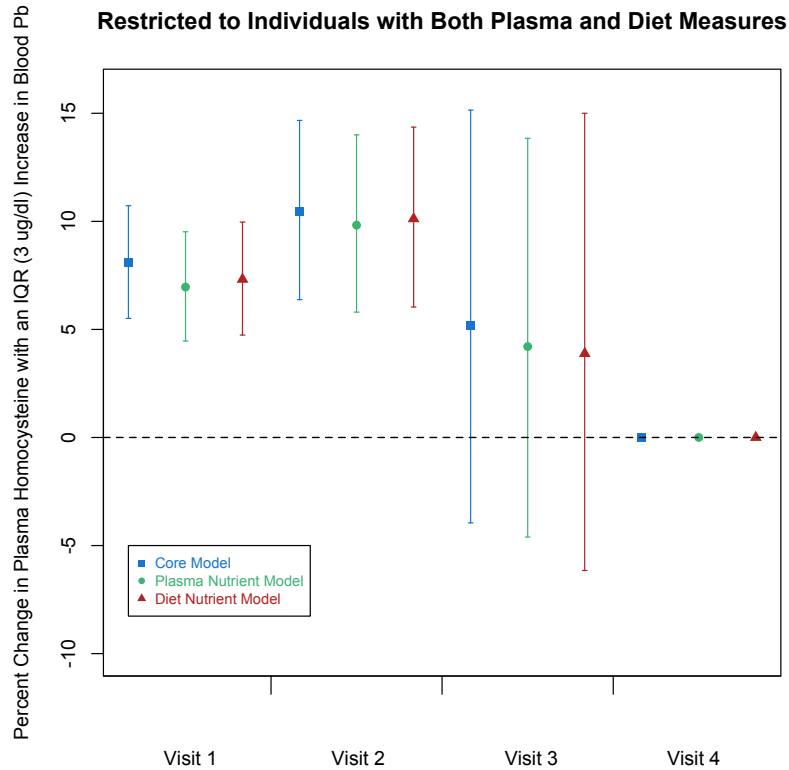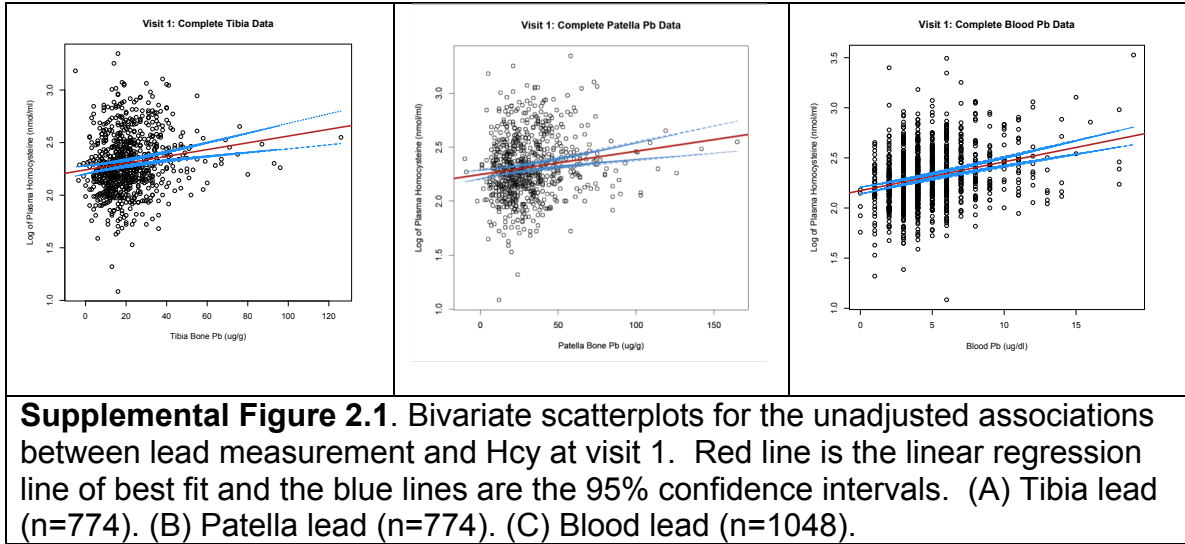
Supplemental Figure 2.2. Blood Pb restricted plasma and diet. *Restrict analysis to only individuals with both plasma and dietary measures. (Visit 1 n=774, visit 2 n= 382, visit 3 n= 65, visit 4 n=0)

CHAPTER III
Research Chapter 2

Genome-wide DNA Methylation Differences Between Late-Onset Alzheimer's Disease and Cognitively Normal Controls in the Human Frontal Cortex

ABSTRACT

Evidence supports a role for epigenetic mechanisms in the pathogenesis of late-onset Alzheimer's disease (LOAD), but little has been done on a genome-wide scale to identify potential sites involved in disease. This study investigates human post-mortem frontal cortex genome-wide DNA methylation profiles between 12 LOAD and 12 cognitively normal age- and gender-matched subjects. Quantitative DNA methylation is determined at 27,578 CpG sites spanning 14,475 genes via the Illumina Infinium HumanMethylation27 BeadArray. Data are analyzed using parallel linear models adjusting for age and gender with empirical Bayes standard error methods. Gene-specific technical and functional validation is performed on an additional 13 matched pair samples, encompassing a wider age range. Analysis reveals 948 CpG sites representing 918 unique genes as potentially associated with LOAD disease status pending confirmation in additional study populations. Across these 948 sites the subtle mean methylation difference between cases and controls is 2.9%. The CpG site with a minimum false discovery rate located in the promoter of the gene Transmembrane Protein 59 (*TMEM59*) is 7.3% hypomethylated in cases.

Methylation at this site is functionally associated with tissue RNA and protein levels of the *TMEM59* gene product. The *TMEM59* gene identified from our discovery approach was recently implicated in Amyloid Precursor Protein post-translational processing, supporting a role for epigenetic change in LOAD pathology. This study demonstrates widespread, modest discordant DNA methylation in LOAD-diseased tissue independent from DNA methylation changes with age. Identification of epigenetic biomarkers of LOAD risk may allow for the development of novel diagnostic and therapeutic targets.

Keywords: DNA methylation, Late Onset Alzheimer's disease, epigenetics, prefrontal cortex

INTRODUCTION

Dementia and Alzheimer's Disease

Worldwide changes in demography are leading to a rapid increase in the numbers of older adults at risk for dementia. Accordingly, the global prevalence of dementia is expected to quadruple from an estimated 35.6 million cases in 2010 to 115.4 million cases in 2050 (International 2010). The global financial burden of dementia in 2010 was $604 billion (US dollars) including direct medical bills, formal social care, and informal care provided by unpaid caregivers (International 2010).

Alzheimer's disease (AD), a progressive, fatal neurodegenerative disease, is the most prevalent form of dementia. Less than two percent of AD cases represent early-onset AD (EOAD) (Bird 2005) defined by disease onset prior to age 60 and genetic mutations in amyloid-β precursor protein (*AβPP*), presenilin-1 (*PSEN-1*), or presenilin-2 (*PSEN-2*) genes (Bertram 2009; Hardy 1997). Mutations in these genes dysregulate the *AβPP* pathway and directly lead to amyloid-β (Aβ) plaque accumulation, a major pathological hallmark of AD.

The remaining vast majority of cases are sporadic, termed Late-Onset Alzheimer's Disease (LOAD) because they manifest symptoms after age 60. Approximately 60% of LOAD cases carry at least one apolipoprotein ε4 allele (*APOE-ε4*), while the global population prevalence of the allele is only approximately 22% (Ashford 2004; Kim et al. 2009). Pooled data on LOAD from recent, collaborative, large genome-wide association studies (GWAS) reported the population attributable risk for APOE variants was between 19% and 35% (Ertekin-Taner 2010). GWAS also identified additional LOAD risk alleles (*CLU*, *PICALM*, *BIN1*, *CR1*, *ABCA7*, *MS4A*, *EPHA1*, *CD33*, *CD2AP*) that contribute added risk in population subsets (Harold et al. 2009; Hollingworth et al. 2011; Lambert et al. 2009; Naj et al. 2011). These risk factor genotypes are neither necessary nor sufficient for LOAD development. Twin studies revealed incomplete concordance (Gatz et al. 1997; Nee and Lippa 1999) and variable

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age on onset (Li et al. 2002) even among monozygotic pairs, highlighting the complex etiology of LOAD. These observations underscore the likely importance of non-genetic factors in LOAD etiology and spurred recent investigations of the epigenetics of AD.

Epigenetics and Alzheimer's Disease

Epigenetics is the study of heritable changes in gene expression that occur without changes to the underlying DNA sequence. Methylation (Maunakea et al. 2010) and hydroxymethylation (Jin et al. 2011) at the 5' site on cytosines in cytosine-guanine (CpG) dinucleotides are important epigenetic modifications associated with gene expression in the human brain. Specific marks distinguish brain regions (Hernandez et al. 2011; Ladd-Acosta et al. 2007) and epigenetic differences in human brain tissues have been associated with such neurological diseases as schizophrenia and bipolar disorder (Mill et al. 2008). Epigenetics is also a mechanism by which environmental exposures can translate to human disease (Dolinoy and Jirtle 2008; Suter and Aagaard-Tillery 2009).

In AD cases lacking highly penetrant genetic susceptibility, the etiology of amyloid dysregulation is not well understood. Altered epigenetic regulation of tau and amyloid processing genes has been observed across multiple brain regions and is a potential mechanism for disease (Barrachina and Ferrer 2009; Tohgi et al. 1999a; Tohgi et al. 1999b). Human post-mortem case-control studies observed global hypomethylation in the entorhinal cortex of AD subjects (Mastroeni et al. 2008) and in the temporal neocortex neuronal nuclei of an AD monozygotic twin relative to their cognitively normal twin (Mastroeni et al. 2009). Evidence for epigenetic involvement in AD pathogenesis spans human studies in various tissues, animal models, and cell culture, and was recently reviewed (2010; Mastroeni et al. 2011; Mill 2011).

Significant transcriptome-wide gene expression differences have been observed between brain tissues of LOAD cases and controls (Loring et al. 2001; Miller et al. 2008). However, previous AD research on DNA methylation as a

regulator of gene expression evaluated DNA methylation at 5' promoter regions of a few candidate genes selected based on *a priori* hypotheses about AD molecular mechanisms. The current research provides a semi-unbiased, quantitative, genome-wide discovery of locations of DNA epigenetic differences in human frontal cortex brain tissue between LOAD cases and controls, which allows for identification of novel disease-associated genes. The gene identified in this study that best distinguished cases and controls was technically validated using an additional sensitive and quantitative method of DNA detection. This mark was also validated using a second population of samples. The functional significance of this DNA methylation mark was further confirmed by gene expression and protein quantification assays.

MATERIALS AND METHODS

Sample Acquisition

The NIA funded Michigan Alzheimer's Disease Center (MADC) (P50AG008671; PI: Sid Gilman) maintains a well-clinically characterized cohort of Alzheimer's disease and cognitively normal control subjects, many of which agreed to participate in autopsy and donated to the MADC Brain Bank. Upon autopsy, each left hemisphere was fixed in 10% neutral formalin for neuropathological diagnosis. The right hemisphere was sectioned coronally, flash frozen, and archived in MADC freezers at -80ºC. Frozen tissue blocks 0.5 cm^3 (50-90 mg) in size were dissected at -20ºC from the mid-frontal gyrus of the frontal lobe and provided for this study. MADC frozen tissues were previously used in high quality expression (Hong et al. 2008; Pan et al. 2007) and proteomic studies (Pan et al. 2007).

Twelve age- and gender- matched pairs of LOAD cases (clinical diagnosis and Braak Score ≥ 4) and controls (clinically confirmed and Braak Score ≤ 2) were used for the genome-wide discovery phase of the project and for gene-specific technical validation. An additional thirteen matched pairs were included in the population validation phase, which included gene-specific DNA

methylation, gene expression, and protein quantification studies. The demographic characteristics of all 50 brains included in this study are described in **Table 3.1**. Post-mortem intervals in hours for AD cases used in the Discovery Set were as follows: 3, 4, 7, 7, 7.75, 8, 8.75, 9, 11, 12, 14, 24. Post-mortem intervals in hours for controls used in the Discovery Set were as follows: 6, 6, 13.5, 14, 17, 18, 18, 18, 19.3, 20.5, 21.25, 24.5. Gray matter for DNA methylation, expression, and protein analysis was excised from the tissue sample and used in this study and vascular lesions were avoided.

DNA Isolation and APOE Genotyping

DNA was extracted from all 25 matched pair samples using the Promega Maxwell Tissue DNA Kit (Madison, WI) according to manufacturer's instructions. APOE genotyping was assayed using the Applied Biosystems TaqMan method (Foster City, CA) according to manufacturer's instructions using the ABI 7900 HT machine (Christensen et al. 2008).

Genome-wide DNA Methylation Discovery

DNA was bisulfite-treated using the Zymo EZ DNA Methylation Kit (Orange, CA) with a modified thermal cycling protocol (98ºC for 10 minutes, 64ºC for 17 hours). Genome-wide DNA methylation was assessed with the Infinium HumanMethylation27 BeadArray (Illumina) performed at the University of Michigan DNA Sequencing Core facility in accordance with manufacturer's instructions and previously published (Bibikova et al. 2009). Six cases and six control samples were randomly applied to each of two 12-sample arrays to avoid biasing case-control differences by batch effect. BeadArrays were imaged using the Illumina BeadArray Reader. Image processing and intensity data extraction are standard components of the BeadScan software that is associated with the BeadArray Reader. The Illumina BeadStudio Software generated percent methylation estimates (beta values) for each probe set based on Cy3 and Cy5 fluorescence intensities. Data was background normalized and exported for further processing.

Statistical Methods for Bead Array

All statistical analysis was performed with the R Statistical Software (version 2.10.1). CpG sites that failed on 10% of samples were not included in subsequent analyses. Linear models adjusting for age and gender were fit across all CpG sites using the limma package (version 3.2.3). As is standard in microarray analyses, empirical Bayesian variance methods were incorporated to site-specific moderated t-tests (Smyth 2004). The linear model used for each individual CpG site was as follows:

% Methylation = β_0 + β_1(Case Status) + β_2(Age) + β_3(Sex)

Top hits by case status were identified by p-values < 0.05 and false discovery rates were calculated. Additional analyses were performed on the top hit. We compared the basic linear model above to a model containing PMI using an F-test. We also compared the R^2 goodness of fit of two simple linear regression models containing only either PMI or Case Status as predictors.

Samples were hierarchically clustered by the single linkage method across the top 26 hits by case status. Positional gene set enrichment analysis was performed using Gene Set Enrichment Analysis (GSEA) to determine statistical over-representation of disease specific epigenetic marks within chromosomal cytogenic bands containing at least 15 genes (Subramanian et al. 2005). Enrichment in promoter and 3'UTR regulatory motifs of disease associated genes was determined by GSEA (Xie et al. 2005). Biological processes and molecular functions associated with LOAD gene lists were established using Gene Ontology (Ashburner et al. 2000).

Gene-Specific DNA Methylation Validation

Site-specific methylation technical (of the original 12 Discovery Set matched pairs) and population (of an additional 13 matched pairs with an expanded age range) validation of the top CpG hit was determined by bisulfite-pyrosequencing on the Qiagen Pyromark MD instrument (Valencia, CA). Using

Pyromark Assay Design Software, a custom pyrosequencing assay was designed to include the two CpG sites present on the Illumina array (Target IDs cg01182697 and cg20793071). This amplicon is located in the promoter of the gene that most statistically distinguished LOAD cases and controls on the Illumina array. Primers were complementary to bisulfite treated DNA in regions without CpG nucleotides (**Table 3.2**). The region of interest was amplified by bisulfite-PCR with the following thermal cycling protocol: 15 minute activation at 95ºC, 45 cycles of 30 second denaturation at 94ºC, 30 second annealing at 55ºC, and 1 minute extension at 72 minutes, followed by a final extension for 10 minutes at 72ºC. Serial dilutions of 100% methylated and unmethylated controls were used to test for any bias in amplification for each assay. Internal bisulfite conversion quality controls were incorporated at original sequence non-CpG cytosines by including C nucleotides in the dispensation order, which should be fully converted to T's following bisulfite treatment.

Incorporation of either a T (for an unmethylated cytosine) or C (for a methylated cytosine) at each CpG provides a quantitative measure for consecutive CpG sites throughout the region sequenced. The level of methylation for each CpG within the target region of analysis was quantified using the Pyro Q-CpG Software. Primers and pyrosequencing assay file information including nucleotide dispensation orders and sequences to analyze are in **Table 3.2**.

Gene Expression

Functional relevance of top methylation marks distinguishing Alzheimer's disease cases and controls was assessed via SYBR green Real Time PCR gene expression assays. RNA was extracted from all 25 matched pair samples using an adjacent portion of the same gray matter sample used for DNA. RNA was extracted using the Qiagen RNeasy Lipid Tissue Kit (Valencia, CA), following homogenization with the Qiagen Tissue Lyser instrument. Assays were designed using Genscript software (Piscataway, NJ). cDNA was generated with the Bio Rad iScript cDNA Synthesis Kit (Hercules, CA) and the primers are listed

in **Table 3.3**. Quantitative PCR assays were run with the iQ SYBR Green Supermix (Bio Rad) on the CFX96 C1000 Thermal Cycler (Bio Rad). CFX Manager software (Bio Rad) was used to determine the threshold cycle (C_T) and perform inter-plate normalizations. C_T values relative to *β-actin* levels were used to compute a fold change between matched pairs. A subset of samples was analyzed on the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array in the University of Michigan Sequencing Core using standard protocols. We determined that *β-actin* is a suitable control in these samples as gene expression of *β-actin* did not differ by case control status at any of the 6 probe sets included in the Affymetrix Array (p-values: 0.13, 0.18, 0.23, 0.43, 0.48, 0.77). Gene expression data were evaluated for normality using R Statistical Software. To determine if higher methylation values were associated with decreased expression of target genes, Spearman correlation coefficients were calculated between CpG methylation (as measured by pyrosequencing) and the expression level data.

Protein Quantification

Adjacent portions of the same 25 matched-pair, gray matter tissue used for DNA were homogenized and protein extracted in Thermo Scientific RIPA buffer (Burlington, Ontario). Protein concentration was quantified using the Thermo Scientific Pierce BSA assay (Burlington, Ontario). Protein (25 ug) was loaded on 10% SDS-polyacrylamide gels for Western Blot analysis. Transmembrane protein 59 antibody was purchased from Novus Biologicals (Littleton, CO) and Transmembrane protein 59 control protein was purchased from OriGene (Rockville, MD). The blots were imaged on the VersaDoc 5000MP instrument (Bio Rad) with Quantity One densitometry software (Bio Rad) under a consistent, constrained area. The levels of Transmembrane protein 59 were standardized to the corresponding tubulin band.

RESULTS

Genome-Wide Descriptive Statistics

DNA was extracted from the frontal cortex of 25 age- and gender-matched LOAD case and control human post-mortem pairs (**Table 3.1**). Sixteen pairs were male and nine pairs were female. LOAD cases and controls did not differ by age (p-value = 0.68). The mean post-mortem interval (PMI) was 12.8 hours, ranging from 3-28 hours. Controls had significantly longer PMI than LOAD cases (p-value=0.0004). The mean number of years in storage was 11.8, ranging from 2-21 years. Cases and controls did not statistically differ in the number of years in storage (p=0.07).

In the Discovery Set, a randomly selected subset of 12 matched pairs ranging in age from 69-95 (mean age 79.8) were analyzed for genome-wide DNA methylation using the Illumina Infinium HumanMethylation27 BeadArray. The BeadArray represented 27,578 CpG sites corresponding to 14,475 unique genes. The average number of CpG sites per gene was 1.9, and 92.0% of CpG sites were within 1000 bp of a transcription start site. CpG sites within CpG islands were overrepresented on the array, as 72.5% of sites were within CpG islands. The average number of CpG sites per sample with Illumina detection p-values greater than 0.05 (considered failing) was 71.1 (range 11-360 sites). CpG sites that failed on more than 2 samples (>10% samples) were not included for further analyses (n= 171 sites).

The global distribution of 5'-cytosine modifications at all CpG sites measured by the array was bimodal, and the distribution of methylation levels was very similar between cognitively normal controls (**Figure 3.1A**) and Alzheimer's disease cases (**Figure 3.1B**). There was a large cluster of sites that had less than 10% methylation (15,735 in controls and 15,619 in AD cases) based on the mean of each group of 12 samples. A second cluster of sites was modified between 75% and 100% (5,226 sites in controls and 5,162 sites in AD cases).

Of the 27,578 sites on the array, 25,380 sites were located in promoter regions as defined within 1,000 bp of a transcription start site. Only 2,198 sites were outside of known promoters. Promoter CpG sites had a median of 5.1%

methylation (IQR= 2.1-5.2) while non-promoter sites had a median of 59.7% methylation (IQR=11.0-84.0). This observation did not depend on AD case status. On the array, 20,006 sites were located within CpG islands and 7,572 sites were outside of CpG islands. CpG island sites had a median methylation of 3.2% (IQR = 1.7-9.6) and non-CpG island sites had 74.6% median methylation (IQR=45.5-85.4). This did not depend on AD case status. Our discovery set of 24 samples ranged in age from 69-95 years (**Table 3.1**), and age was an important predictor of methylation. There were 2,416 sites and 2,227 unique genes associated with age (based on a p-value of 0.05). Of these sites, 1,294 were hypermethylated with increasing age and 1,122 were hypomethylated with age. The top 25 CpG sites associated with age among controls are listed in **Table 3.4**.

Alzheimer's Disease-Specific Results

Following age and gender adjusted linear fit models with empirical Bayseian standard error adjustments, 948 CpG sites representing 918 unique genes were significantly associated with AD case status (based on p-value of 0.05). Among these 948 sites, the maximum mean methylation difference between AD cases and controls was 19.5% at a CpG site 249 base pairs from the predicted TSS of C21orf56 on chromosome 21 (cases 34.8% methylated vs. controls 15.9% methylated). The mean observed disease specific methylation difference across the 958 sites was 2.9% (IQR=0.88-4.2).

The top 26 autosomal CpG sites distinguishing cases and controls (as defined by FDR) are depicted in a heatmap (**Figure 3.2**). Samples clustered on case status with the exception of two control samples. One of those controls was the oldest control subject in the study at 94 years of age. The top 25 CpG sites that were significantly different by case status are outlined in **Table 3.5**.

Gene ontology analysis of the top 958 disease specific sites revealed hypermethylation in several molecular functions and biological processes associated with transcription. The top 10 molecular functions enriched for

hypermethylation with AD cases were RNA polymerase II transcription factor activity (**Figure 3.3A**), RNA binding, GTPase regulator activity, cytokine binding, DNA binding, lyase activity, ATPase activity, transcription factor activity, and nucleoside triphosphatase activity. Similarly, the top 10 biological processes associated with hypermethylation were nucleic acid metabolic process, DNA replication, regulation of nucleic acid metabolism, regulation of DNA dependent transcription, regulation of RNA metabolic process, regulation of cell cycle, DNA dependent transcription, positive regulation of RNA metabolic process, DNA metabolic process, RNA biosynthetic process, and nervous system development.

Hypomethylation was enriched at functions and processes related to membrane transport and protein metabolism. The top 10 molecular functions associated with hypomethylation in AD cases are electron carrier activity, cation transmembrane transporter activity, metal ion transmembrane transporter activity, enzyme binding, rhodopsin-like receptor activity, cation channel activity, integrin binding, phosphoric ester hydrolase activity, G-protein coupled receptor activity, and peptidase activity. The top 10 biological processes associated with hypomethylation in LOAD cases were carboxylic acid metabolic processes (**Figure 3.3B**), organic acid metabolic process, biosynthetic process, cation transport, nitrogen compound metabolic process, amine metabolic process, negative regulation of developmental process, programmed cell death, apoptosis, and anti-apoptosis.

Several promoter and 3' UTR regulatory binding motifs were enriched in the disease associated gene list. Hypermethylation in LOAD cases was observed in genes containing binding site motifs for transcription factors *POU3F2* (p-value < 0.001) and *HOXA4* (p-value=0.004), and microRNAs *MIR-9* (p-value = 0.002), *MIR-518C* (p-value < 0.001), *MIR-1* (p-value=0.025), and *MIR-326* (p-value=0.019). Genes containing *MIR-140* (p-value = 0.04) and *NFE2* (p-value=0.019) motifs were hypomethylated in LOAD cases.

Positional gene set analysis of the 958 disease associated CpG sites identified enrichment of hypomethylation at the chromosomal location19q13

(Normalized Enrichment Score (NES) = 1.24), in cytogenetic band region where the *APOE* gene is located (Kim et al. 2009). In addition, hypermethylation in LOAD cases was seen at 19q13 (NES= -0.72), a candidate location for genetic linkage with LOAD (Wijsman et al. 2004). We visually identified broad regions of altered methylation in AD brains compared to control brains (**Figure 3.4**). These include the p-arm of chromosome 14 and distal q-arm of chromosome 3 (hypomethylated in AD brains compared to control brains) and the p-arms of chromosomes 10 and 17 (hypermethylated in AD brains compared to control brains). Chromosome 15 had the highest density of observed disease-specific methylation differences.

We checked the list of disease specific hits identified with the BeadArray for genes known to be associated with AD. There were two CpG sites on the array corresponding to each of the following genes: *ABCA7*, *APOE*, *ABPP*, *BACE1*, *BDNF*, *BIN1*, *CD2AP*, *CD33*, *CLU*, *CR1*, *EPHA1*, *MAPT*, *MS4A6A*, *PICALM*, *PSEN2*, and *TOMM40*. There was one site representing *PSEN1* on the array and this site was associated with AD (Cases mean methylation = 1.6%, Controls mean methylation = 2.6%; p-value=0.034; cg11490446). Gene expression of the probe set for *PSEN1* at exon 2 differed by case status based on the results of the Affymetrix gene expression array (probe 207782_s_at, p-value = 0.0076, fdr = 0.35). The Spearman correlation coefficient linking expression of this gene expression probe set and methylation measured by the Illumina BeadArray is -0.61 (pvalue=0.0014). AD cases were less methylated and had higher expression of this probe. There was no difference gene expression in the other five probe sets for *PSEN1*.   One of the two sites corresponding to *EPHA1* was associated with hypermethylation with age (p-value = 0.029; cg02376703). One of the two sites associated with *PSEN2* was associated with hypomethylation with age (p-value = 0.030; cg25514304). The remaining CpG sites within LOAD candidate genes were not associated with differential methylation by case status or age.

We also assessed known human imprinted genes for their association with AD, focusing on CpG sites located within Differentially Methylated Regions (DMRs) (Choufani et al. 2011). The Illumina array contains 10 sites in the DMR for *DIRAS3*, 8 sites each for *PLAGL1* and *GNAS*, 7 sites for *ZIM2*, 4 sites for *PEG10*, 3 sites for *PEG3*, 2 sites for *MEST*, and 1 site each for the genes *GRB10*, *KCNQ1*, and *SNURF*. One of the sites in the DMR for *DIRAS3* was more highly methylated in AD cases (43.4%) than controls (38.5%) (p-value = 0.024; cg21808053). One of the sites in the DMR for *GNAS* was hypomethylated with age among controls (p-value = 0.012; cg21625881) and the site for *KCNQ1* was hypermethylated with age (p-value = 0.023; cg27119222).

Gene-Specific Results

After adjusting for age and sex, the highest ranking site (FDR = 0.36, p-value = 0.000013) associated with LOAD was a CpG site upstream of Transmembrane Protein 59 (*TMEM59*). *TMEM59* is responsible for post-translational glycosylation of *ABPP* and leads to retention of *ABPP* in the Golgi apparatus (Ullrich et al. 2010). AD cases had 7.3% lower methylation at *TMEM59* than controls, and this difference was more profound in older subjects (**Figure 3.5A**). Methylation of *TMEM59* was significantly associated with age in cases relative to controls (p-value=0.013). In a second *TMEM59* model, we added PMI as a predictor and compared the goodness of fit of the two nested models using an F-test. PMI did not improve the model fit and PMI is not a statistically significant predictor of methylation at the *TMEM59* site. In a simple linear regression model with Case Status as the only predictor of *TMEM59* methylation, the model fit R^2 was 0.597. The model fit R^2 with PMI as the only predictor was 0.132.

The methylation findings were technically validated by pyrosequencing a single assay containing both CpG sites from the Illumina array that were associated with *TMEM59*. The CpG density 1000 bp flanking the top hit on either side is 1.5%. Pyrosequencing the original 24 discovery samples confirmed the difference between cases and controls at *TMEM59* was 2.7% (**Figures 3.5B and**

3.5D). Methylation was again associated with age in cases relative to controls in the technical validation (p-value=0.0084). Pyrosequencing of an additional 26 matched pair samples across an expanded younger age range (61-94) confirmed the age-associated reduction in methylation (p-value-0.0022), and the association with AD case status was not statistically significant at an alpha of 0.05 (p-value = 0.088) (**Figure 3.5C**).

We determined expression levels of *TMEM59* at four points along the 8 exon transcript (**Figure 3.6A**) (including the beginning, end, and two middle sites) to functionally validate the DNA methylation results with the *TMEM59* gene. At the four locations along the transcript that were assayed by real time PCR, controls had lower RNA expression levels than cases (**Figure 3.6B**). For the first assay on the transcript, AD cases had 24.9% higher expression (fold change 1.33; p-value 0.0013). For two assays in the middle of the transcript, AD cases had 20.5% (fold change 1.26; p-value 0.0071) and 28.3% (fold change 1.40; p-value 0.056) higher expression. The assay at the end of the transcript showed AD cases to have 21.5% (fold change 1.27; p-value-0.0036) higher expression than controls. DNA methylation and RNA expression were negatively correlated at *TMEM59* (Spearman correlation coefficient = -0.274, p-value = 0.0083).

To further investigate the functional implications of the observed DNA methylation and gene expression differences of *TMEM59*, we measured the protein levels by Western blot in the full set of 25 case brains and 25 control brains. No differences were observed for the full length 36 kDa protein (p-value=0.68) (**Figure 3.6C**), but AD cases had reduced levels of a shorter protein that was also bound by the antibody specific for the C-terminus of *TMEM59* (p-value=0.040) (**Figures 3.6C, 3.6E**). The quantity of shorter protein was associated with age (**Figure 3.6D**).

DISCUSSION

We performed a genome-wide, semi-unbiased quantitative comparison of frontal cortex DNA methylation from age- and gender-matched LOAD cases and

controls. The CpG site most strongly associated with case status was located in the promoter region of *TMEM59*, a gene recently implicated in Alzheimer's disease pathogenesis (Ullrich et al. 2010). This gene is involved in the post-translational modification of Amyloid Precursor Protein (*AβPP*) and causes retention of *AβPP* in the Golgi apparatus (Ullrich et al. 2010). The magnitude of methylation difference at this site between cases and controls was very modest (7.3% difference using the Illumina array), but the direction of association was confirmed using an alternate method of DNA methylation detection (2.7% difference using pyrosequencing). In an expanded population including a higher number of younger cases and controls, an interaction between age and case status was detected. Thus, age modifies the association between disease status and methylation. There was not a primary association between case status and methylation when the younger population was included. In the original sample of LOAD cases and controls, *TMEM59* DNA methylation levels corresponded to functional changes in *TMEM59* gene expression. LOAD cases had lower methylation and higher expression of *TMEM59* than control samples. No differences in the level of the full length *TMEM59* protein were observed between cases and controls; however a smaller protein that bound the *TMEM59* antibody was significantly higher in controls. This *TMEM59* protein size pattern is consistent with the *TMEM59* control protein lysate. The shorter protein may represent a proteolytic product of the full length protein. The shorter protein is approximately 17 kDa, which could also be consistent with translation of an alternative RNA transcript beginning at exon 5 of the *TMEM59* gene. The observed differences in protein expression levels are consistent with epigenetic regulation. Further molecular research is needed to better understand the gene expression and protein regulation and potential role of DNA methylation at this site.

Well-studied genes known to be involved in AD pathogenesis or identified through GWAS for genetic association with LOAD were largely not associated with disease-specific DNA methylation differences in this study. A notable exception was *PSEN1*, which was modestly hypomethylated in LOAD cases.

Consistent with previous human post-mortem tissue studies, *PSEN1* showed very low levels of methylation in our population (Siegmund et al. 2007). Here, LOAD cases had reduced DNA methylation that was associated with increased *PSEN1* gene expression, suggesting the DNA methylation change may be functional at this site. Studies in mice and neuroblastoma cell lines demonstrate that *PSEN1* gene expression is regulated by DNA methylation at specific promoter CpG sites and this regulation depends on B vitamin availability (Fuso et al. 2011). The correlation between DNA methylation and gene expression in our study support the cell line and mouse model findings. The Illumina HumanMethylation27 BeadArray platform used in this study also allowed for discovery of novel gene associations with AD. For example, methylation change was observed within the DMR for the imprinted gene, *DIRAS3*. Genomic imprinting in Alzheimer's disease is a potential mechanism to explain epidemiological parent-of-origin inheritance observations(Fallin et al. 2011; Mosconi et al. 2011).

Greater than 900 genes (6% of genes featured on the array) were differentially methylated by case status after adjusting for age and gender. Overall, the disease related methylation effect size (2.9%) was relatively modest, and the global methylation distributions of AD cases and controls were similar. Together these findings suggest that DNA methylation may play a role in LOAD and the individual effects at each CpG site may be subtle. The magnitude and absolute number of DNA methylation changes observed in this study are consistent with previous reports in the literature performed on candidate gene subsets. In a case-control study of prefrontal cortex DNA methylation of twelve genes, only two genes were associated with AD status and the differences in methylation were less than 10% (Wang et al. 2008).

Gene set enrichment analysis revealed key patterns in the identified set of disease associated CpG sites. First, gene ontology analysis showed hypermethylation of genes involved in transcription and DNA replication, while membrane transporters were hypomethylated. Second, hypermethylation was

enriched at genes containing *POU3F2* binding motifs. *POU3F2* is a transcription factor critical in central nervous system development that regulates Nestin gene expression, a protein important for radial axon growth (Jin et al. 2009). Third, positional analysis showed hypomethylation with case status at 19q13 and hypermethylation at 19p13, cytogenic band regions genetically linked with AD (Kim et al. 2009; Wijsman et al. 2004).

LOAD cases and control sample groups were similar with respect length of time in storage, but LOAD cases had shorter PMI then control samples (p-value = 0.0004). During PMI, samples may be exposed to damaging lower pH conditions and higher temperatures where enzymes may be active. PMI was not a significant confounder at the *TMEM59* site, however, PMI may be a factor at other specific locations throughout the DNA methylome.

This study measured genome-wide DNA methylation differences between LOAD case and control subjects aged 69 to 95. Across this relatively short age range, DNA methylation was associated with age at over 2,400 CpG sites, representing more than 8% of the sites on the BeadArray. Both hyper and hypomethylation was observed. Previous studies have observed global hypomethylation with age in the brain (Pogribny and Vanyushin 2010), but gene-specific studies of aging and methylation have noted varying patterns (Siegmund et al. 2007). These results further support age as an important covariate to consider in statistical models of DNA methylation in late life.

Age is a major factor in epigenetic change in the brain (Hernandez et al. 2011), potentially confounding or modifying disease specific associations. In a study of cerebral cortex DNA from gestation to 104 years of age, eight of fifty loci showed increases in methylation through late life and two sites presented changes suggestive of an acceleration of age-related change in a subset of samples with LOAD (Siegmund et al. 2007). Additional evidence supports increased age-dependent epigenetic drift with disease. In LOAD prefrontal cortex samples representing a 30 year age range, an age-specific epigenetic drift was more prominent among cases compared to controls. The average

94

methylation in promoters of *MTHFR* and *APOE* increased by 6.8%, while control samples decreased with age by 10.6% (Wang et al. 2008).

Cultured cells can potentially have very different epigenetic profiles than primary cells as an artifact of growth in culture (Smiraglia et al. 2001), and thus use of primary human frontal cortex tissue is a strength of this study. DNA methylation is brain region specific and greater differences have been seen between the cerebellum and cortex regions than by sex, age, post-mortem interval, race, or cause of death (Ladd-Acosta et al. 2007). This study consistently used frontal cortex tissue because of its role in advanced AD. As with many studies of epigenomic, transcriptomic, or proteomic changes in the human brain, the tissue samples represented populations of mixed cell types, an important limitation, which may have resulted in attenuated effects. Epigenomes are cell type specific (Lister et al. 2009; Maunakea et al. 2010), and brain cell types have different roles in AD (Selkoe 2001). The AD brain has an active changing cell population including neuronal loss and glial activation (Akiyama et al. 2000) that may in part be responsible for the observed results. DNA methylation data, however, was not enriched in inflammatory mediators, which would have supported changes due to gliosis. This study considered brain region specific methylation and as epigenomic platforms require lower input DNA amounts, future research may be able to also consider cell type specific changes.

Results from large DNA methylome and transcriptome maps of the human brain suggest that intragenic CpG sites rather than promoter CpG islands may better correlate with transcription (Maunakea et al. 2010). The genome-wide sites included on the Illumina Infinium HumanMethylation27 BeadArray are more likely to be located within promoter region CpG islands. Important methylation events located elsewhere throughout the genome would be missed using this platform and may be included in future research utilizing alternative technologies.

These results must be interpreted with caution because this study had a small sample size relative to the large number of CpG site comparisons and the

magnitude of observed methylation differences between LOAD cases and controls was moderate. The results from the array were technically validated at the top CpG site, but it is not clear whether this observation will be consistent across populations. While a small study, we identified modest DNA methylation differences as a potential event in LOAD.

FIGURES
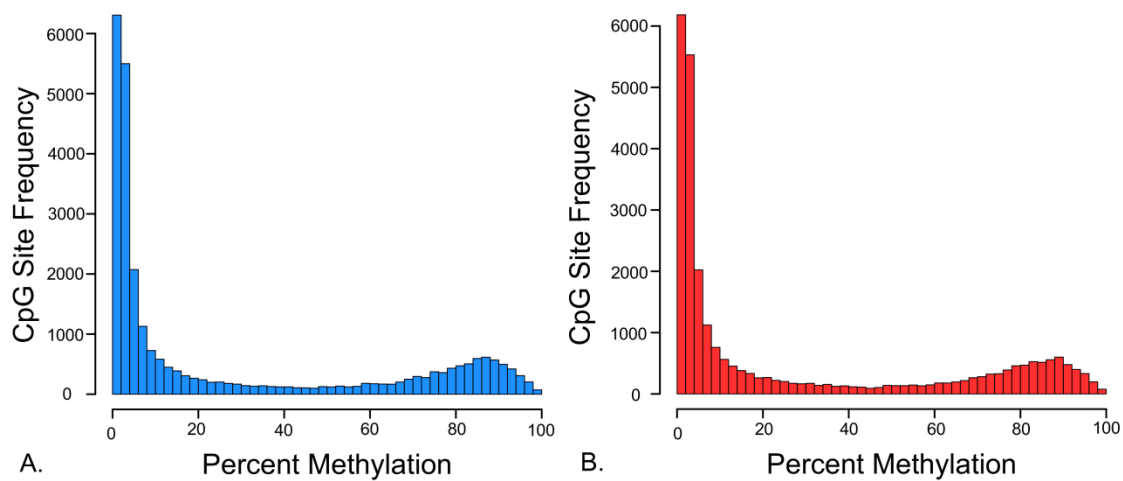
Figure 3.1. Mean percent methylation frequency distribution of the Discovery Set of 12 cognitively normal control samples (A) and 12 Alzheimer's disease cases (B) across the 27,578 CpG sites on the Illumina HumanMethylation27 BeadArray.
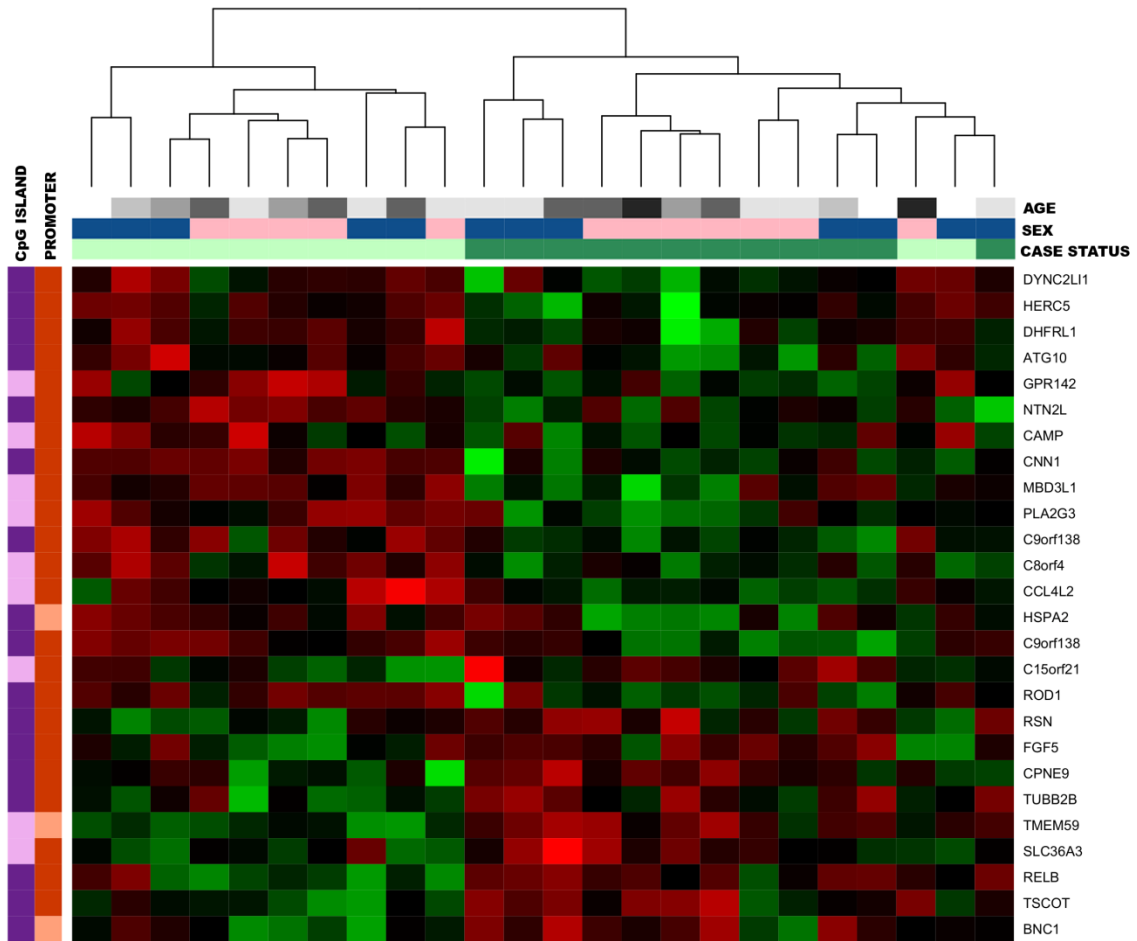
Figure 3.2. Hierarchical clustering heatmap of the Discovery Set top 26 autosomal CpG loci associated with late-onset Alzheimer's disease (LOAD) case/control status after adjusting for sex and age. Green represents hypermethylation in LOAD cases and red represents hypomethylation in cases. Horizontal color bars at the top refer to the age, sex, and case status of the sample. In the Case Status color bar, light green represents control samples and dark green represents LOAD cases. For sex, female is light pink and male is dark blue. In the age panel, black represents ages 91-95, darkest gray 86-90, medium gray 81-85, light gray 76-80, lightest gray 71-75, and white represents ages 66-70. Vertical color bars on the left refer to the CpG island and promoter status of the CpG sites. In the CpG island bar, dark purple represents sites within CpG islands and light purple represents sites outside of CpG islands. In the promoter bar, dark orange represents sites within promoter regions and light orange represents sites outside of promoter regions.
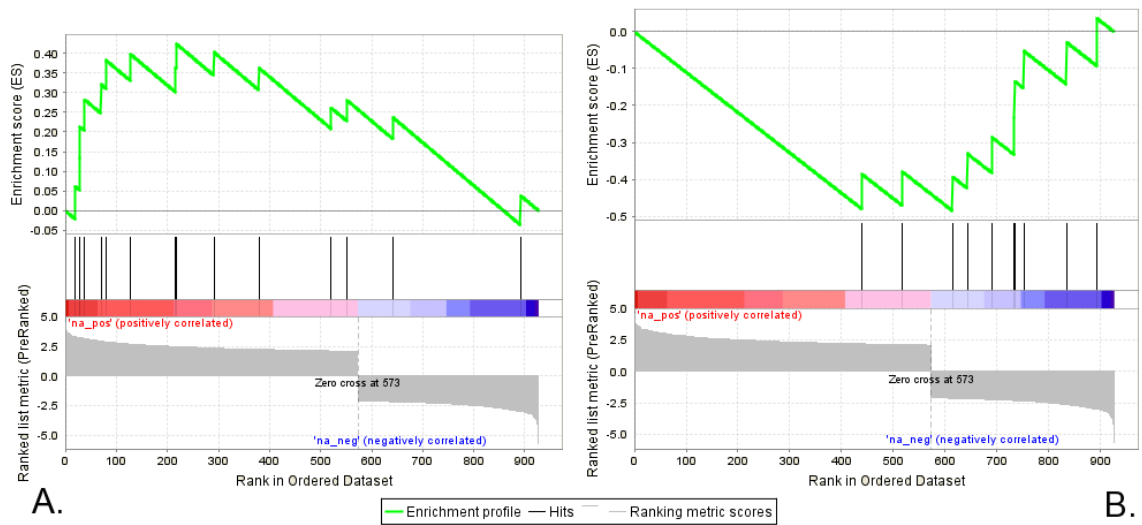
Figure 3.3. Discovery Set gene set enrichment analysis plots. (A) Genes associated with RNA polymerase II transcription factor activity molecular function were hypermethylated in LOAD cases relative to controls (p-value = 0.013). (B) Genes associated with carboxylic acid metabolic biological processes were hypomethylated in LOAD cases relative to controls (p-value= 0.013).
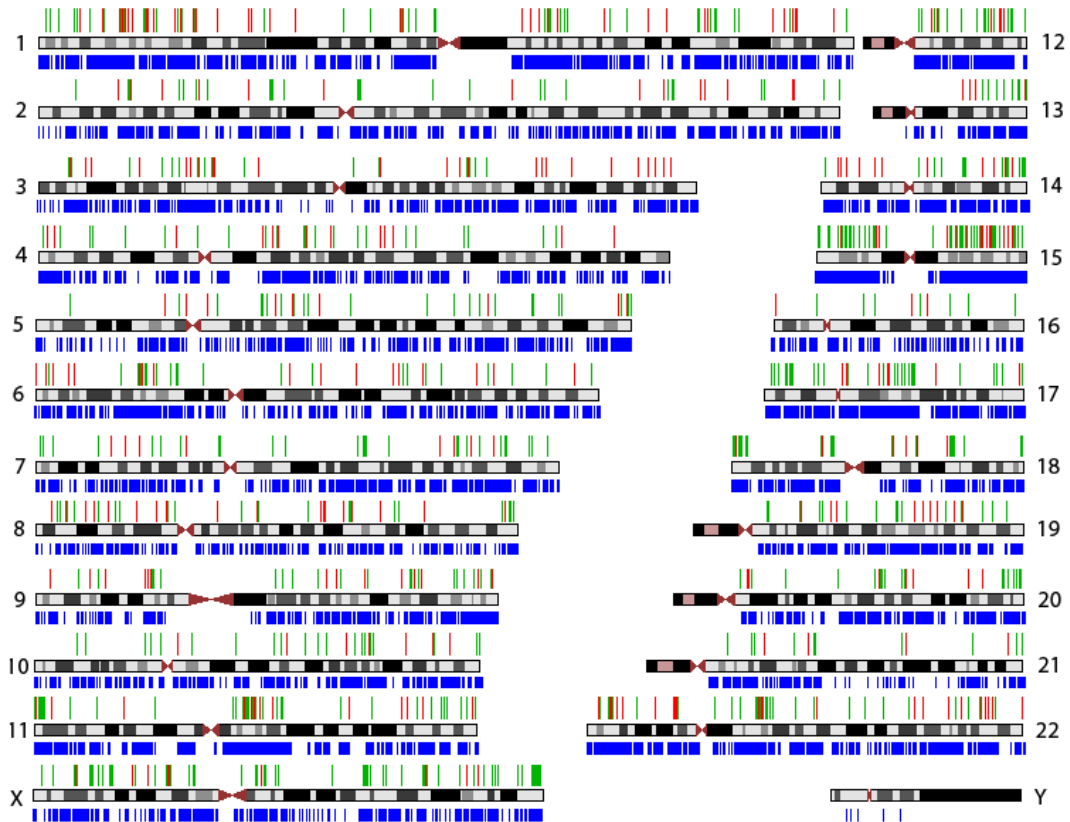
Figure 3.4. Human chromosome ideogram in black. Distribution of CpG sites featured on the Illumina HumanMethylation27 BeadArray is below the chromosomes in blue. Distribution of CpG sites that were significantly associated with late-onset Alzheimer's disease (LOAD) in the Discovery Set are above the ideograms. Green represents hypermethylation with LOAD status. Red represents hypomethylation with LOAD status.

C8 : TAATAAYGAAGGGGATTTGTTTTAYGAGTTAGTATATATGGTGTAAAT
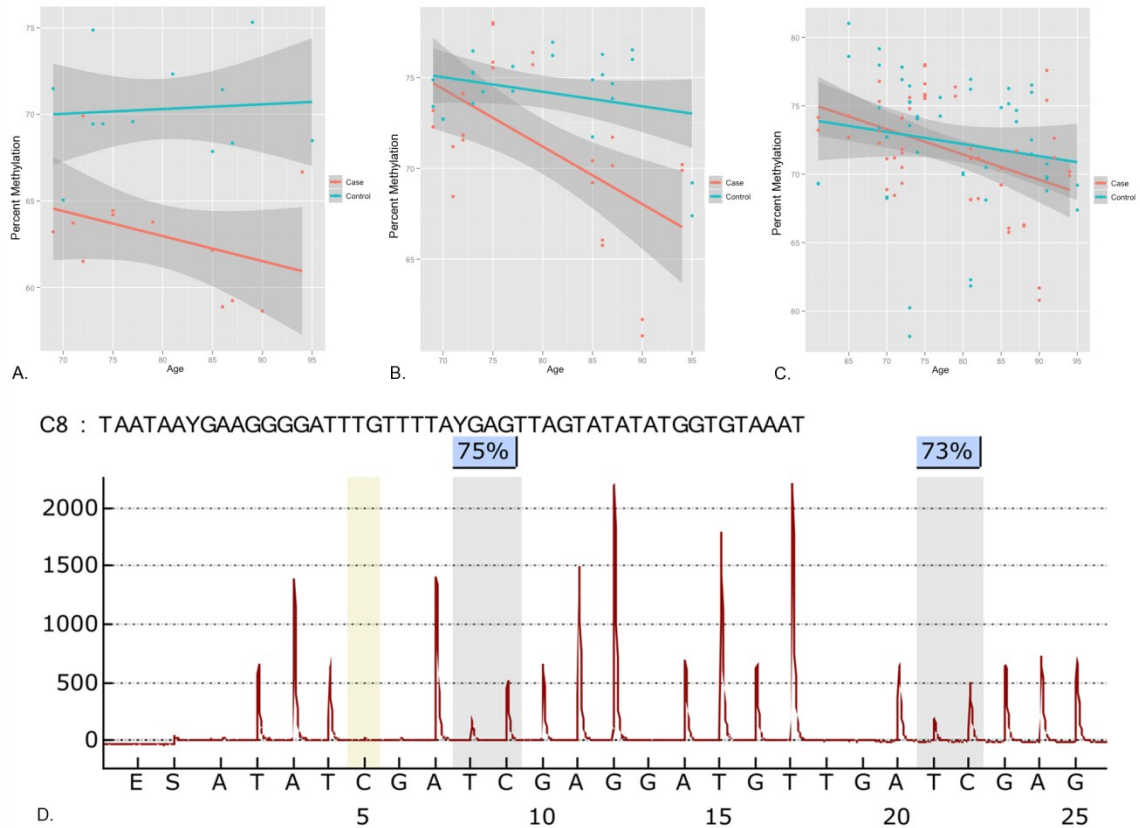
Figure 3.5. Methylation upstream of the *TMEM59* gene. (A) Percent methylation by age and case status (Late-Onset Alzheimer's Disease cases in red; Controls in blue). Data from the Illumina HumanMethylation27 BeadArray. (B) Age vs. percent methylation bisulfite-pyrosequencing technical validation data of original 24 samples run in duplicate in the Discovery Set. (C) Age vs. percent methylation bisulfite pyrosequencing of Discovery Set 24 samples plus 26 additional population validation samples run in duplicate. (D) A representative bisulfite-pyrosequencing assay pyrogram for 2 CpG sites in the promoter of *TMEM59*.
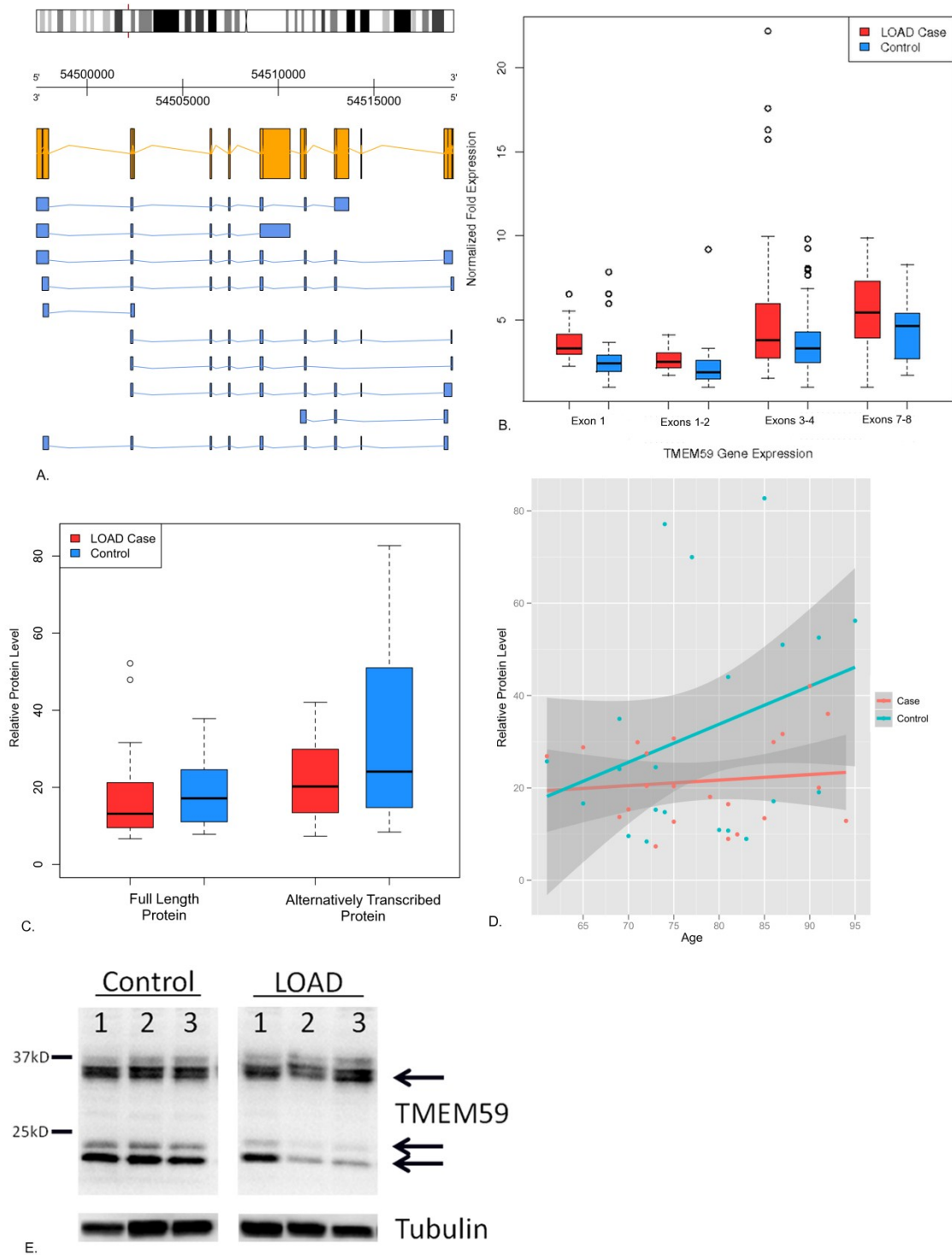
Figure 3.6. Functional validation of observed DNA methylation differences for *TMEM59*, a gene involved in the post-translational modification of Amyloid Precursor Protein (*ABPP*). (A) *TMEM59* is located on chromosome 1 and is transcribed on the reverse strand. The reference sequence mRNA is yellow.

Predicted alternative isoforms are in blue. (B) Boxplot of *TMEM59* gene expression by Q-PCR in the Discovery Set. Two-sample t-test between cases and controls were all statistically significant (exon 1 p-value=0.0013; exons 1-2 p-value=0.0071; exons 3-4 p-value=0.0036, exons 7-8 p-value=0.0083) (C) Boxplot of relative protein levels of *TMEM59* in the Discovery Set plus an additional 26 validation samples. Paired t-tests did not reflect case specific differences for the full length protein (p-value=0.68), while the shorter protein fragment was significantly lower in AD cases (p-value=0.040). (D) Levels of the shorter *TMEM59* protein fragment as a function of age. (E) Representative western blot image of *TMEM59* protein expression in controls and AD cases 1-3 representing identical exposures of the same gel. No differences were detected between AD and controls for full length *TMEM59* protein based on case status, but the levels of the *TMEM59* shorter proteins were reduced in AD cases. These shorter proteins were also observed in the TMEM59 control protein lysate.

TABLES

Table 3.1. Study population mean demographics by case status. Range is provided in parentheses.

Characteristic	LOAD Cases		Cognitively Normal Controls	
	Discovery Set (n=12)	Full Set (n=25)	Discovery Set (n=12)	Full Set (n=25)
Neuropathological Diagnosis	High Likelihood AD n=11 Intermediate Likelihood n=1	High Likelihood AD n=21 Intermediate Likelihood n=4	Control n=11 Other n=1	Control n=24 Other n=1
Braak Stage	4.7 (2-6)	4.8 (2-6)	1.3 (1-2)	1.5 (1-2)
Age	79.6 (69-94)	78.2 (61-94)	79.9 (69-95)	78.3 (61-95)
Sex				
Female	6	9	6	9
Male	6	16	6	16
Post Mortem Interval (hours)	9.6 (3-24)	9.5 (3-27.5)	16.3 (6-24.5)	16.0 (5-28)
Years in Storage	10.75 (5-17)	10.5 (3-18)	13.2 (3-20)	13.2 (2-21)
Race	Caucasian (n=12)	Caucasian (n=25)	Caucasian (n=12)	Caucasian (n=25)
Age of onset	69.1 (59-78)		NA	NA
MMSE	11.7 (0-28) missing=1	12.9 (0-30) missing=2	26.8 (25-28) missing=10	26 (24-28) missing=16
# Years from Diagnosis to Death	5.1 (1-12)	5.2 (1-12)	NA	NA
APOE Genotype				
2/2	2	2	3	6
2/3	1	1	0	1
2/4	0	1	0	0
3/3	3	7	6	13
3/4	5	13	1	3
4/4	1	1	1	1
Missing	0	0	1	1

Table 3.2. Pyrosequencing assay information.

Gene	Sequence 5 – 3'
TMEM59	
Forward Primer	GGGTAGGTATATAGAATTATATTTGGTATT
Reverse Primer Biotinylated	AAATTTCTACACACCCCTACTACA
Sequencing Primer	AATAGATTATATTTTGTAAAAGAA
Dispensation Order	ATATCGATCGAGGATGTTGATCGAG
Sequence to Analyze	TAATAAYGAAGGGGATTTGTTTTAYGAGTTAGTATATATGGTGTAAAT

Table 3.3. Primer sequences for gene expression QPCR assays.

Gene	Sequence 5' – 3'
TMEM59 Exon 1	
Forward Primer	TGACTCGGTCTTGGGTGATA
Reverse Primer	TCTTCCTTAGGGTAGGTGTGC
TMEM59 Exons 1-2	
Forward Primer	GGGCCTGTCAGTTGACCTAC
Reverse Primer	CTGCAACCTCTCTGACATGC
TMEM59 Exons 3-4	
Forward Primer	GAACAACTTATGTCCCTGATGC
Reverse Primer	CGTCATCGGCTTGAAGATAA
TMEM59 Exons 7-8	
Forward Primer	TCCTCTCGGTGATGGTATTG
Reverse Primer	TCAGCTTCTCAGAGGGAACA
B-actin	
Forward Primer	TGCTATCCAGGCTGTGCTAT
Reverse Primer	AGTCCATCACGATGCCAGT

Table 3.4. CpG sites differentially methylated with age among cognitively normal controls (Discovery Set, Age 69-94).Beta coefficient can be interpreted as the rate of change in methylation per year across the years studied.

Rank	Associated Gene	Beta Coefficient	p-value	Distance to Transcription Start Site	CpG Island	Chromosome	Biological Description
1	DNAI2	0.441288	1.08E-05	714	FALSE	17	Dynein intermediate polypeptide 2; axonemal;. Function in microtubule motor activity.
2	ECM2	0.453176	3.85E-05	717	FALSE	9	Extracellular matrix protein 2 precursor. Function in integrin binding and cell-matrix adhesion
3	UNQ689	0.409188	1.39E-04	991	FALSE	4	hypothetical protein LOC401138
4	C3	0.262665	1.52E-04	680	FALSE	19	Complement component 3 precursor. Function in acylation-stimulating protein cleavage in innate immune response
5	OSMR	0.25763	2.10E-04	404	TRUE	5	oncostatin M receptor. Role in cell proliferation and cell surface linked signal transduction
6	MEG3	-0.45638	2.15E-04	NA	TRUE	14	Predicted gene from GNOMON
7	GLO1	0.238369	2.36E-04	480	TRUE	6	glyoxalase I. Role in zinc ion binding, lyase activity in carbohydrate metabolism and antiapoptosis.
8	CRNN	0.295641	2.50E-04	167	FALSE	1	Hypothetical protein LOC49860. Tumor-related protein.
9	SF3B2	-0.16749	2.92E-04	484	TRUE	11	splicing factor 3B subunit 2
10	PIK3C2B	0.221434	3.22E-04	164	FALSE	1	phosphoinositide-3-kinase; class 2; beta polypeptide. Role in intracellular signaling cascade
11	RIBC2	0.226036	3.68E-04	126	TRUE	22	RIB43A domain with coiled-coils 2. Synonym C22orf11
12	CCDC74B	0.230644	3.71E-04	1015	FALSE	2	hypothetical protein LOC91409
13	C20orf77	0.622206	6.42E-04	605	TRUE	20	hypothetical protein LOC58490
14	C9orf112	0.530497	6.84E-04	317	TRUE	9	hypothetical protein LOC92715
15	LCE1B	0.388947	7.51E-04	1310	FALSE	1	late cornified envelope 1B Role in epidermal differentiation complex 2A
16	SFRS11	0.36302	7.59E-04	1480	FALSE	1	Splicing factor p54. Nucleic acid binding and nuclear mRNA splicing
17	SLC18A2	0.055023	8.92E-04	275	TRUE	10	solute carrier family 18 (vesicular monoamine); member 2. Vesicle monoamine transporter type 2.
18	PAPPA	-0.21564	1.07E-03	204	FALSE	9	pregnancy-associated plasma protein A preproprotein. Insulin-like growth factor dependent IGF binding protein.
19	FIGNL1	0.64861	1.08E-03	599	TRUE	7	fidgetin-like 1 ATP binding nucleoside-triphosphatase activity
20	NMT1	-0.08842	1.10E-03	285	TRUE	17	N-myristoyltransferase 1
21	VAMP5	-0.28073	1.13E-03	492	TRUE	2	vesicle-associated membrane protein 5 (myobrevin). Role in vesicle-mediated transport, myogenesis, and cell differentiation
22	FLJ33641	0.634	1.20E-03	974	FALSE	5	hypothetical protein LOC202309
23	DVL3	0.40801	1.21E-03	580	TRUE	3	dishevelled 3. Kinase activity. Role in nervous system development.
24	C20orf4	-0.15153	1.22E-03	250	TRUE	20	hypothetical protein LOC25980
25	IGF2	0.534292	1.25E-03	NA	TRUE	11	insulin-like growth factor 2

Table 3.5. Table of the 25 CpG sites most significantly differentially methylated by AD case status (Discovery Set).

Rank	Associated Gene	% Methylation Cases	% Methylation Controls	p-value	Distance to Transcription Start Site	CpG Island	Chromosome	Biological Description
1	*TMEM59*	63.03	70.30	1.32E-05	1339	FALSE	1	APP post-translational glycolytic processing
2	ATG10	8.16	5.59	1.97E-04	197	TRUE	5	Autophagy
3	C9orf138	85.53	77.88	2.08E-04	47	TRUE	9	Hypothetical Protein
4	CPNE9	4.76	6.33	4.43E-04	549	TRUE	3	Copine-like protein
5	RELB	36.40	45.75	5.68E-04	470	TRUE	19	reticuloendotheliosis viral oncogene homolog B
6	C9orf138	68.36	57.60	8.92E-04	406	TRUE	9	Hypothetical Protein
7	PLA2G3	45.95	37.03	9.66E-04	488	FALSE	22	Phospholipase A2
8	DHFRL1	6.41	4.41	1.08E-03	511	TRUE	3	Hypothetical Protein
9	MBD3L1	16.33	11.98	1.08E-03	141	FALSE	19	Methyl-CpG binding domain protein 3-like
10	RSN	2.37	2.85	1.37E-03	698	TRUE	12	Restin isoform a. Intermediate filament associated protein
11	OTUD5	22.39	18.53	1.53E-03	232	TRUE	X	Hypothetical Protein
12	TUBB2B	7.58	10.50	1.53E-03	494	TRUE	6	Tubulin, beta polypeptide paralog. Microtubule associated.
13	NTN2L	7.63	4.97	1.59E-03	159	TRUE	16	Netrin 2-like. Structural molecule, axon guidance.
14	GPR142	90.60	88.52	1.64E-03	237	FALSE	17	Signal transduction
15	TSCOT	55.93	62.95	1.65E-03	498	TRUE	9	Thymic stromal co-transporter
16	IL2RG	67.12	60.80	1.70E-03	88	FALSE	X	Interleukin 2 receptor, gamma precursor.
17	BNC1	39.63	48.20	1.86E-03	NA	TRUE	15	Zinc finger protein basonuclin. Metal ion binding.
18	HERC5	2.57	1.43	1.91E-03	108	TRUE	4	Cyclin-E binding protein 1.
19	SLC36A3	73.03	80.25	2.18E-03	203	FALSE	5	Proton/amino acid transporter 3
20	DYNC2LI1	4.44	3.05	2.21E-03	24	TRUE	2	Dynein 2 light intermediate chain
21	SLC7A3	10.20	6.88	2.32E-03	208	TRUE	X	Cationic amino acid transporter
22	FGF5	4.24	5.70	2.36E-03	544	TRUE	4	Fibroblast growth factor 5
23	CAMP	92.62	89.57	2.53E-03	284	FALSE	3	Cathelicidin antimicrobial peptide
24	CNN1	13.81	9.18	2.59E-03	92	TRUE	19	Caloonin 1. Calmodulin binding
25	C15orf21	87.01	88.96	2.76E-03	7	FALSE	15	Dresden prostate carcinoma 2

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CHAPTER IV
Research Chapter 3

An integrated analysis of genome-wide RNA expression and DNA methylation in late-onset Alzheimer's disease and neuropathological controls

ABSTRACT

Introduction: Spontaneous, neurodegenerative, late-onset Alzheimer's disease (LOAD) is associated with aberrations in brain gene expression. Recent studies have observed dysregulation of epigenetic mechanisms, such as DNA methylation, but the association of gene expression with these marks in disease tissue is unknown. Gene expression analysis in conjunction with DNA methylation mapping provides insight into the mechanisms of LOAD.

Research: To investigate the potential epigenetic regulation of gene expression in LOAD, we evaluated the human frontal cortex of 11 cases of LOAD and 12 cognitively normal controls. We measured genome-wide RNA gene expression using the Affymetrix U133A Plus 2.0 Array and DNA methylation using the Illumina HumanMethylation 27K BeadArray. We performed validation of X specific loci.

Results: After adjusting for age and sex, there were 176 probe sets (145 unique genes) distinguishing gene expression of LOAD cases and controls (FDR<0.1, p-value<3.3×10^{-4}). Of these, 76.7% were down-regulated in LOAD, including the statistically top five genes *DUSP16*, *ERICH1*, *ESF1*, *PTPRF*, and *RNBP1*. Among the genes where expression was associated with LOAD (p-value<0.05), DNA methylation and gene expression were correlated at 151 genes. Positive (47.7%) and inverse (52.3%) associations were observed between gene expression and DNA methylation.

Conclusions: LOAD case and control samples differed by RNA expression levels and relatively few (7.2%) of those differences were correlated with DNA methylation. Among genes with correlated DNA methylation and gene expression, positive and negative associations were observed with approximately equal frequencies. Integrated genome-wide analyses of DNA methylation and RNA gene expression provide a functional molecular signature of LOAD and suggest novel sites for disease biomarker development.

Keywords: Alzheimer's disease, epigenetics, DNA methylation, frontal cortex, gene expression

INTRODUCTION

Alzheimer's disease (AD) is a fatal neurodegenerative disease that affects over five million people in the United States. It is the sixth leading cause of death across all ages and the prevalence is rising (Alzheimer's Association 2011). AD is characterized by two neuropathologies: β-amyloid (Aβ) plaques and tau neurofibrillary tangles (NFT). Highly penetrant genetic mutations in the Aβ production pathway account for approximately 2% of AD cases, termed early-onset AD (EOAD) cases (Bird 2005). The remaining 98% of AD cases manifest symptoms after age 55 and are termed late-onset AD (LOAD) cases. Genome-wide association studies reveal genetic risk factors for LOAD that are neither necessary nor sufficient to cause disease. LOAD risk is associated with polymorphisms in the apolipoprotein E4 (*APOE ε4*), *ABCA7, BIN1, CD2AP, CD33, CLU, CR1, EPHA1, MS4A4A* and *PICALM* genes (Harold et al. 2009; Hollingworth et al. 2011; Lambert et al. 2009; Naj et al. 2011). The combined population attributable fraction (PAF) for these multiple genetic risk factors is 0.50, even after adjusting for *APOE* ε4 dose (Naj et al. 2011). Thus, LOAD risk may be conferred through an interaction of external factors with genetic risk factors through gene-environment interactions or epigenetic modifications.

In LOAD, where genetic determinants confer only partial risk, Aβ and NFT pathways may be dysregulated through epigenetic mechanisms. Epigenetics refers to heritable changes in gene expression that do not involve changes to the underlying DNA sequence. Recent evidence suggests age-related changes in candidate gene methylation, such as upstream of Microtubule Associated Protein Tau (*MAPT*) in post-mortem human brain tissue (Tohgi et al. 1999). Striking disease-specific differences however, have not been consistently detected in promoter CpG island regions of Aβ and NFT candidate genes (Barrachina and Ferrer 2009). Newer research investigates non-canonical disease pathways, including cellular protein translation. For example, AD samples from the parietal and prefrontal cortex were hypermethylated in the promoter regions of nucleolar rRNA genes (Pietrzak et al. 2011), coinciding with reduced ribosomal activity that has been observed with LOAD.

To better understand the etiology of LOAD and the pathogenic processes, mapping the more complete epigenetic signature of disease is an important emerging area of research (Bakulski et al. 2012a). Recent work from our research group has broadened the targeted pathway approach to examine genome-wide DNA methylation in LOAD (Bakulski et al. 2012a; Bakulski et al. 2012b). DNA methylome studies in LOAD brain tissues suggest widespread, yet modest DNA methylation changes associated with both age and disease (Bakulski et al. 2012b). DNA methylation change is apparent, but the functional implications of the observed epigenetic marks have not yet been determined.

LOAD cases and controls have been classified based on their differential gene expression profiles in post-mortem brain tissues. For example, studies of gene expression by brain region in AD indicate significant dysregulation beyond the Aβ and NFT pathways (Kong et al. 2011). Expression levels of ceramide fatty acid processing genes including *ASMas*, *NSMass2*, and *GALC* were upregulated in the brains of AD subjects (Filippov et al. 2012). Changes in gene expression may also be at the center of hippocampal-dependent memory and corresponding deficiencies in AD. For example, hippocampal neurons derived

from AD transgenic mice had reduced expression of activity-induced CREB-dependent genes, which was reversible with expression of CREB-regulated transcription coactivator 1 (C*rtc1*) (Saura 2012). Transcriptome profiles of AD and controls suggest AD brain tissues have increased expression of broad markers of chronic inflammation, cell adhesion, cell proliferation, and protein synthesis, as well as downregulation of signal transduction, energy metabolism, stress response, synaptic vesicle synthesis and function, calcium binding, and structural proteins (Loring et al. 2001). The factors responsible for these broad gene expression changes are not clear. In order to understand the etiology of LOAD, we move upstream in the LOAD course and investigate the regulation of the LOAD transcriptome, controlled in part through the epigenome.

Recent individual gene studies have underscored the strengths in analyzing matched epigenetic and gene expression data in AD. The candidate gene, peptidyl-prolyl cis/trans isomerase (*Pin1*), was examined in human peripheral blood mononuclear cells of 60 matched LOAD and control samples (Arosio et al. 2012). *Pin1* promoter DNA methylation had a modest reduction (8%) and increased gene expression (74%) in LOAD (Arosio et al. 2012). A second example involves the Aβ A4 precursor protein-binding family A member 2 (*APBA2*) gene implicated in Aβ production. In primary rat cortical neurons, expression of A*pba2* was reduced with promoter DNA methylation at a location 7-120 base pairs upstream of the transcription start site (TSS) (Hao et al. 2012). An integrated analysis of observed DNA methylation and previously published RNA-seq expression was performed on a human cell line expressing the Swedish mutation of *APP* (APP-sw) and it revealed three potential gene sites (*CTIF*, *NXT2*, and *DDR2*) where DNA methylation and RNA expression were correlated (Sung et al. 2011). These results are promising, but cell lines have distinct epigenetic profiles from primary tissue (Smiraglia et al. 2001) and cell lines engineered with EOAD mutations are not adequate models for spontaneous LOAD. Previous studies show that analyses incorporating both epigenetic and gene expression data can be very useful (Sartor et al. 2011). Research using

primary human brain tissue in LOAD is needed to characterize the connection between DNA methylation and gene expression on a genome-wide scale.

Here we utilize whole genome approaches to interrogate both gene expression and DNA methylation profiles in LOAD cases and controls to identify potential functional relationships between epigenetic patterns and disease status. First, human post-mortem frontal cortex samples from LOAD and neuropathologically normal control subjects are assessed for gene expression profiles. Next, we combined gene expression data with DNA methylation results in the same samples. Identification of genes with disease-specific gene expression differences that are correlated with DNA methylation changes support an emerging role of epigenetics in LOAD.

Results

Gene expression results.

Gene expression in the frontal cortex was measured in 11 LOAD cases and 12 cognitively normal controls using the Affymetrix GeneChip Human Genome U133 Plus 2.0 Array. The array features 54,675 probe sets representing 20,722 unique gene ids. The mean expression level across LOAD and controls was 4.73 and it ranged from 1.75 to 14.25.

After adjusting for age and sex, site-specific expression values were compared between cases and controls. The *DUSP16* (dual specificity phosphatase 16) gene transcript distinguished LOAD cases and controls with the greatest statistical significance (p-value= $8.85*10^{-7}$, FDR= 0.0316, log(2) fold change: -0.913). *DUSP16* was down-regulated in LOAD, as were the next eight statistically significant genes with strongest association with LOAD status (**Figure 4.1**). These top nine genes (*DUSP16, ERICH1, ESF1, PTPRF, FNBP1, RBM4, JMJD1C, LUC7L3*, and *RBM25*) were down-regulated in AD case samples with an average of -1.18 log(2) fold change. Three of the top nine genes were RNA splicing regulators (*RBM4, LUC7L3, RBM25*).

The top 25 probesets that differentiated cases and controls at a false discovery rate of 0.033 are listed in **Table 4.1**. Twenty-two of the top 25 genes (88%) (*DUSP16, ERICH1, ESF1, PTPRF, FNBP1, RBM4, JMJD1C, LUC7L3, RBM25, NUPL1, TCF25, UBXN4, CDC42BPA, CCAR1, ZNF37A, BRD4, EIF5B, LIMA1, ZFHX4, SMARCA4 , MYO6, CHAF1A*) were down-regulated in LOAD. Three of the top 5 genes (12%) (*SYTL4, DPYSL3*, and *LOC100505875*) were up-regulated in LOAD.

Overall, there were 176 probe sets (146 unique genes) distinguishing LOAD cases and controls at a false discovery rate of less than 0.1 (p-value$<3.2 \times 10^{-4}$) (**Supplemental Table 4.1**). The log(2)fold change between cases and controls in these probe sets ranged from -1.98 to 1.72. Forty-one (23.9%) of these probe sets were up-regulated in LOAD, while 135 (76.7%) were down-regulated in LOAD. The normalized expression of the top 176 sites is illustrated in a heatmap of using Ward's hierarchical clustering methods and the maximum distance function (**Figure 4.2**). Cases and controls are separated into two column clusters and the probeset rows separate into two groups based on up and down regulation with disease. These 176 probesets also distinguish cases and controls when using Principal Component Analysis. Principal component analysis (PCA) is a dimension reduction tool to reduce multiple correlated variables to linearly uncorrelated variables. When data were restricted to probe sets that were differentially expressed according to LOAD status (n=176), PCA was an effective visualization strategy. The first principal component described 67.2% of the variance and the second principal component described 9.5% of the variance (**Figure 4.3A**). PCA on the 176 probe sets associated with LOAD (FDR <0.1), clustered samples by LOAD case and control (**Figure 4.3B).** The heatmap and the PCA plot illustrate that these 176 probesets together are markers of LOAD case/control status in these samples.

To examine whether these differences observed between AD cases and controls were due to disease-specific changes in expression or changing cell-type populations, we incorporated publicly available cell-type specific data from

the NCBI Gene Expression Omnibus (GEO). We combined GEO data from embryonic stem cell-derived neuronal precursor cells (NPC) and astrocytes with our own AD case and control data. We used a follow-up heatmap to plot the expression levels at the original 176 probesets we found to distinguish cases and controls alongside the expression at those sites in NPC and astrocyte cells (**Supplemental Figure 4.1**). At these 176 sites, NPC and astrocyte cells were separated into two cell-type clusters. NPC and astrocytes did not cluster with AD cases or controls however, and the embryonic stem cell-derived data displayed greater range in gene expression change than our data.

To test whether there were biological patterns in the group of genes that were found to be different in expression between LOAD cases and controls, we used the software, LRpath, developed by the National Center for Integrative Biomedical Informatics (NCIBI) (Sartor et al. 2009). LRpath is a program that runs gene set enrichment testing using logistic regression on the full list of genes and associated p-values. We used LRpath to calculate overrepresentation among our top expression hits in gene ontology terms (biological processes and molecular functions), cytogenic bands, transcription factor targets, and miRNA targets. We observed down-regulation of 92 gene ontology biological processes at p<0.01 (FDR<0.1). The top fifteen biological processes that are decreased in LOAD are listed in **Table 4.2** and the full list is in **Supplemental Table 4.2**.   Nine processes were at false discovery rates < 0.002: synaptic transmission, transmission of nerve impulses, cell-cell signaling, oxidative phosphorylation, regulation of synaptic plasticity, regulation of transmission of nerve impulse, cellular respiration, regulation of neurological system process, neurotransmitter transport, and regulation of neuronal synaptic plasticity. Similarly, we observed enrichment of 348 gene ontology biological processes in the genes with increased expression in AD cases (p-value<0.01, FDR<0.1). The top fifteen biological processes that are increased in LOAD are listed in **Table 4.3** and the full list is in **Supplemental Table 4.3**. The top nine were translational elongation, defense response, immune response, innate immune response, inflammatory response, response to biotic stimulus, response to other organism, activation of

immune response, and regulation of immune response at false discovery rates < $4*10^{-7}$.   The results for gene ontology molecular functions were similar to the results for 77 significantly up and 79 down regulated biological processes (**Supplemental Tables 4.4 and 4.5**, respectively).

Genes differentially expressed in LOAD were also enriched in specific transcription factor targets, microRNA targets, and cytogenic bands. Reduced-expression in LOAD was associated with 17 transcription factors (**Supplemental Table 4.6**) including the top five: NRSF_01, NMYC_01, CREB_01, CREBP1CJUN_01, and MEIS1BHOXA9_02. Over-expression in LOAD was associated with the following six transcription factors NFKAPPAB_01, NKX22_01, OCT_C, PBX1_01, HOXA3_01, and CREL_01 (**Supplemental Table 4.7**). Genes targets associated with three microRNAs were over-expressed in LOAD (mir-506, mir-124, mir-433) and genes under-expressed in LOAD were enriched in binding for three microRNAs (mir-129-5p, mir-185, and mir-328) (**Supplemental Tables 4.8 and 4.9**, respectively). Among genes with higher expression in LOAD, 32 cytogenic bands were enriched, including the top five: 7p13-p12, 17p13, 4q25, 10p12.31, and 1q25 (**Supplemental Table 4.10**). Across genes with lower expression in LOAD, 33 cytogenic bands were enriched, including the top five: 16p13.3, 10p11.2, 8p23, 12p12.3, 8p21.3 (**Supplemental Table 4.11**).

Expression-Methylation

To test the extent to which DNA methylation was associated with the observed LOAD gene expression differences, we linked genome-wide gene expression and DNA methylation data from the same samples. DNA methylation data was generated using the Illumina Infinium HumanMethylation27 BeadArray (Bakulski et al. 2012b). We assessed combined methylation and expression at 2,094 Entrez gene IDs (**Figure 4.4**). These 2,094 genes had RNA expression associated with LOAD status (p-value <0.05) and DNA methylation data was available.

Across these 2,094 genes, we tested whether DNA methylation was correlated with gene expression. At 151 genes (7.2% of genes tested), DNA methylation and gene expression were significantly correlated (Pearson correlation test p-value<0.05). Among the 151 correlated sites, 79 (52.3%) represented the canonical, inverse association between DNA methylation and gene expression. Conversely, 72 (47.7%) represented non-canonical, positive correlation between DNA methylation and gene expression. Pearson correlation values ranged from -0.62 to 0.68 across these significantly correlated sites. Among the 151 DNA methylation and RNA expression correlated sites, 81 (53.6%) genes had higher methylation in LOAD and 70 (46.4%) had lower methylation in LOAD (**Figure 4.5A**). Similarly, 86 (57%) had higher expression in LOAD and 65 (43%) had lower expression (**Figure 4.5B**)

Significant differences in methylation levels between LOAD cases and controls were observed at 24 of the 151 DNA methylation-gene expression correlated sites (15.9%), after adjusting for age and sex (p-value<0.05) (**Table 4.4**). These 24 sites had significant disease-specific differences in DNA methylation, gene expression, and correlated methylation-expression values. Canonical, inverse correlation was observed at the following 17 genes (70.8% of 24 genes with significant methylation differences): *ARHGAP15, PTAFR, FAM122C, SNX20, MKNK1, PSEN1, PMP2, CDC42EP3, PLD5, SKI, GPR34, TP53TG5, WWTR1, CATSPERG, PARVG, PPP1R3B,* and *IFI16*. Non-canonical, positive correlation between DNA methylation and gene expression was observed at 7 (29.2%) of sites: *TMPO, SERPINH1, PSMB2, ITGAM, MED12, OLFML2B,* and *NACC2.* The first 9 genes with significant disease-specific differences in DNA methylation, gene expression, and correlated methylation-expression are illustrated in scatter plots in **Figure 4.6** and the remaining 15 genes are in **Supplemental Figure 4.2**.

To test whether the genes with correlated expression and methylation values shared common biological pathways, we again performed gene set enrichment analysis using LRpath software. We uploaded the Pearson's

correlation test p-values corresponding to the correlations between the 2,094 genes with linked RNA expression and DNA methylation data. We observed enrichment at the cytogenic bands across DNA methylation and RNA expression correlated genes: 17p11.2, 14q24.3, 16q22.1, 11q23.3, 6p21.2, and 19p13.3, as shown in the circos plot (**Figure 4.7**). We also observed transcription factor binding site enrichment at CEBPA_01, OCT1_05, GFI1_01, EVI1_06, MEF2_02, OCT1_01, NCX_01, PAX4_03, USF_C, GATA1_01, HAND1E47_01, PAX3_01, FOXO3_01, and COUP_01. The following microRNA targets were enriched among the correlated DNA methylation and RNA expression sites: mir-342-3p, mir-215, mir-192, mir-455-5p, mir-300, mir-488, and mir-142-3p. 171 biological processes were enriched, including the top ranked twelve: protein deubiquitination, protein modification by small protein removal, NK-kappB import into nucleus, regulation of NF-kappaB import into nucleus, negative regulation of axonogenesis, ion membrane transport, regulation of transcription factor import into nucleus, microtubule depolymerization, positive regulation of protein ubiquitination, nuclear-transcribed mRNA catabolic process, mRNA catabolic process, and positive regulation of ligase activity.

Discussion

LOAD is a complex disease involving multiple physiological changes that are evident in post-mortem pathology. Known genetic risk factors are responsible for only approximately half of the risk of LOAD disease initiation and progression (Naj et al. 2011). We interrogated the gene expression changes associated with disease and an epigenetic gene expression regulator. DNA methylation changes have been observed in LOAD, but the function of DNA methylation as a biomarker or potential mechanism of disease with gene expression implications had not been well characterized. We compared frontal cortex genome-wide RNA expression and DNA methylation profiles of LOAD cases and neuropathology-confirmed controls.   Tissues were acquired on autopsy and nucleic acid samples were analyzed using array-based methods. We identified genes where expression was correlated with DNA methylation and where there were

differences by disease status. These observations support a potential functional role for DNA methylation in LOAD at a subset of genes.

First, using frontal cortex tissue, we identified discordant gene expression in LOAD. We identified a subset of genes that were associated with LOAD where expression of samples clustered by case status.   These genes also differentiated NPC and astrocyte cell lines, which clustered separately from post-mortem samples. The majority of genes altered in LOAD were repressed in the disease samples. Across the entire gene set, the biological processes synaptic plasticity and transmission of nerve impulse were down-regulated in LOAD, while immune-related responses were up-regulated in LOAD. These findings are consistent with the current understanding of LOAD pathogenesis. In a novel analysis of genes with altered expression in LOAD, we integrated gene expression and DNA methylation data to test the role of DNA methylation in disease RNA changes. Seven percent of genes with altered expression in LOAD were correlated with DNA methylation levels. Gene expression varies with disease and DNA methylation may regulate the expression changes at a subset of genes.

A handful of identified genes have previously been implicated in LOAD and in cognitive changes. Specifically, we found reduced gene expression of *ERICH1* in LOAD. Copy number variation within the *ERICH1* gene was previously associated with intra-extradimensional set shifting (IED) domain on the CANTAB cognition test (Need et al. 2009). We also observed downregulation of formin binding protein 1 (*FNBP1*) in LOAD samples. *FNBP1* was previously shown to be downregulated in blood mononuclear cells of AD subjects (Maes et al. 2007). *FNBP1* is implicated in endocytosis and cellular trafficking and protein processing. Further, we observed reduced expression of *PTPRF*. AD pathogenesis is associated with abnormal autophagy and *PTPRF* binds PTPσ, an autophagic phosphatase (Martin et al. 2011).

The gene expression probe set which best distinguished LOAD cases and controls was associated with *DUSP16* (dual specificity phosphatase 16). *DUSP16* is a member of a family of proteins that catalyse the inactivation of mitogen-activated protein kinase (*MAPK*). *MAPK* plays a role in long term potentiation (LTP) of neurons (English and Sweatt 1997). The *p38 MAPK* pathway is involved in tau pathology in AD (Johnson and Bailey 2003) and has been targeted for AD treatment (Munoz and Ammit 2010). In addition, *RBM4* was downregulated in LOAD in our study. *RBM4* is a splice factor that interacts with an intronic element for *MAPT* and stimulates tau exon 10 inclusion (Kar et al. 2006). *RMB25* and *LUC7L3* are also splice factors that were downregulated in LOAD in our study. Dysregulation of splicing factors could have widespread downstream effects for neurodegenerative disease (Licatalosi and Darnell 2006).

Previous research has performed transcriptome array analysis on LOAD cases and controls in the neocortex and found a large number of genes with divergent disease gene expression with wide-ranging physiological functions (Tan et al. 2010). Among the genes that were associated with LOAD (FDR<0.1), we observed 77% were down-regulated in gene expression with LOAD. Similarly, Maes *et al.* found of the 942 genes with 2-fold differential change in AD blood mononuclear cells relative to controls, 87% were downregulated and only 13% were upregulated in AD (Maes et al. 2007). Research has looked at genetics as a driver of gene expression differences. A previous genome-wide gene expression study in Alzheimer's disease linked SNP data with transcript levels (Webster et al. 2009), and found that ~5% of transcripts had correlation between expression profiles and genotypes that could distinguish LOAD cases and controls. Results from our study suggest that a similar level of genes with a altered expression (7% of genes) are correlated with DNA methylation levels. The current paper is the first study, that we are aware of, to examine the association between DNA methylation and gene expression in LOAD on a genome-wide scale. Future research may integrate genetics, transcriptomics, and epigenomics for a more complete understanding of gene regulation in LOAD.

A limitation of cross-sectional research, such as this, is that causal relationships cannot be inferred. Later work will be able to study the time course of the DNA methylation and gene expression associations observed here. Further, LOAD is characterized by neuronal loss (Coleman and Flood 1987) and glial cell activation (Mattson 2004). Changing cell type populations may be responsible for these observations and future work could identify the cell type specific DNA methylation and gene expression patterns.

GWAS studies estimate that known genetic LOAD risk alleles confer a combined population attributable fraction of 0.5 (Naj et al. 2011). Additional risk genotypes may be discovered, but the remaining risk likely results from environmental exposures and an interaction of genetic and environmental factors, which have been historically understudied. Epigenetics represents an important intersection of genes and environment. By identifying new candidate areas of epigenetic and gene expression variability with LOAD, this research provides the groundwork for future studies in environmental and genetic susceptibility to LOAD. LOAD research may increasingly consider a combined gene-environment paradigm.

Materials and Methods

Sample ascertainment. The Michigan Alzheimer's Disease Center (MADC) (P50AG008671; PI: Henry Paulson) followed a clinically characterized cohort of Alzheimer's disease and cognitively normal control subjects. Many subjects (and legal care-givers) consented to autopsy and donated to the MADC Brain Bank. Diagnoses were neuropathologically confirmed using Braak and Reagan scoring in the left hemispheres. The right hemispheres were coronally sectioned, flash frozen, and archived at -80ºC. For the current study, frozen tissue blocks from the mid-frontal gyrus of the frontal lobe were dissected at -20ºC. MADC frozen tissues were previously used in high quality DNA methylation (Bakulski et al. 2012b), expression (Hong et al. 2008; Pan et al. 2007), and proteomic studies (Pan et al. 2007).

Samples were eligible for the current study if neuropathology confirmed the diagnosis and if the subjects were at least 60 years old at death. The MADC Brain Bank had 98 eligible LOAD cases (Braak Score ≥ 4) and 39 controls (Braak Score ≤ 2). Twelve gender- and age- (+/- 2 years) matched pairs of LOAD cases and controls were generated randomly, without replication. The population characteristics of both sets of samples used in the current study are described in **Table 4.5**. This human subjects study was approved by the Institutional Review Board of the University of Michigan Medical School.

DNA and RNA isolation. Gray matter free of vascular lesions was selected from the tissue blocks. DNA and RNA were isolated from adjoining tissues in all 25 matched pairs. DNA was extracted according to manufacturer's instructions using the Maxwell Tissue DNA Kit (Promega, AS1030). Tissue was homogenized with the TissueLyser II (Qiagen, 85300) and total RNA was extracted using the RNeasy Lipid Tissue Mini Kit (Qiagen, 74804).

Transcriptome-wide RNA expression. RNA from the twelve case-control pairs was interrogated for genome-wide expression analysis. RNA quality was assessed using the Agilent 2100 Bioanalyzer and quantified using the Thermo Scientific NanoDrop Spectrophotometer 2000. Samples with RNA quality greater than 3.0 RNA Integrity Number (RIN) and absorbance ratios A260/A280 greater than 2.0 were used for further study. One AD case sample did not meet this set of criteria and was not used for further RNA study. The quality of RNA did not differ by LOAD cases and controls (mean RIN=6.0, p-value=0.8). 5 ug of RNA was used for cDNA synthesis using a T7-Oligo(dT) promoter primer. An *in vitro* transcription (IVT) reaction produced biotin-labeled cRNA that was then fragmented. At the University of Michigan Affymetrix Core facility, cRNA was hybridized to the GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix, 900470) using the 16-hour hybridization protocol. Each sample was hybridized to its own chip. The probe array was washed and stained according to the GeneChip Expression Wash, Stain and Scan User Manual (Affymetrix, 702731). The probe array was scanned using the Affymetrix Scanner 3000 instrument and

the image files were analyzed for probe intensities. The GeneChip provided data on 20,722 unique genes using 54,675 probe sets.

Affymetrix Data Processing. All further data processing and statistical analyses were done in R Statistical Software (version 2.18). Data was background corrected, log2 transformed, and quantile normalized with Robust Multichip Average (RMA) methods (Gautier et al. 2004). Using the affyQCReport and simpleaffy packages, quality control between the chips was high.

Gene specific differential expression and significance by AD case status was assessed using parallel linear models and empirical Bayesian variance methods at site-specific moderated t-tests (Smyth 2004). The following parallel linear models were fit at each CpG site that adjusted for subject's age and sex.

Expression = β_0 + β_1(AD Case Status) +β_2(Age) + β_3(Sex)

Probesets with AD Case Status β values that are significant at false discovery <0.1 are listed in a table and are used for data visualization. A heatmap with mean normalized expression values was used to cluster samples and probesets. To further illustrate differences between gene expression profiles in LOAD cases and controls, principle component analysis was used. Across all probes on the GeneChip, the first three principal components explain 67% of variance.

For each probeset, the Entrez gene ID, corresponding AD Case Status β p-value, and T-test statistic for direction of association were uploaded to the LRpath gene set enrichment testing program (Sartor et al. 2009). LRpath was used to test for enrichment in gene ontology terms, cytogenic bands, transcription factor binding sites, and microRNA binding sites.

We also downloaded publicly available cell-type specific gene expression data from NCBI's Gene Expression Omnibus (GEO). Gene expression in embryonic stem cell derived neuronal precursor cells (GSE7178) and astrocytes

(GSE5080) were plotted in a heatmap alongside the top hits distinguishing LOAD cases and controls.

Genome-wide DNA methylation. One µg of DNA from the twelve case-control pairs in the Discovery Phase was bisulfite-treated according to manufacturer's instructions with the EZ DNA Methylation Kit (Zymo, D5001). The Illumina recommended extended bisulfite thermocycling protocol (98ºC 10 minutes, hold at 64ºC for 17 hours) was followed. Bisulfite-treated DNA was prepped and applied to the Infinium HumanMethylation27 BeadArray (Illumina, WG-311-2201) by the University of Michigan DNA Sequencing Core facility using methods published by Illumina researchers (Bibikova et al. 2009). Across 27,578 probe sets, the BeadChip primarily targets CpG sites in the promoter regions of 14,475 genes and 110 miRNAs. Batch effects can potentially bias experimental differences, so six cases and six control samples were randomly applied to each of the 12-sample BeadChips. Fluorescent intensities were imaged using the Illumina BeadArray Reader and the associated BeadScan software was used for image processing and data extraction. Data were background normalized and percent methylation estimates (beta values) were calculated for each probe set. Data were exported to R for further processing.

Illumina Data Processing. The Illumina GenomeStudio software scored CpG sites for individual samples as failing based on fluorescence. CpG sites that failed on greater than 10% of samples (n=171 sites) were excluded from analyses. Tests for differences in methylation by epidemiological characteristics were performed using the limma BioConductor R package. The following parallel linear models were fit at each CpG site that adjusted for subject's age and sex.

% Methylation = β_0 + β_1(AD Case Status) + β_2(Age) + β_3(Sex)

Data integration and statistical methods. We filtered the list of genes differentially expressed between LOAD cases and controls and selected only the probe sets that met an expression difference threshold of p<0.05 (n=4,063 probe sets). We removed 750 probe sets that did not map to Entrez gene ID's

(n=3,313). To reduce the data to a single expression value per unique gene ID, if greater than one probe set was differentially expressed for a given gene, we selected the probe with the highest expression value. The set of unique Affymetrix gene ID's that were associated with LOAD case status included 2,768 probe sets.

We filtered the Illumina methylation data to only include CpG sites within 1500 bp of a transcription start site with a known Entrez gene ID (n=25,811). In the case of multiple Illumina probes for a given gene ID, we selected the CpG site with the lowest p-value for the association between LOAD cases and controls (n=13,865).

We then matched the Affymetrix expression data and the Illumina methylation data by Entrez gene IDs. This yielded a total of 2,094 unique gene ID's where Illumina and Affymetrix matched and where the Affymetrix probe set was statistically significantly associated with LOAD case status (**Figure 4.4**). The Pearson's correlation between gene expression and DNA methylation was calculated for each gene. We performed LRpath gene set enrichment analysis on the 2,094 gene ID's with linked expression and methylation data.
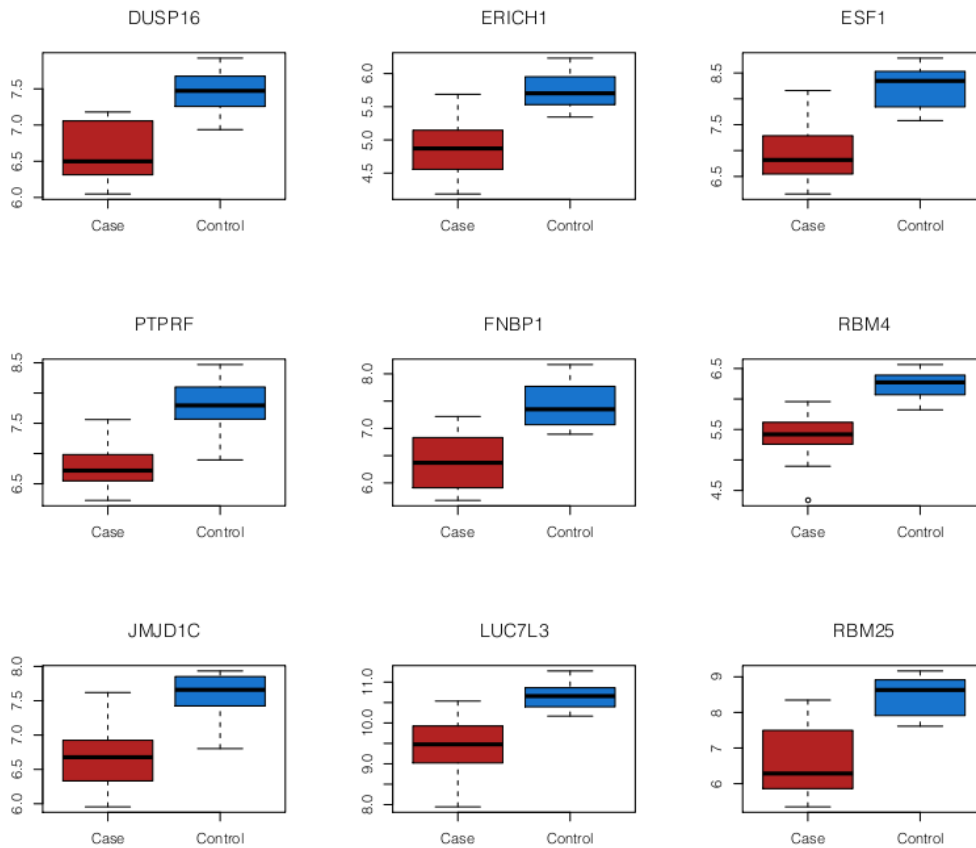
Figure 4.1. Boxplots of gene expression of the 9 top genes that differ by LOAD case or control status. LOAD cases display reduced gene expression at all of 9 of the genes.
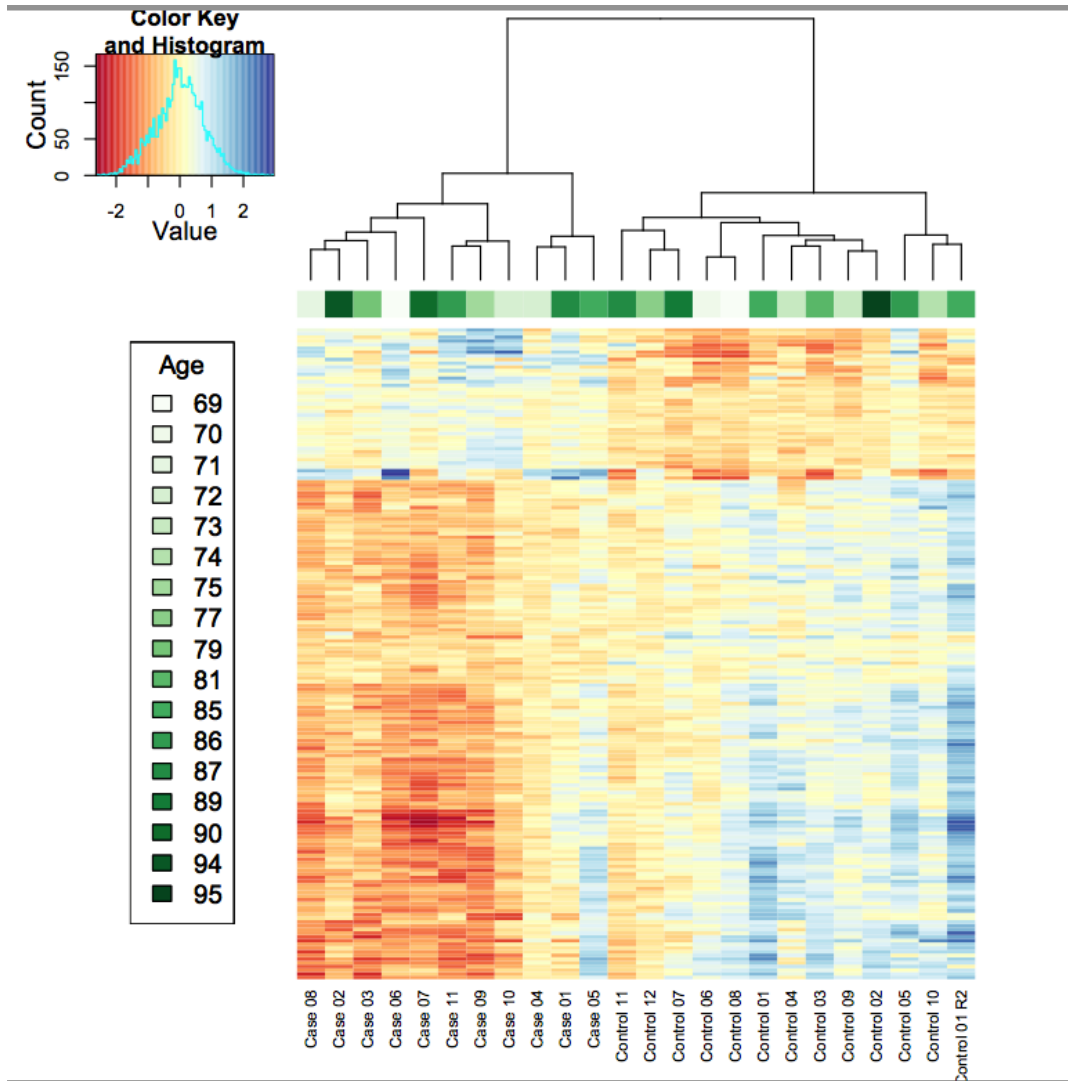
Figure 4.2. Heatmap of all of the 176 sites on the Affymetrix gene expression array (FDR < 0.1) associated with LOAD case status. This plot used maximum distance and Ward's hierarchical clustering methods. Data has been normalized to the mean expression value per probeset.
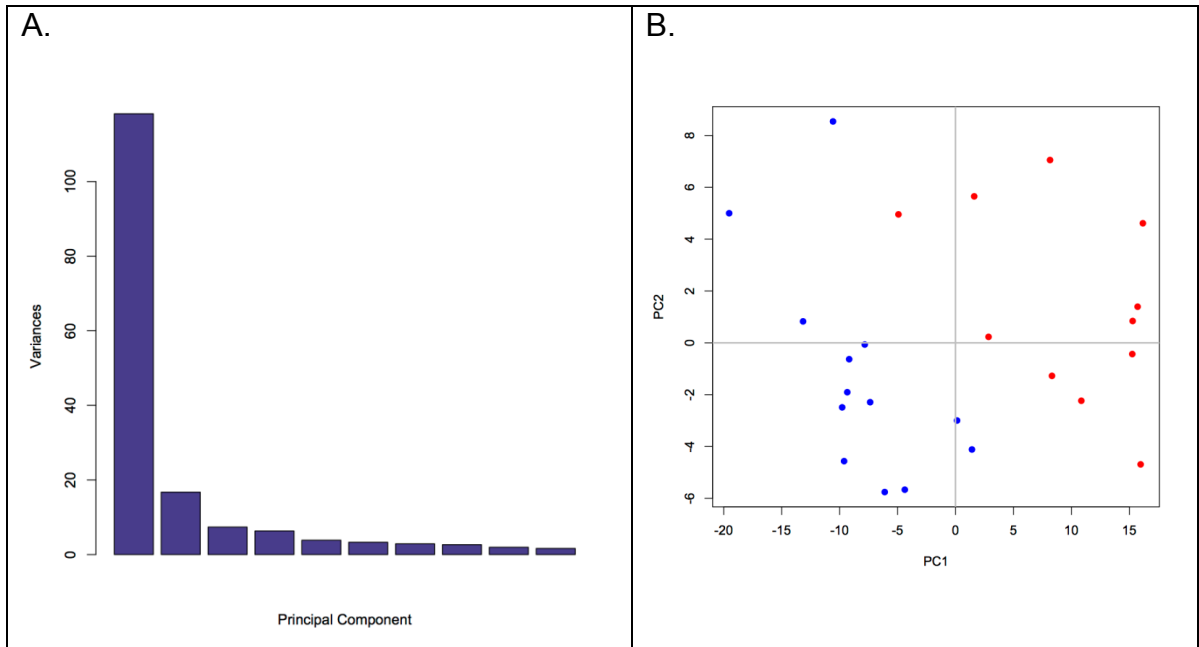
Figure 4.3. Principal Component Analysis. (A) Principal component loading histogram. (B). Principal component analysis scree plot for the top 176 probesets (FDR < 0.1).
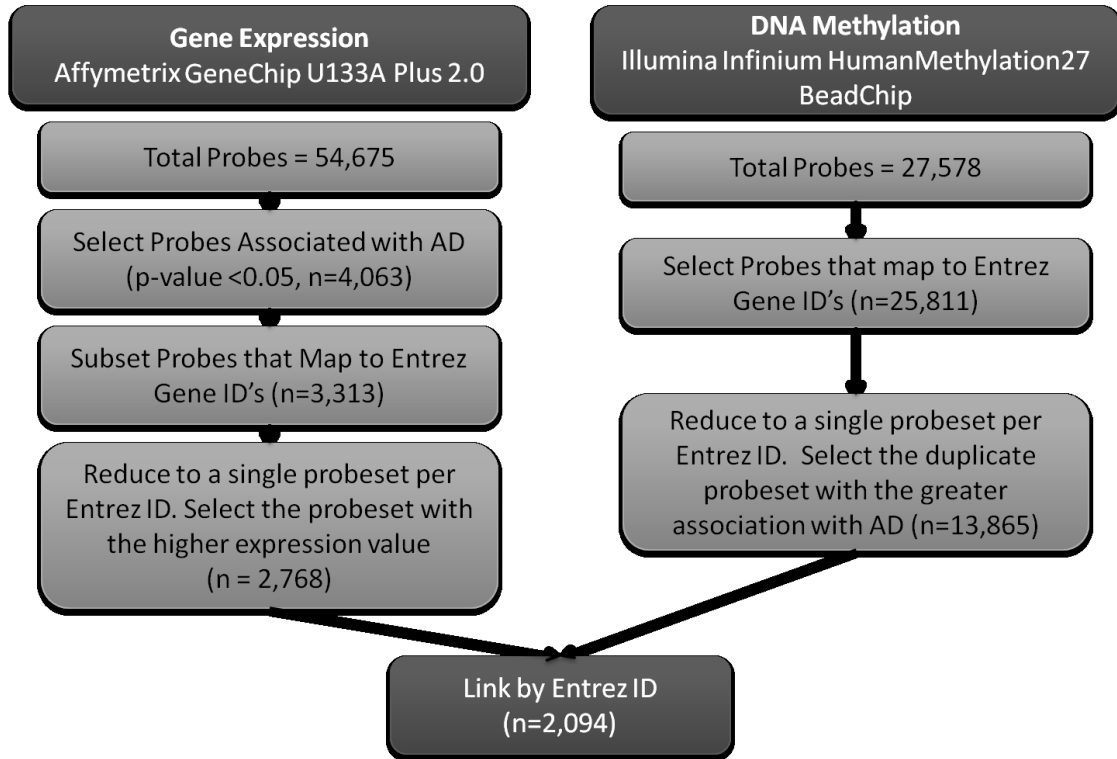
Figure 4.4. Gene-expression and DNA methylation linked data analysis pipeline.
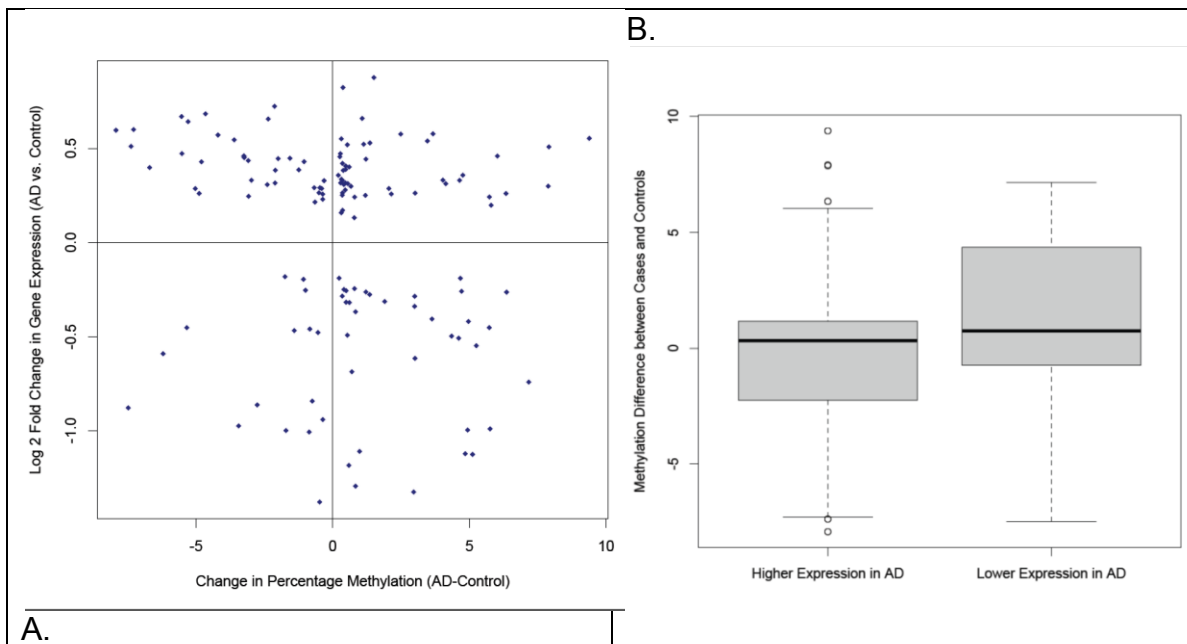
Figure 4.5 (A) Scatterplot of the 133 genes that displayed discordant gene expression and DNA methylation between LOAD cases and controls. The top of the figure represents genes less expressed in AD (n=50) and the bottom of the figure represents genes upregulated (n=84) in LOAD. The left of the figure shows genes more highly methylated in LOAD and the right shows less

135

methylation in LOAD. (B) Boxplot of the differences in methylation between AD and controls separated by the direction of change in expression.
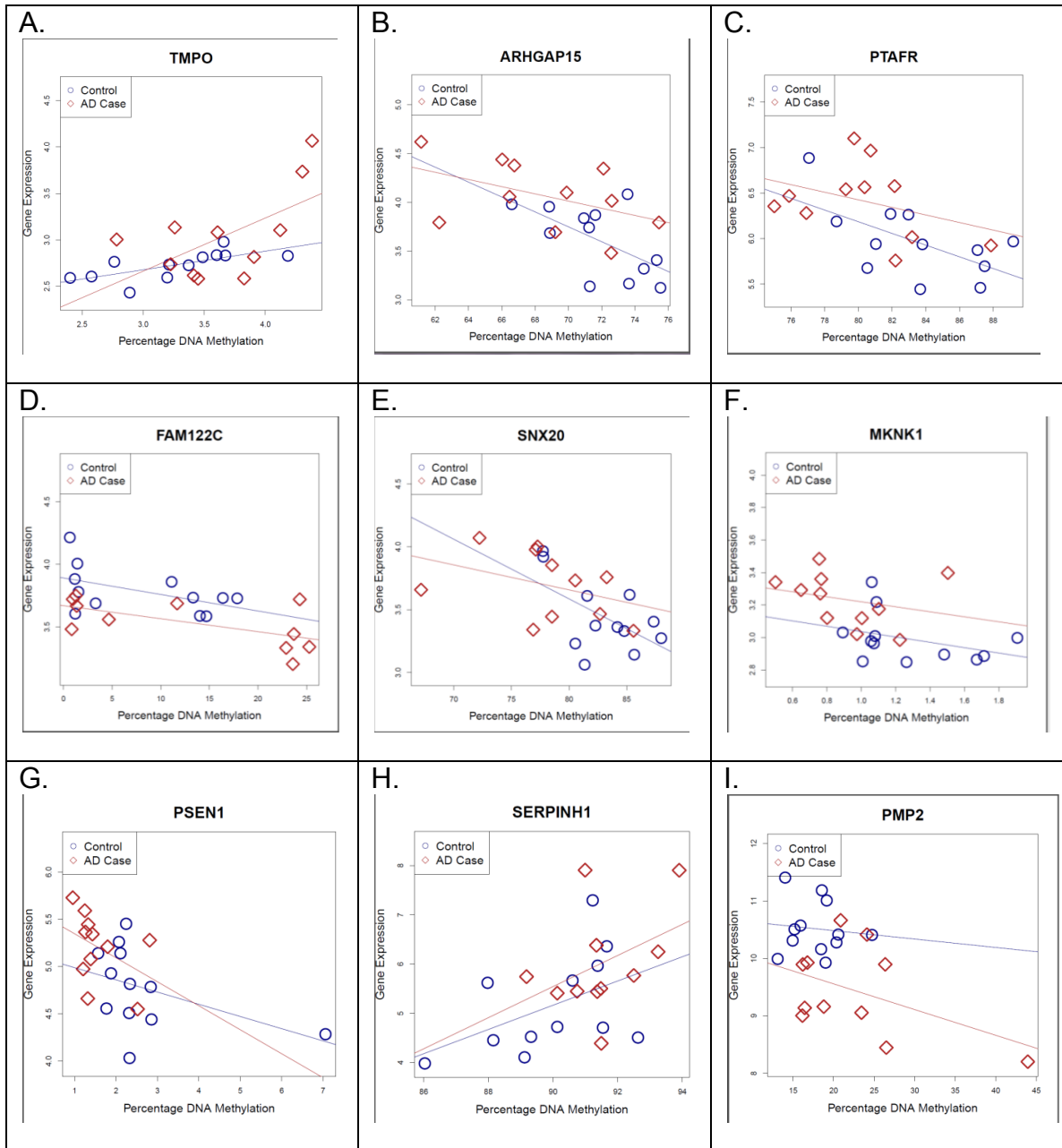
Figure 4.6. Scatter plots of 9 genes: expression vs. methylation.
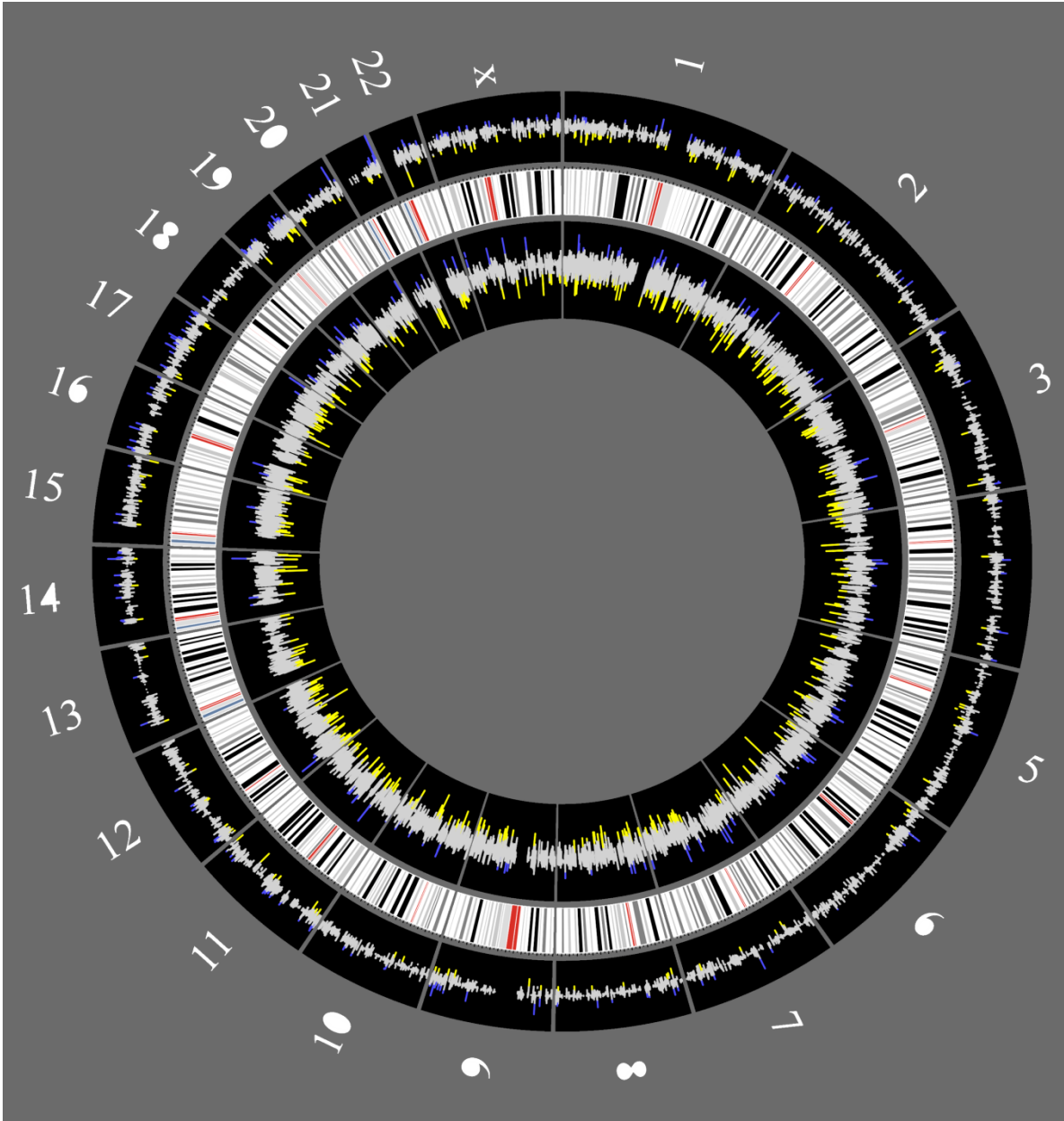
Figure 4.7. Circos plot: Locations of gene expression change and DNA methylation change.

TABLES

Table 4.1. Affymetrix expression differences between LOAD cases and controls (n=25) after adjusting for age and sex.

Rank	Affymetrix ID	Entrez ID	Gene Symbol	Gene Name	Chromosome Location	Average Expression	Fold Change	Log2 Fold Change	T-test Statistic	P-value	Adjusted P-value
1	1558740_s_at	80824	DUSP16	dual specificity phosphatase 16	12p13.2	7.11	0.531	-0.913	-6.63	8.85E-07	0.0316
2	227016_at	157697	ERICH1	glutamate-rich 1	8p23.3	5.37	0.529	-0.917	-6.26	2.09E-06	0.0316
3	218859_s_at	51575	ESF1	ESF1, nucleolar pre-rRNA processing protein, homolog (S. cerevisiae)	20p12.1	7.72	0.393	-1.349	-6.16	2.65E-06	0.0316
4	200635_s_at	5792	PTPRF	protein tyrosine phosphatase, receptor type, F	1p34	7.35	0.488	-1.034	-6.12	2.96E-06	0.0316
5	213940_s_at	23048	FNBP1	formin binding protein 1	9q34	7.03	0.425	-1.233	-5.87	5.33E-06	0.0316
6	213718_at	5936	RBM4	RNA binding motif protein 4	11q13	5.86	0.537	-0.897	-5.85	5.57E-06	0.0316
7	224933_s_at	221037	JMJD1C	jumonji domain containing 1C	10q21.3	7.19	0.504	-0.990	-5.83	5.96E-06	0.0316
8	208835_s_at	51747	LUC7L3	LUC7-like 3 (S. cerevisiae)	17q21.33	10.14	0.399	-1.327	-5.78	6.59E-06	0.0316
9	212027_at	58517	RBM25	RNA binding motif protein 25	14q24.3	7.73	0.264	-1.919	-5.74	7.27E-06	0.0316
10	241425_at	9818	NUPL1	nucleoporin like 1	13q12.13	5.70	0.558	-0.842	-5.73	7.60E-06	0.0316
11	213311_s_at	22980	TCF25	transcription factor 25 (basic helix-loop-helix)	16q24.3	8.47	0.374	-1.420	-5.71	7.89E-06	0.0316
12	212007_at	23190	UBXN4	UBX domain protein 4	2q21.3	8.97	0.421	-1.247	-5.66	8.88E-06	0.0316
13	214464_at	8476	CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	1q42.11	8.32	0.303	-1.722	-5.64	9.27E-06	0.0316
14	227703_s_at	94121	SYTL4	synaptotagmin-like 4	Xq21.33	6.31	2.538	1.344	5.64	9.32E-06	0.0316
15	224736_at	55749	CCAR1	cell division cycle and apoptosis regulator 1	10q21.3	8.02	0.446	-1.165	-5.64	9.47E-06	0.0316
16	228711_at	7587	ZNF37A	zinc finger protein 37A	10p11.2	7.10	0.584	-0.775	-5.61	1.01E-05	0.0316
17	201430_s_at	1809	DPYSL3	dihydropyrimidinase-like 3	5q32	5.01	1.937	0.954	5.61	1.01E-05	0.0316
18	226054_at	23476	BRD4	bromodomain containing 4	19p13.1	7.45	0.538	-0.894	-5.59	1.05E-05	0.0316
19	201026_at	9669	EIF5B	eukaryotic translation initiation factor 5B	2q11.2	7.24	0.465	-1.104	-5.58	1.10E-05	0.0316
20	230781_at	100505875	LOC100505875	uncharacterized LOC100505875	NA	4.56	2.494	1.318	5.52	1.26E-05	0.0322
21	222457_s_at	51474	LIMA1	LIM domain and actin binding 1	12q13	5.52	0.432	-1.212	-5.51	1.28E-05	0.0322
22	219779_at	79776	ZFHX4	zinc finger homeobox 4	8q21.11	6.64	0.627	-0.674	-5.50	1.33E-05	0.0322
23	212520_s_at	6597	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	19p13.2	7.19	0.427	-1.229	-5.49	1.35E-05	0.0322
24	203215_s_at	4646	MYO6	myosin VI	6q13	7.26	0.253	-1.984	-5.47	1.41E-05	0.0322
25	203975_s_at	10036	CHAF1A	chromatin assembly factor 1, subunit A (p150)	19p13.3	4.89	0.674	-0.569	-5.45	1.51E-05	0.0330

Table 4.2. Among genes with lower gene expression in AD cases vs. controls, the following are the top 15 biological processes that are down-regulated based on LR-Path.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	synaptic transmission	353	4.92E-12	2.92E-09	223, 5368, 627, 6529, 7425, 9379
2	transmission of nerve impulse	411	6.52E-12	3.38E-09	223, 5368, 627, 6529, 7425, 9379
3	cell-cell signaling	700	5.95E-07	6.03E-05	223, 4826, 5368, 627, 6529, 7425, 9379, 9547, 9636
4	oxidative phosphorylation	94	3.10E-06	1.81E-04	155066, 4704
5	regulation of synaptic plasticity	53	8.57E-06	4.23E-04	627, 7425
6	regulation of transmission of nerve impulse	132	2.83E-05	1.10E-03	627, 6529, 7425
7	cellular respiration	94	2.90E-05	1.10E-03	4704
8	regulation of neurological system process	142	4.41E-05	1.57E-03	627, 6529, 7425
9	neurotransmitter transport	96	5.54E-05	1.93E-03	6529, 9379
10	regulation of neuronal synaptic plasticity	32	5.96E-05	2.06E-03	7425
11	regulation of synaptic transmission	121	7.69E-05	0.003	627, 6529, 7425
12	respiratory electron transport chain	59	8.08E-05	0.003	4704
13	regulation of neurotransmitter levels	82	1.13E-04	0.003	223, 9379
14	nucleotide-excision repair, DNA damage removal	21	1.16E-04	0.003	2073
15	ATP synthesis coupled electron transport	51	1.89E-04	0.005	4704

Table 4.3. Among genes with higher gene expression in AD cases vs. controls, the following are the top 15 biological processes that are up-regulated based on LR-Path.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	translational elongation	101	4.07E-20	1.69E-16	11224, 1937, 2197, 25873, 6135, 6154, 6173, 6176, 6188, 6193, 6207, 6222, 6232
2	defense response	661	1.16E-16	2.42E-13	10219, 10410, 1050, 10581, 11326, 12, 23643, 241, 2532, 2919, 313, 3440, 3487, 3588, 3600, 3823, 4057, 4615, 4688, 51191, 58191, 6039, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7305, 8519, 929, 9332, 9450
3	immune response	660	3.86E-15	5.34E-12	10346, 10410, 10581, 11326, 23643, 2669, 2919, 3108, 3109, 3122, 3588, 3600, 4057, 4615, 4688, 4860, 51191, 54209, 563, 58191, 604, 6398, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719, 8519, 929, 9450
4	response to wounding	598	1.07E-14	1.11E-11	10219, 1050, 11326, 12, 1462, 2162, 23643, 241, 2697, 2919, 313, 3399, 3440, 3487, 3587, 3588, 3600, 4615, 4814, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7423, 929, 9332, 9450
5	innate immune response	201	8.28E-13	6.68E-10	10410, 10581, 11326, 23643, 4615, 4688, 51191, 58191, 7097, 7098, 710, 7100, 712, 713, 714, 718, 8519, 929, 9450
6	inflammatory response	365	9.65E-13	6.68E-10	10219, 1050, 11326, 12, 23643, 241, 2919, 313, 3440, 3487, 3588, 3600, 4615, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 929, 9332, 9450
7	response to biotic stimulus	442	8.34E-12	3.85E-09	10049, 10346, 10410, 10581, 11080, 1373, 1937, 23643, 3315, 3440, 3587, 3600, 3665, 3669, 4057, 51191, 6283, 7079, 7097, 7098, 7100, 7132, 8519, 871, 929
8	response to other organism	354	5.69E-11	2.36E-08	10346, 10410, 10581, 1373, 1937, 23643, 3315, 3440, 3587, 3600, 3665, 3669, 4057, 51191, 6283, 7079, 7097, 7098, 7100, 7132, 8519, 929
9	activation of immune response	110	7.45E-11	2.81E-08	11326, 1997, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719
10	regulation of immune response	245	8.89E-10	3.08E-07	11326, 1997, 3600, 604, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719
11	response to bacterium	213	1.42E-09	4.53E-07	1373, 23643, 3587, 4057, 6283, 7079, 7097, 7098, 7100, 7132, 929
12	I-kappaB kinase/NF-kappaB cascade	171	2.35E-09	6.98E-07	23643, 2697, 3965, 4615, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132
13	regulation of toll-like receptor signaling pathway	9	2.97E-09	8.21E-07	7097, 7098, 7100
14	positive regulation of toll-like receptor signaling pathway	7	7.11E-09	1.85E-06	7097, 7098, 7100
15	humoral immune response	80	8.56E-09	2.09E-06	11326, 4057, 54209, 710, 712, 713, 714, 718, 9450

Table 4.4. Gene expression and methylation correlation.

Rank	Entrez ID	Gene Symbol	Affymetrix ID	Illumina ID	Log2 Expression Fold Change	Expression Difference P-Value	Percent Difference in Methylation	Methylation Difference P-Value	Pearson Correlation Coefficient	Pearson Correlation Test P-Value
1	7112	TMPO	209754_s_at	cg23264519	0.309	0.041	0.411	0.046	0.617	0.002
2	55843	ARHGAP15	218870_at	cg23627134	0.457	0.003	-3.241	0.016	-0.614	0.002
3	5724	PTAFR	227184_at	cg18468844	0.436	0.011	-3.088	0.031	-0.591	0.003
4	159091	FAM122C	239047_at	cg16510010	-0.259	0.003	4.715	0.016	-0.586	0.003
5	124460	SNX20	228869_at	cg27081230	0.261	0.016	-4.881	0.016	-0.567	0.005
6	8569	MKNK1	1560720_at	cg15445332	0.230	0.002	-0.362	0.029	-0.540	0.008
7	5663	PSEN1	207782_s_at	cg11490446	0.430	0.008	-1.044	0.034	-0.534	0.009
8	871	SERPINH1	207714_s_at	6.250186	0.878	0.041	1.507	0.026	0.525	0.010
9	5375	PMP2	206826_at	cg21649520	-1.124	0.000	4.849	0.024	-0.521	0.011
10	5690	PSMB2	231323_at	3.890297	0.402	0.027	0.606	0.007	0.509	0.013
11	3684	ITGAM	205786_s_at	cg00833777	0.509	0.030	7.910	0.004	0.503	0.015
12	10602	CDC42EP3	209288_s_at	cg05469695	0.446	0.040	-1.993	0.027	-0.491	0.017
13	200150	PLD5	1563933_a_at	cg12613383	0.657	0.002	-2.358	0.042	-0.488	0.018
14	6497	SKI	213755_s_at	cg06382459	-0.263	0.032	1.217	0.011	-0.484	0.019
15	2857	GPR34	223620_at	cg22835805	0.670	0.031	-5.528	0.006	-0.464	0.026
16	27296	TP53TG5	207482_at	cg14226064	-0.407	0.029	3.634	0.006	-0.461	0.027
17	25937	WWTR1	202133_at	cg12507125	0.725	0.010	-2.123	0.004	-0.454	0.030
18	9968	MED12	203506_s_at	cg21693321	0.242	0.037	5.736	0.029	0.449	0.032
19	57828	CATSPERG	231261_at	cg18996334	-0.189	0.042	0.234	0.043	-0.448	0.032
20	64098	PARVG	233510_s_at	cg19863740	0.385	0.004	-2.099	0.035	-0.445	0.033
21	25903	OLFML2B	213125_at	cg20172280	0.242	0.043	0.803	0.022	0.443	0.034
22	79660	PPP1R3B	222662_at	cg24727203	0.601	0.004	-7.281	0.011	-0.441	0.035
23	138151	NACC2	212993_at	cg12004206	0.421	0.022	0.358	0.044	0.441	0.035
24	3428	IFI16	208966_x_at	cg21406461	0.598	0.030	-7.925	0.039	-0.438	0.036

Table 4.5. Study population characteristics.

	LOAD Cases	Neuropathologically Normal Controls
N	11	12
Age mean(range)	80 (69-94)	79.9 (69-95)
Sex	6 Females 5 Males	6 Females 6 Males
Ethnicity	Caucasian	Caucasian
Braak Stage mean	4.75	1.33
Years in storage mean (range)	11 (5-17)	13.2 (3-20)

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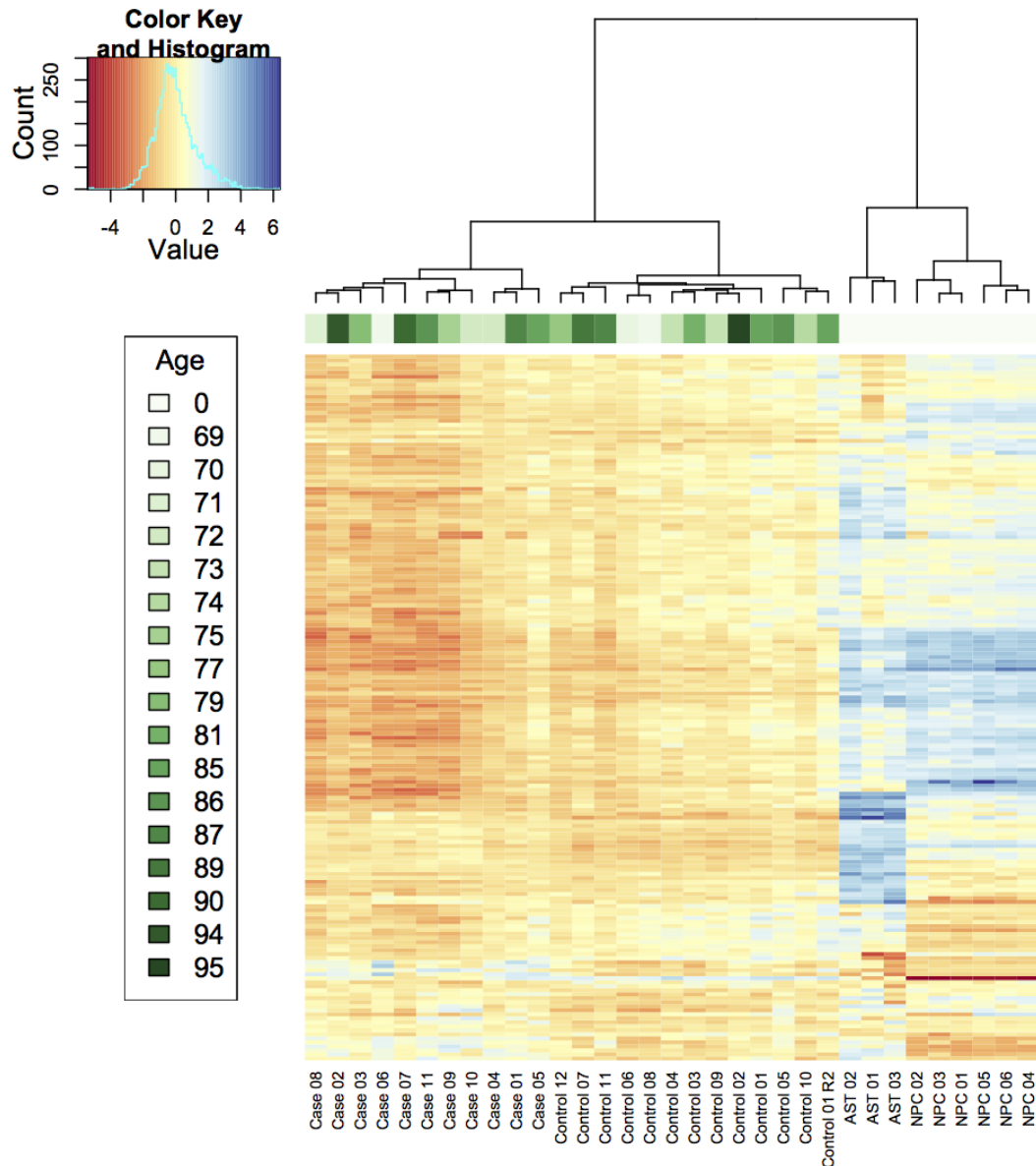
Supplemental Figure 4.1. Heatmap of all of the 176 sites on the Affymetrix gene expression array (FDR < 0.1) associated with LOAD case status. Publicly available data from embryonic stem cell derived neuronal precursor cells (GSE7178) and astrocytes (GSE5080) have been included. This plot used maximum distance and Ward's hierarchical clustering methods and normalized to the mean expression value per probeset.
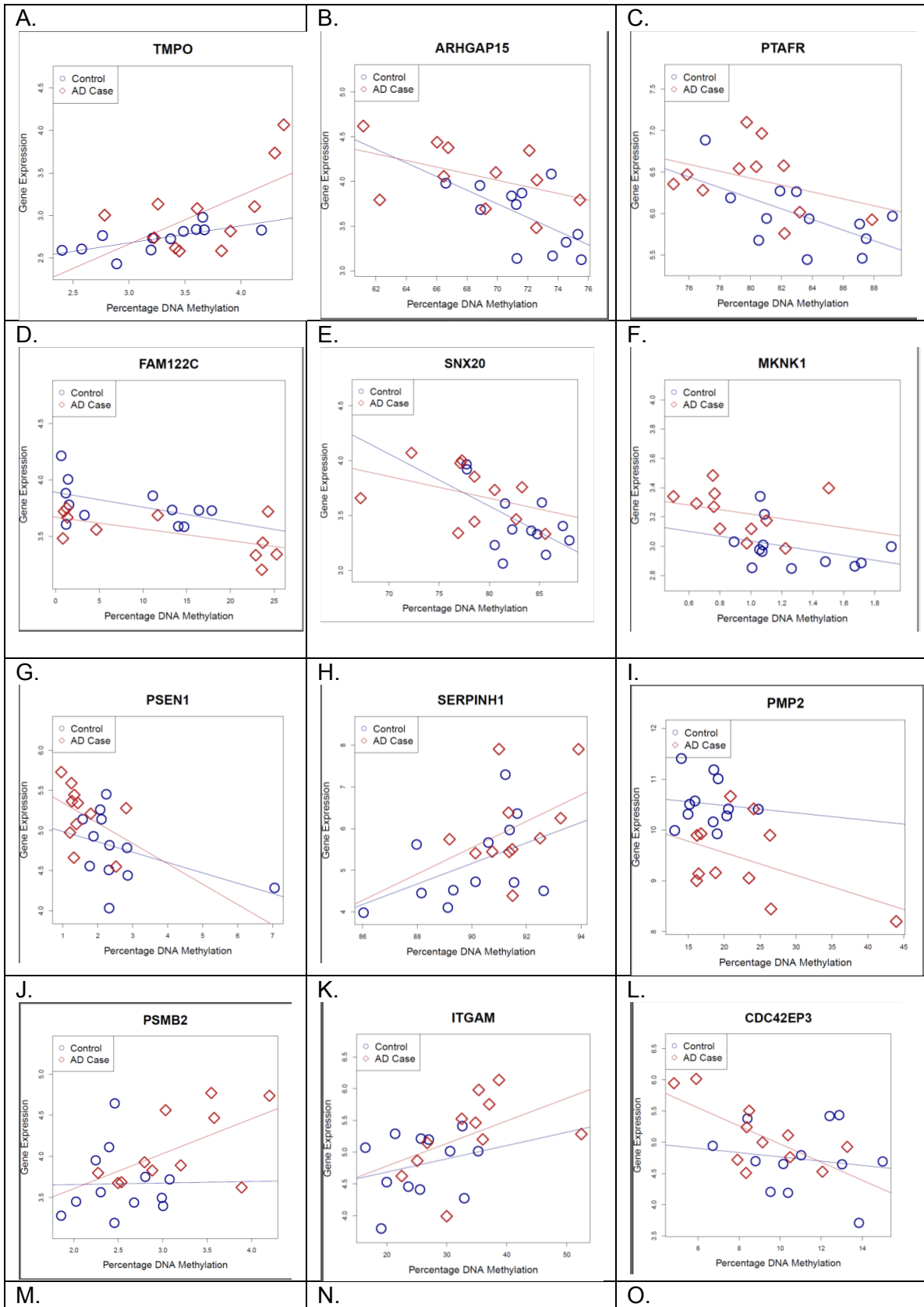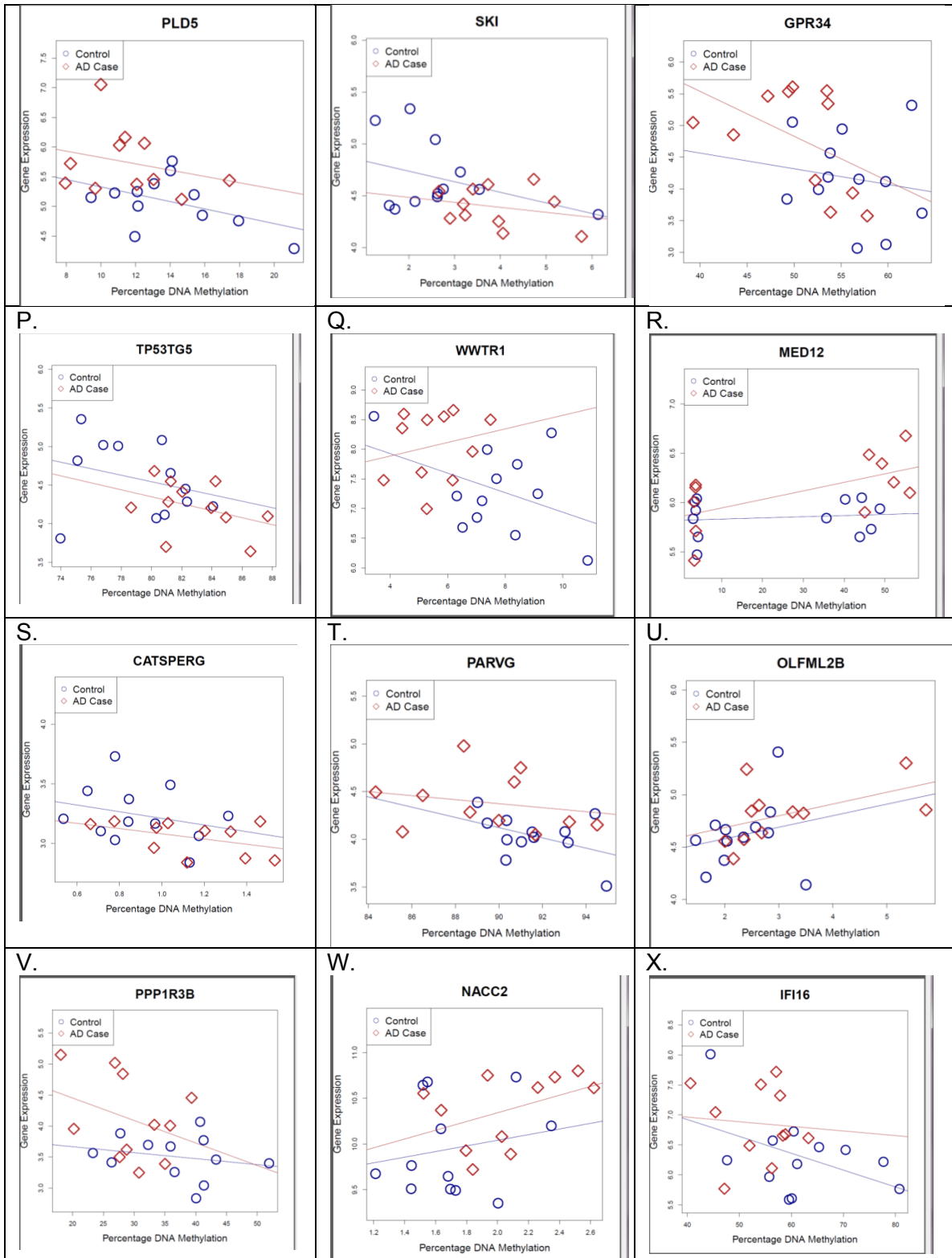
Supplemental Figure 2. Scatterplots of expression vs. methylation for the 23 genes significantly associated with AD via expression (p<0.05) and methylation (p<0.05), and methylation and expression are significantly correlated (Pearson's p<0.05).

SUPPLEMENTAL TABLES

Supplemental Table 4.1. Gene expression probesets associated with AD case vs. controls at FDR <0.1 (n=176)

Rank	Affymetrix ID	Entrez ID	Gene Symbol	Gene Name	Chromosome Location	Average Expression	Fold Change	Log2 Fold Change	T-test Statistic	P-value	Adjusted P-value
1	1558740_s_at	80824	DUSP16	dual specificity phosphatase 16	12p13.2	7.11	0.531	-0.913	-6.63	8.85E-07	0.0316
2	227016_at	157697	ERICH1	glutamate-rich 1	8p23.3	5.37	0.529	-0.917	-6.26	2.09E-06	0.0316
3	218859_s_at	51575	ESF1	ESF1, nucleolar pre-rRNA processing protein, homolog (S. cerevisiae)	20p12.1	7.72	0.393	-1.349	-6.16	2.65E-06	0.0316
4	200635_s_at	5792	PTPRF	protein tyrosine phosphatase, receptor type, F	1p34	7.35	0.488	-1.034	-6.12	2.96E-06	0.0316
5	213940_s_at	23048	FNBP1	formin binding protein 1	9q34	7.03	0.425	-1.233	-5.87	5.33E-06	0.0316
6	213718_at	5936	RBM4	RNA binding motif protein 4	11q13	5.86	0.537	-0.897	-5.85	5.57E-06	0.0316
7	224933_s_at	221037	JMJD1C	jumonji domain containing 1C	10q21.3	7.19	0.504	-0.990	-5.83	5.96E-06	0.0316
8	208835_s_at	51747	LUC7L3	LUC7-like 3 (S. cerevisiae)	17q21.33	10.14	0.399	-1.327	-5.78	6.59E-06	0.0316
9	212027_at	58517	RBM25	RNA binding motif protein 25	14q24.3	7.73	0.264	-1.919	-5.74	7.27E-06	0.0316
10	241425_at	9818	NUPL1	nucleoporin like 1	13q12.13	5.70	0.558	-0.842	-5.73	7.60E-06	0.0316
11	213311_s_at	22980	TCF25	transcription factor 25 (basic helix-loop-helix)	16q24.3	8.47	0.374	-1.420	-5.71	7.89E-06	0.0316
12	212007_at	23190	UBXN4	UBX domain protein 4	2q21.3	8.97	0.421	-1.247	-5.66	8.88E-06	0.0316
13	214464_at	8476	CDC42BPA	CDC42 binding protein kinase alpha (DMPK-like)	1q42.11	8.32	0.303	-1.722	-5.64	9.27E-06	0.0316
14	227703_s_at	94121	SYTL4	synaptotagmin-like 4	Xq21.33	6.31	2.538	1.344	5.64	9.32E-06	0.0316
15	224736_at	55749	CCAR1	cell division cycle and apoptosis regulator 1	10q21.3	8.02	0.446	-1.165	-5.64	9.47E-06	0.0316
16	228711_at	7587	ZNF37A	zinc finger protein 37A	10p11.2	7.10	0.584	-0.775	-5.61	1.01E-05	0.0316
17	201430_s_at	1809	DPYSL3	dihydropyrimidinase-like 3	5q32	5.01	1.937	0.954	5.61	1.01E-05	0.0316
18	226054_at	23476	BRD4	bromodomain containing 4	19p13.1	7.45	0.538	-0.894	-5.59	1.05E-05	0.0316
19	201026_at	9669	EIF5B	eukaryotic translation initiation factor 5B	2q11.2	7.24	0.465	-1.104	-5.58	1.10E-05	0.0316
20	230781_at	100505875	LOC100505875	uncharacterized LOC100505875	NA	4.56	2.494	1.318	5.52	1.26E-05	0.0322
21	222457_s_at	51474	LIMA1	LIM domain and actin binding 1	12q13	5.52	0.432	-1.212	-5.51	1.28E-05	0.0322
22	219779_at	79776	ZFHX4	zinc finger homeobox 4	8q21.11	6.64	0.627	-0.674	-5.50	1.33E-05	0.0322
23	212520_s_at	6597	SMARCA4	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	19p13.2	7.19	0.427	-1.229	-5.49	1.35E-05	0.0322
24	203215_s_at	4646	MYO6	myosin VI	6q13	7.26	0.253	-1.984	-5.47	1.41E-05	0.0322
25	203975_s_at	10036	CHAF1A	chromatin assembly factor 1, subunit A (p150)	19p13.3	4.89	0.674	-0.569	-5.45	1.51E-05	0.0330
26	232595_at	NA	NA	NA	NA	3.49	1.360	0.444	5.41	1.66E-05	0.0349
27	222628_s_at	51455	REV1	REV1 homolog (S. cerevisiae)	2q11.1-q11.2	7.19	0.530	-0.916	-5.37	1.81E-05	0.0366
28	200842_s_at	2058	EPRS	glutamyl-prolyl-tRNA synthetase	1q41	7.79	0.367	-1.444	-5.36	1.88E-05	0.0366
29	222792_s_at	29080	CCDC59	coiled-coil domain containing 59	12q21.31	6.50	0.566	-0.822	-5.34	1.98E-05	0.0366
30	214375_at	8496	PPFIBP1	PTPRF interacting protein, binding protein 1 (liprin beta 1)	12p12.1	6.27	0.440	-1.185	-5.33	2.03E-05	0.0366
31	220727_at	54207	KCNK10	potassium channel, subfamily K, member 10	14q31.3	6.30	0.577	-0.794	-5.30	2.16E-05	0.0366
32	206726_at	27306	HPGDS	hematopoietic prostaglandin D synthase	4q22.3	3.32	1.353	0.436	5.29	2.23E-05	0.0366
33	242233_at	NA	NA	NA	NA	5.52	0.603	-0.730	-5.28	2.25E-05	0.0366
34	202379_s_at	4820	NKTR	natural killer-tumor recognition sequence	3p23-p21	9.16	0.476	-1.070	-5.28	2.28E-05	0.0366

35	214911_s_at	6046	BRD2	bromodomain containing 2	6p21.3	8.26	0.467	-1.098	-5.25	2.47E-05	0.0386
36	216563_at	23253	ANKRD12	ankyrin repeat domain 12	18p11.22	7.50	0.389	-1.363	-5.22	2.64E-05	0.0399
37	211993_at	65125	WNK1	WNK lysine deficient protein kinase 1	12p13.3	6.85	0.344	-1.540	-5.20	2.76E-05	0.0399
38	219387_at	55704	CCDC88A	coiled-coil domain containing 88A	2p16.1	8.08	0.286	-1.807	-5.16	3.05E-05	0.0399
39	226176_s_at	84132	USP42	ubiquitin specific peptidase 42	7p22.1	6.36	0.510	-0.971	-5.16	3.09E-05	0.0399
40	223185_s_at	79365	BHLHE41	basic helix-loop-helix family, member e41	12p12.1	4.14	0.464	-1.108	-5.15	3.10E-05	0.0399
41	209945_s_at	2932	GSK3B	glycogen synthase kinase 3 beta	3q13.3	5.25	0.703	-0.509	-5.14	3.19E-05	0.0399
42	229353_s_at	64710	NUCKS1	nuclear casein kinase and cyclin-dependent kinase substrate 1	1q32.1	10.51	0.526	-0.926	-5.14	3.20E-05	0.0399
43	225041_at	54737	MPHOSPH8	M-phase phosphoprotein 8	13q12.11	8.89	0.376	-1.410	-5.14	3.24E-05	0.0399
44	225565_at	1385	CREB1	cAMP responsive element binding protein 1	2q34	5.33	0.552	-0.857	-5.13	3.27E-05	0.0399
45	222122_s_at	57187	THOC2	THO complex 2	Xq25-q26.3	7.11	0.462	-1.114	-5.13	3.28E-05	0.0399
46	213298_at	4782	NFIC	nuclear factor I/C (CCAAT-binding transcription factor)	19p13.3	5.44	0.447	-1.161	-5.10	3.55E-05	0.0422
47	209230_s_at	26471	NUPR1	nuclear protein, transcriptional regulator, 1	16p11.2	7.06	2.413	1.271	5.08	3.74E-05	0.0432
48	222540_s_at	51773	RSF1	remodeling and spacing factor 1	11q14.1	8.25	0.398	-1.328	-5.07	3.79E-05	0.0432
49	222620_s_at	64215	DNAJC1	DnaJ (Hsp40) homolog, subfamily C, member 1	10p12.31	7.29	0.385	-1.378	-5.04	4.08E-05	0.0442
50	1569594_a_at	9147	NEMF	nuclear export mediator factor	14q22	7.97	0.429	-1.221	-5.04	4.08E-05	0.0442
51	229586_at	80205	CHD9	chromodomain helicase DNA binding protein 9	16q12.2	7.65	0.476	-1.072	-5.04	4.13E-05	0.0442
52	208963_x_at	3992	FADS1	fatty acid desaturase 1	11q12.2-q13.1	7.71	0.556	-0.847	-5.00	4.52E-05	0.0467
53	226975_at	55599	RNPC3	RNA-binding region (RNP1, RRM) containing 3	1p21	6.65	0.393	-1.346	-5.00	4.53E-05	0.0467
54	229163_at	55450	CAMK2N1	calcium/calmodulin-dependent protein kinase II inhibitor 1	1p36.12	7.38	0.567	-0.820	-4.99	4.69E-05	0.0472
55	1555495_a_at	10283	CWC27	CWC27 spliceosome-associated protein homolog (S. cerevisiae)	5q12.3	8.27	0.566	-0.820	-4.97	4.85E-05	0.0472
56	200702_s_at	57062	DDX24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	14q32	7.59	0.358	-1.480	-4.97	4.88E-05	0.0472
57	225946_at	11228	RASSF8	Ras association (RalGDS/AF-6) domain family (N-terminal) member 8	12p12.3	6.58	1.665	0.736	4.96	5.04E-05	0.0472
58	209376_x_at	9169	SCAF11	SR-related CTD-associated factor 11	12q12	7.02	0.468	-1.095	-4.96	5.09E-05	0.0472
59	208994_s_at	9360	PPIG	peptidylprolyl isomerase G (cyclophilin G)	2q31.1	7.81	0.406	-1.299	-4.95	5.10E-05	0.0472
60	213850_s_at	9169	SCAF11	SR-related CTD-associated factor 11	12q12	7.86	0.460	-1.122	-4.94	5.27E-05	0.0480
61	201183_s_at	1108	CHD4	chromodomain helicase DNA binding protein 4	12p13	7.21	0.461	-1.118	-4.93	5.35E-05	0.0480
62	232323_s_at	55761	TTC17	tetratricopeptide repeat domain 17	11p11.2	6.38	0.469	-1.092	-4.92	5.60E-05	0.0494
63	209088_s_at	29855	UBN1	ubinuclein 1	16p13.3	6.99	0.418	-1.257	-4.91	5.75E-05	0.0497
64	215049_x_at	9332	CD163	CD163 molecule	12p13.3	5.22	3.284	1.715	4.90	5.85E-05	0.0497
65	222616_s_at	10600	USP16	ubiquitin specific peptidase 16	21q22.11	6.39	0.329	-1.606	-4.89	5.92E-05	0.0497
66	213729_at	55660	PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)	2q23.3	6.87	0.460	-1.120	-4.89	5.99E-05	0.0497
67	231061_at	NA	NA	NA	NA	5.42	0.540	-0.888	-4.88	6.11E-05	0.0499
68	217728_at	6277	S100A6	S100 calcium binding protein A6	1q21	8.34	1.537	0.620	4.87	6.26E-05	0.0500
69	203729_at	2014	EMP3	epithelial membrane protein 3	19q13.3	5.56	1.977	0.983	4.86	6.47E-05	0.0500
70	227298_at	401264	FLJ37798	uncharacterized LOC401264	6p12.3	6.06	0.750	-0.414	-4.86	6.48E-05	0.0500
71	229635_at	1.01E+08	LOC100505702	uncharacterized LOC100505702	NA	4.25	2.236	1.161	4.86	6.50E-05	0.0500

72	208685_x_at	6046	BRD2	bromodomain containing 2	6p21.3	8.27	0.487	-1.038	-4.84	6.73E-05	0.0511
73	217832_at	10492	SYNCRIP	synaptotagmin binding, cytoplasmic RNA interacting protein	6q14-q15	7.35	1.764	0.819	4.82	7.16E-05	0.0536
74	208772_at	NA	NA	NA	NA	8.03	0.579	-0.788	-4.80	7.56E-05	0.0557
75	219437_s_at	29123	ANKRD11	ankyrin repeat domain 11	16q24.3	6.84	0.376	-1.411	-4.79	7.75E-05	0.0557
76	238595_at	NA	NA	NA	NA	5.86	0.487	-1.037	-4.79	7.77E-05	0.0557
77	238584_at	79781	IQCA1	IQ motif containing with AAA domain 1	2q37.3	4.26	0.467	-1.099	-4.78	7.85E-05	0.0557
78	35436_at	2801	GOLGA2	golgin A2	9q34.11	6.16	0.495	-1.015	-4.76	8.20E-05	0.0569
79	1558965_at	51317	PHF21A	PHD finger protein 21A	11p11.2	3.12	1.419	0.505	4.76	8.24E-05	0.0569
80	241458_at	NA	NA	NA	NA	4.63	1.432	0.518	4.76	8.38E-05	0.0569
81	220946_s_at	29072	SETD2	SET domain containing 2	3p21.31	3.30	0.550	-0.862	-4.75	8.53E-05	0.0569
82	214843_s_at	23032	USP33	ubiquitin specific peptidase 33	1p31.1	6.00	0.600	-0.738	-4.75	8.59E-05	0.0569
83	236869_at	NA	NA	NA	NA	4.33	0.553	-0.854	-4.74	8.74E-05	0.0569
84	207542_s_at	358	AQP1	aquaporin 1 (Colton blood group)	7p14	6.41	2.433	1.283	4.74	8.75E-05	0.0569
85	201914_s_at	11231	SEC63	SEC63 homolog (S. cerevisiae)	6q21	6.13	0.384	-1.380	-4.73	8.93E-05	0.0575
86	221210_s_at	80896	NPL	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	1q25	3.93	2.039	1.028	4.72	9.21E-05	0.0581
87	203181_x_at	6733	SRPK2	SRSF protein kinase 2	7q22-q31.1	9.55	0.496	-1.011	-4.71	9.43E-05	0.0581
88	233080_s_at	55660	PRPF40A	PRP40 pre-mRNA processing factor 40 homolog A (S. cerevisiae)	2q23.3	6.77	0.490	-1.029	-4.70	9.70E-05	0.0581
89	209466_x_at	5764	PTN	pleiotrophin	7q33	8.46	0.411	-1.282	-4.69	9.81E-05	0.0581
90	208663_s_at	7267	TTC3	tetratricopeptide repeat domain 3	21q22.2	8.39	0.298	-1.745	-4.69	9.98E-05	0.0581
91	211737_x_at	5764	PTN	pleiotrophin	7q33	9.37	0.445	-1.168	-4.68	1.02E-04	0.0581
92	214314_s_at	9669	EIF5B	eukaryotic translation initiation factor 5B	2q11.2	6.17	0.551	-0.861	-4.68	1.02E-04	0.0581
93	206929_s_at	4782	NFIC	nuclear factor I/C (CCAAT-binding transcription factor)	19p13.3	7.19	0.499	-1.002	-4.67	1.03E-04	0.0581
94	209127_s_at	9733	SART3	squamous cell carcinoma antigen recognized by T cells 3	12q24.1	6.13	0.417	-1.260	-4.67	1.03E-04	0.0581
95	212382_at	6925	TCF4	transcription factor 4	18q21.1	7.25	0.387	-1.371	-4.67	1.04E-04	0.0581
96	225590_at	57630	SH3RF1	SH3 domain containing ring finger 1	4q32.3	5.92	0.595	-0.749	-4.67	1.04E-04	0.0581
97	226782_at	253512	SLC25A30	solute carrier family 25, member 30	13q14.13	5.11	1.364	0.448	4.67	1.05E-04	0.0581
98	204964_s_at	8082	SSPN	sarcospan (Kras oncogene-associated gene)	12p11.2	6.29	0.497	-1.008	-4.66	1.06E-04	0.0581
99	208942_s_at	7095	SEC62	SEC62 homolog (S. cerevisiae)	3q26.2	9.81	0.596	-0.746	-4.65	1.08E-04	0.0581
100	232677_at	NA	NA	NA	NA	4.55	0.603	-0.731	-4.65	1.09E-04	0.0581
101	208993_s_at	9360	PPIG	peptidylprolyl isomerase G (cyclophilin G)	2q31.1	8.28	0.463	-1.111	-4.64	1.10E-04	0.0581
102	208610_s_at	23524	SRRM2	serine/arginine repetitive matrix 2	16p13.3	7.99	0.326	-1.619	-4.64	1.11E-04	0.0581
103	216520_s_at	7178	TPT1	tumor protein, translationally-controlled 1	13q14	11.68	1.383	0.468	4.64	1.12E-04	0.0581
104	201224_s_at	10250	SRRM1	serine/arginine repetitive matrix 1	1p36.11	8.23	0.543	-0.882	-4.64	1.12E-04	0.0581
105	225730_s_at	25917	THUMPD3	THUMP domain containing 3	3p25.3	5.40	0.463	-1.111	-4.64	1.12E-04	0.0581
106	242916_at	11064	CNTRL	centriolin	9q33.2	4.22	0.575	-0.799	-4.64	1.13E-04	0.0581
107	212570_at	23052	ENDOD1	endonuclease domain containing 1	11q21	5.80	0.496	-1.012	-4.63	1.14E-04	0.0581
108	215338_s_at	4820	NKTR	natural killer-tumor recognition sequence	3p23-p21	7.00	0.510	-0.970	-4.61	1.20E-04	0.0609
109	211996_s_at	NA	NA	NA	NA	10.44	0.621	-0.686	-4.60	1.24E-04	0.0615
110	230180_at	10521	DDX17	DEAD (Asp-Glu-Ala-Asp) box helicase 17	22q13.1	5.80	0.510	-0.971	-4.60	1.24E-04	0.0615

111	1569302_at	85459	KIAA1731	KIAA1731	11q21	5.21	0.508	-0.976	-4.59	1.27E-04	0.0625
112	208676_s_at	5036	PA2G4	proliferation-associated 2G4, 38kDa	12q13.2	7.93	0.718	-0.478	-4.59	1.28E-04	0.0625
113	218659_at	55252	ASXL2	additional sex combs like 2 (Drosophila)	2p24.1	6.85	1.483	0.568	4.57	1.32E-04	0.0634
114	211948_x_at	23215	PRRC2C	proline-rich coiled-coil 2C	1q23.3	8.90	0.557	-0.843	-4.57	1.33E-04	0.0634
115	225377_at	55684	C9orf86	chromosome 9 open reading frame 86	9q34.3	7.78	0.408	-1.293	-4.57	1.33E-04	0.0634
116	1555913_at	54856	GON4L	gon-4-like (C. elegans)	1q22	4.58	0.643	-0.636	-4.57	1.34E-04	0.0634
117	221745_at	10238	DCAF7	DDB1 and CUL4 associated factor 7	17q23.3	5.94	0.641	-0.642	-4.56	1.36E-04	0.0635
118	1556211_a_at	NA	NA	NA	NA	4.88	0.300	-1.737	-4.55	1.41E-04	0.0654
119	214305_s_at	23451	SF3B1	splicing factor 3b, subunit 1, 155kDa	2q33.1	6.57	0.510	-0.971	-4.54	1.44E-04	0.0663
120	209579_s_at	8930	MBD4	methyl-CpG binding domain protein 4	3q21.3	8.48	0.529	-0.918	-4.52	1.50E-04	0.0683
121	228801_at	94101	ORMDL1	ORM1-like 1 (S. cerevisiae)	2q32	4.16	0.678	-0.561	-4.51	1.53E-04	0.0692
122	239894_at	1E+08	LOC100128511	uncharacterized LOC100128511	10p12.31	3.94	0.614	-0.703	-4.51	1.55E-04	0.0694
123	202845_s_at	10928	RALBP1	ralA binding protein 1	18p11.3	8.73	1.381	0.466	4.50	1.58E-04	0.0696
124	201085_s_at	6651	SON	SON DNA binding protein	21q22.1-q22.2	7.75	0.356	-1.492	-4.50	1.58E-04	0.0696
125	206826_at	5375	PMP2	peripheral myelin protein 2	8q21.3-q22.1	10.04	0.459	-1.124	-4.47	1.70E-04	0.0739
126	201024_x_at	9669	EIF5B	eukaryotic translation initiation factor 5B	2q11.2	9.48	0.524	-0.934	-4.47	1.70E-04	0.0739
127	203186_s_at	6275	S100A4	S100 calcium binding protein A4	1q21	5.35	1.685	0.753	4.46	1.74E-04	0.0742
128	242835_s_at	728730	LOC728730	uncharacterized LOC728730	2p22.1	5.29	0.380	-1.395	-4.46	1.75E-04	0.0742
129	239154_at	NA	NA	NA	NA	5.39	0.578	-0.790	-4.46	1.76E-04	0.0742
130	232617_at	1520	CTSS	cathepsin S	1q21	5.85	1.904	0.929	4.46	1.77E-04	0.0742
131	203761_at	6503	SLA	Src-like-adaptor	8q22.3-qter	6.22	1.909	0.933	4.45	1.78E-04	0.0742
132	238893_at	338758	LOC338758	uncharacterized LOC338758	12q21.33	7.45	0.506	-0.983	-4.45	1.79E-04	0.0742
133	204999_s_at	22809	ATF5	activating transcription factor 5	19q13.3	3.64	0.670	-0.577	-4.44	1.84E-04	0.0758
134	241955_at	25831	HECTD1	HECT domain containing E3 ubiquitin protein ligase 1	14q12	5.29	0.413	-1.275	-4.43	1.89E-04	0.0770
135	213509_x_at	8824	CES2	carboxylesterase 2	16q22.1	7.20	0.716	-0.482	-4.42	1.92E-04	0.0778
136	218454_at	79887	PLBD1	phospholipase B domain containing 1	12p13.1	4.02	1.365	0.449	4.42	1.95E-04	0.0786
137	209258_s_at	9126	SMC3	structural maintenance of chromosomes 3	10q25	5.46	0.386	-1.372	-4.41	2.00E-04	0.0794
138	201730_s_at	7175	TPR	translocated promoter region (to activated MET oncogene)	1q25	6.69	0.426	-1.233	-4.41	2.01E-04	0.0794
139	231729_s_at	828	CAPS	calcyphosine	19p13.3	4.69	2.011	1.008	4.40	2.02E-04	0.0794
140	1552326_a_at	220136	CCDC11	coiled-coil domain containing 11	18q21.1	4.19	1.442	0.528	4.39	2.11E-04	0.0822
141	208095_s_at	NA	NA	NA	NA	6.95	0.353	-1.502	-4.38	2.12E-04	0.0822
142	208879_x_at	24148	PRPF6	PRP6 pre-mRNA processing factor 6 homolog (S. cerevisiae)	20q13.33	6.57	0.430	-1.218	-4.38	2.16E-04	0.0832
143	212994_at	57187	THOC2	THO complex 2	Xq25-q26.3	6.17	0.506	-0.984	-4.37	2.22E-04	0.0850
144	244154_at	80821	DDHD1	DDHD domain containing 1	14q21	4.89	0.722	-0.471	-4.36	2.25E-04	0.0853
145	222020_s_at	50863	NTM	neurotrimin	11q25	6.37	0.508	-0.977	-4.35	2.33E-04	0.0863
146	204787_at	11326	VSIG4	V-set and immunoglobulin domain containing 4	Xq12-q13.3	5.92	2.774	1.472	4.34	2.35E-04	0.0863
147	209715_at	23468	CBX5	chromobox homolog 5	12q13.13	7.32	0.564	-0.825	-4.34	2.36E-04	0.0863
148	242974_at	961	CD47	CD47 molecule	3q13.1-q13.2	5.50	1.343	0.426	4.34	2.38E-04	0.0863
149	214129_at	9659	PDE4DIP	phosphodiesterase 4D interacting protein	1q12	6.28	1.945	0.960	4.34	2.38E-04	0.0863
150	208930_s_at	3609	ILF3	interleukin enhancer binding factor 3, 90kDa	19p13.2	5.53	0.452	-1.147	-4.34	2.39E-04	0.0863

151	219507_at	51319	RSRC1	arginine/serine-rich coiled-coil 1	3q25.32	5.74	0.360	-1.473	-4.33	2.41E-04	0.0863
152	223138_s_at	170506	DHX36	DEAH (Asp-Glu-Ala-His) box polypeptide 36	3p13-q23	5.53	0.471	-1.085	-4.33	2.41E-04	0.0863
153	222737_s_at	29117	BRD7	bromodomain containing 7	16q12	7.03	0.513	-0.962	-4.33	2.42E-04	0.0863
154	1554470_s_at	29068	ZBTB44	zinc finger and BTB domain containing 44	11q24.3	4.40	1.524	0.608	4.33	2.43E-04	0.0864
155	226416_at	90459	ERI1	exoribonuclease 1	8p23.1	5.18	1.401	0.486	4.33	2.46E-04	0.0868
156	206167_s_at	395	ARHGAP6	Rho GTPase activating protein 6	Xp22.3	5.74	1.485	0.570	4.32	2.48E-04	0.0870
157	204061_at	5613	PRKX	protein kinase, X-linked	Xp22.3	5.49	2.377	1.249	4.32	2.51E-04	0.0870
158	202844_s_at	10928	RALBP1	ralA binding protein 1	18p11.3	7.35	0.470	-1.090	-4.32	2.51E-04	0.0870
159	203645_s_at	9332	CD163	CD163 molecule	12p13.3	4.50	2.827	1.499	4.30	2.59E-04	0.0892
160	224631_at	NA	NA	NA	NA	6.65	0.447	-1.162	-4.30	2.64E-04	0.0901
161	217869_at	51144	HSD17B12	hydroxysteroid (17-beta) dehydrogenase 12	11p11.2	9.37	0.726	-0.461	-4.29	2.70E-04	0.0918
162	235409_at	23269	MGA	MAX gene associated	15q14	5.91	0.491	-1.026	-4.28	2.78E-04	0.0937
163	224856_at	2289	FKBP5	FK506 binding protein 5	6p21.31	6.81	1.951	0.964	4.27	2.81E-04	0.0940
164	212120_at	23433	RHOQ	ras homolog family member Q	2p21	8.27	1.367	0.451	4.27	2.86E-04	0.0940
165	241769_at	NA	NA	NA	NA	5.38	0.541	-0.888	-4.27	2.86E-04	0.0940
166	226189_at	3696	ITGB8	integrin, beta 8	7p21.1	9.05	1.469	0.555	4.26	2.87E-04	0.0940
167	223797_at	114224	PRO2852	uncharacterized protein PRO2852	NA	6.19	1.467	0.553	4.26	2.87E-04	0.0940
168	208710_s_at	8943	AP3D1	adaptor-related protein complex 3, delta 1 subunit	19p13.3	8.12	0.627	-0.674	-4.26	2.91E-04	0.0946
169	214055_x_at	23215	PRRC2C	proline-rich coiled-coil 2C	1q23.3	8.85	0.553	-0.855	-4.25	2.99E-04	0.0961
170	242728_at	NA	NA	NA	NA	4.54	1.474	0.560	4.25	2.99E-04	0.0961
171	224605_at	401152	C4orf3	chromosome 4 open reading frame 3	4q26	8.04	0.611	-0.711	-4.25	3.00E-04	0.0961
172	201606_s_at	11137	PWP1	PWP1 homolog (S. cerevisiae)	12q23.3	6.49	0.486	-1.040	-4.24	3.02E-04	0.0961
173	221043_at	NA	NA	NA	NA	3.57	0.621	-0.687	-4.23	3.12E-04	0.0987
174	213328_at	4750	NEK1	NIMA (never in mitosis gene a)-related kinase 1	4q33	6.24	0.463	-1.111	-4.23	3.16E-04	0.0987
175	227221_at	64393	ZMAT3	zinc finger, matrin-type 3	3q26.32	6.74	1.315	0.395	4.22	3.17E-04	0.0987
176	239946_at	NA	NA	NA	NA	4.77	1.382	0.467	4.22	3.18E-04	0.0987

Supplemental Table 4.2. Among genes with lower expression in Alzheimer's, several biological processes were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	synaptic transmission	353	4.92E-12	2.92E-09	223, 5368, 627, 6529, 7425, 9379
2	transmission of nerve impulse	411	6.52E-12	3.38E-09	223, 5368, 627, 6529, 7425, 9379
3	cell-cell signaling	700	5.95E-07	6.03E-05	223, 4826, 5368, 627, 6529, 7425, 9379, 9547, 9636
4	oxidative phosphorylation	94	3.10E-06	1.81E-04	155066, 4704
5	regulation of synaptic plasticity	53	8.57E-06	4.23E-04	627, 7425
6	regulation of transmission of nerve impulse	132	2.83E-05	1.10E-03	627, 6529, 7425
7	cellular respiration	94	2.90E-05	1.10E-03	4704
8	regulation of neurological system process	142	4.41E-05	1.57E-03	627, 6529, 7425
9	neurotransmitter transport	96	5.54E-05	1.93E-03	6529, 9379
10	regulation of neuronal synaptic plasticity	32	5.96E-05	2.06E-03	7425
11	regulation of synaptic transmission	121	7.69E-05	0.003	627, 6529, 7425
12	respiratory electron transport chain	59	8.08E-05	0.003	4704
13	regulation of neurotransmitter levels	82	1.13E-04	0.003	223, 9379
14	nucleotide-excision repair, DNA damage removal	21	1.16E-04	0.003	2073
15	ATP synthesis coupled electron transport	51	1.89E-04	0.005	4704
16	mitochondrial ATP synthesis coupled electron transport	51	1.89E-04	0.005	4704
17	generation of a signal involved in cell-cell signaling	178	1.92E-04	0.005	4826, 627, 7425, 9379
18	signal release	178	1.92E-04	0.005	4826, 627, 7425, 9379
19	oxygen transport	5	2.37E-04	0.006	3049
20	mitochondrial electron transport, NADH to ubiquinone	37	4.13E-04	0.01	4704
21	tRNA metabolic process	113	5.68E-04	0.013	26995, 54938, 80222
22	cerebellar cortex formation	9	8.19E-04	0.017	
23	synaptic vesicle transport	31	1.12E-03	0.023	
24	cellular amino acid metabolic process	227	1.17E-03	0.023	10157, 2954, 54938, 80222
25	ncRNA metabolic process	230	1.32E-03	0.026	26995, 5393, 54938, 6839, 80222
26	cerebellar cortex morphogenesis	12	1.50E-03	0.028	
27	synaptic vesicle exocytosis	14	1.52E-03	0.029	
28	nucleobase, nucleoside and nucleotide metabolic process	551	1.55E-03	0.029	155066, 254272, 3704, 482, 56342, 79077, 8382
29	aspartate family amino acid catabolic process	6	1.70E-03	0.03	10157
30	negative regulation of synaptic transmission, GABAergic	7	1.91E-03	0.033	6529
31	generation of precursor metabolites and energy	324	2.01E-03	0.033	155066, 4704, 7425
32	cellular amino acid catabolic process	68	2.32E-03	0.037	10157, 2954
33	neurotransmitter secretion	47	2.36E-03	0.038	9379
34	DNA dealkylation	6	2.40E-03	0.038	
35	DNA dealkylation involved in DNA repair	6	2.40E-03	0.038	
36	regulation of megakaryocyte differentiation	14	0.003	0.039	8364, 8366
37	fear response	18	0.003	0.039	627
38	L-amino acid import	8	0.003	0.041	6529
39	lysosome organization	24	0.003	0.043	53, 84067
40	nucleoside phosphate metabolic process	529	0.004	0.049	155066, 254272, 3704, 482, 56342, 79077, 8382
41	nucleotide metabolic process	529	0.004	0.049	155066, 254272, 3704, 482, 56342, 79077, 8382
42	ATP hydrolysis coupled proton transport	9	0.004	0.051	155066
43	energy coupled proton transport, against electrochemical gradient	9	0.004	0.051	155066
44	inner cell mass cell proliferation	5	0.004	0.053	27339
45	glutamate secretion	19	0.004	0.053	627
46	subpallium development	11	0.004	0.053	220, 585
47	ATP metabolic process	96	0.004	0.056	155066, 482
48	transcription-coupled nucleotide-excision repair	5	0.004	0.057	2073

49	striatum development	9	0.004	0.058	220, 585
50	cellular amine metabolic process	306	0.005	0.061	10157, 223, 2954, 54938, 80222
51	electron transport chain	109	0.005	0.061	4704
52	ATP synthesis coupled proton transport	38	0.005	0.061	155066
53	energy coupled proton transport, down electrochemical gradient	38	0.005	0.061	155066
54	ATP biosynthetic process	86	0.005	0.061	155066, 482
55	establishment of melanosome localization	10	0.005	0.064	585
56	RNA processing	557	0.005	0.064	23070, 26995, 27339, 5393, 56342, 6100, 6839, 85437, 9360
57	amine metabolic process	403	0.005	0.065	10157, 223, 2954, 54938, 80222
58	aerobic respiration	34	0.005	0.065	
59	amine catabolic process	78	0.006	0.071	10157, 2954
60	second-messenger-mediated signaling	261	0.006	0.072	56342, 8364, 8366
61	RNA methylation	11	0.006	0.072	23070
62	oxidation reduction	614	0.006	0.073	10157, 220, 223, 242, 4704, 728294, 7923
63	nucleobase, nucleoside and nucleotide biosynthetic process	290	0.007	0.077	155066, 482, 56342, 8382
64	nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	290	0.007	0.077	155066, 482, 56342, 8382
65	cellular amino acid and derivative metabolic process	355	0.007	0.079	10157, 223, 2954, 54938, 80222
66	regulation of adenylate cyclase activity	96	0.007	0.079	56342
67	establishment of pigment granule localization	11	0.008	0.083	585
68	amino acid activation	45	0.008	0.083	54938, 80222
69	tRNA aminoacylation	45	0.008	0.083	54938, 80222
70	tRNA aminoacylation for protein translation	45	0.008	0.083	54938, 80222
71	dicarboxylic acid metabolic process	42	0.008	0.084	
72	flagellum assembly	7	0.008	0.084	585
73	flagellum organization	7	0.008	0.084	585
74	small molecule catabolic process	419	0.008	0.084	10157, 254272, 2954, 79077
75	purine nucleotide biosynthetic process	246	0.008	0.084	155066, 482, 56342, 8382
76	cognition	115	0.008	0.084	585, 627, 6529
77	nucleoside triphosphate metabolic process	244	0.008	0.085	155066, 254272, 482, 79077, 8382
78	GPI anchor biosynthetic process	33	0.009	0.087	79087, 80235
79	purine nucleotide metabolic process	422	0.009	0.088	155066, 254272, 482, 56342, 8382
80	RNA modification	49	0.009	0.088	23070, 26995
81	melanosome localization	14	0.009	0.088	585
82	regulation of cAMP biosynthetic process	111	0.009	0.089	56342
83	vesicle docking involved in exocytosis	25	0.009	0.091	
84	regulation of cytokinesis	9	0.009	0.091	585
85	cerebellar Purkinje cell differentiation	6	0.009	0.091	
86	cerebellar Purkinje cell layer formation	6	0.009	0.091	
87	cerebellar Purkinje cell layer morphogenesis	6	0.009	0.091	
88	GPI anchor metabolic process	34	0.01	0.093	79087, 80235
89	regulation of lyase activity	99	0.01	0.095	56342
90	cAMP biosynthetic process	113	0.01	0.096	56342
91	regulation of cyclase activity	98	0.01	0.099	56342
92	cerebellar cortex development	14	0.01	0.099	

Supplemental Table 4.3. Among genes with higher expression in Alzheimer's, several biological processes were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	translational elongation	101	4.07E-20	1.69E-16	11224, 1937, 2197, 25873, 6135, 6154, 6173, 6176, 6188, 6193, 6207, 6222, 6232
2	defense response	661	1.16E-16	2.42E-13	10219, 10410, 1050, 10581, 11326, 12, 23643, 241, 2532, 2919, 313, 3440, 3487, 3588, 3600, 3823, 4057, 4615, 4688, 51191, 58191, 6039, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7305, 8519, 929, 9332, 9450
3	immune response	660	3.86E-15	5.34E-12	10346, 10410, 10581, 11326, 23643, 2669, 2919, 3108, 3109, 3122, 3588, 3600, 4057, 4615, 4688, 4860, 51191, 54209, 563, 58191, 604, 6398, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719, 8519, 929, 9450
4	response to wounding	598	1.07E-14	1.11E-11	10219, 1050, 11326, 12, 1462, 2162, 23643, 241, 2697, 2919, 313, 3399, 3440, 3487, 3587, 3588, 3600, 4615, 4814, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7423, 929, 9332, 9450
5	innate immune response	201	8.28E-13	6.68E-10	10410, 10581, 11326, 23643, 4615, 4688, 51191, 58191, 7097, 7098, 710, 7100, 712, 713, 714, 718, 8519, 929, 9450
6	inflammatory response	365	9.65E-13	6.68E-10	10219, 1050, 11326, 12, 23643, 241, 2919, 313, 3440, 3487, 3588, 3600, 4615, 604, 60675, 6283, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 929, 9332, 9450
7	response to biotic stimulus	442	8.34E-12	3.85E-09	10049, 10346, 10410, 10581, 11080, 1373, 1937, 23643, 3315, 3440, 3587, 3600, 3665, 3669, 4057, 51191, 6283, 7079, 7097, 7098, 7100, 7132, 8519, 871, 929
8	response to other organism	354	5.69E-11	2.36E-08	10346, 10410, 10581, 1373, 1937, 23643, 3315, 3440, 3587, 3600, 3665, 3669, 4057, 51191, 6283, 7079, 7097, 7098, 7100, 7132, 8519, 929
9	activation of immune response	110	7.45E-11	2.81E-08	11326, 1997, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719
10	regulation of immune response	245	8.89E-10	3.08E-07	11326, 1997, 3600, 604, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719
11	response to bacterium	213	1.42E-09	4.53E-07	1373, 23643, 3587, 4057, 6283, 7079, 7097, 7098, 7100, 7132, 929
12	I-kappaB kinase/NF-kappaB cascade	171	2.35E-09	6.98E-07	23643, 2697, 3965, 4615, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132
13	regulation of toll-like receptor signaling pathway	9	2.97E-09	8.21E-07	7097, 7098, 7100
14	positive regulation of toll-like receptor signaling pathway	7	7.11E-09	1.85E-06	7097, 7098, 7100
15	humoral immune response	80	8.56E-09	2.09E-06	11326, 4057, 54209, 710, 712, 713, 714, 718, 9450
16	positive regulation of immune response	155	9.07E-09	2.09E-06	11326, 1997, 3600, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719
17	positive regulation of intracellular protein kinase cascade	222	1.03E-08	2.24E-06	2697, 3059, 3965, 4615, 6275, 6283, 6398, 7098, 7105, 7132, 7423
18	regulation of response to stimulus	533	1.94E-08	4.02E-06	10488, 11326, 1997, 23411, 285, 2874, 3600, 4615, 604, 6188, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7423
19	positive regulation of immune system process	268	2.77E-08	5.36E-06	11326, 1997, 3600, 4860, 604, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719, 7423
20	defense response to bacterium	96	2.84E-08	5.36E-06	4057, 6283, 7097, 7098, 7100, 7132
21	positive regulation of I-kappaB kinase/NF-kappaB cascade	115	4.25E-08	7.67E-06	2697, 3965, 4615, 6275, 6283, 6398, 7098, 7105, 7132
22	humoral immune response mediated by circulating immunoglobulin	37	4.47E-08	7.73E-06	710, 712, 713, 714, 718
23	positive regulation of response to stimulus	276	5.08E-08	8.43E-06	10488, 11326, 1997, 3600, 7097, 7098, 710, 7100, 712, 713, 7132, 714, 718, 719, 7423
24	complement activation	37	7.95E-08	1.24E-05	11326, 710, 712, 713, 714, 718
25	toll-like receptor signaling pathway	22	8.06E-08	1.24E-05	7097, 7098, 7100
26	regulation of I-kappaB kinase/NF-kappaB cascade	127	8.46E-08	1.25E-05	2697, 3965, 4615, 6275, 6283, 6398, 7098, 7105, 7132
27	regulation of cytokine production	199	9.39E-08	1.34E-05	11326, 1997, 4615, 604, 7097, 7098, 7100, 718, 719, 929
28	innate immune response-activating signal transduction	25	1.02E-07	1.41E-05	7097, 7098, 7100
29	pattern recognition receptor signaling pathway	24	1.11E-07	1.49E-05	7097, 7098, 7100
30	activation of innate immune response	26	1.24E-07	1.61E-05	7097, 7098, 7100
31	cytokine production	226	2.25E-07	2.83E-05	11326, 1997, 4615, 4860, 604, 7097, 7098, 7100, 718, 719, 929
32	positive regulation of JNK cascade	23	2.34E-07	2.86E-05	7098
33	positive regulation of signal transduction	275	3.05E-07	3.58E-05	2697, 3059, 3965, 4615, 4734, 6275, 6283, 6398, 7098, 7105, 7132, 7423
34	positive regulation of tumor necrosis factor production	14	3.11E-07	3.58E-05	7097, 7098, 929
35	positive regulation of signaling process	280	3.59E-07	3.97E-05	2697, 3059, 3965, 4615, 4734, 6275, 6283, 6398, 7098, 7105, 7132, 7423
36	interleukin-6 production	44	3.63E-07	3.97E-05	4615, 7097, 7098
37	complement activation, classical pathway	27	4.92E-07	5.24E-05	710, 712, 713, 714, 718
38	positive regulation of cytokine production	104	5.08E-07	5.28E-05	4615, 7097, 7098, 7100, 718, 719, 929
39	positive regulation of interleukin-6 production	23	7.62E-07	7.54E-05	4615, 7097, 7098
40	regulation of interleukin-12 production	23	9.39E-07	9.07E-05	7097, 7098
41	positive regulation of stress-activated protein kinase	29	1.08E-06	1.02E-04	7098

	signaling cascade				
42	multi-organism process	772	1.17E-06	1.08E-04	10346, 10410, 10488, 1050, 10581, 1373, 1937, 1981, 200186, 23411, 23643, 23764, 285, 3315, 3440, 358, 3587, 3600, 3665, 3669, 4057, 4734, 51191, 5422, 5696, 6283, 6993, 7079, 7097, 7098, 7100, 7132, 8519, 929
43	interleukin-12 production	24	1.31E-06	1.15E-04	7097, 7098
44	positive regulation of signaling pathway	376	1.35E-06	1.15E-04	1950, 2697, 3059, 3965, 4615, 4734, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132, 7423
45	positive regulation of cell communication	408	1.64E-06	1.15E-04	1950, 2697, 3059, 3965, 4615, 4734, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132, 7423
46	response to molecule of bacterial origin	133	1.78E-06	1.15E-04	1373, 23643, 3587, 7079, 7097, 7100, 7132, 929
47	regulation of response to stress	321	1.87E-06	1.15E-04	23411, 2874, 3600, 4615, 604, 6188, 7097, 7098, 710, 7100, 7132, 718, 7423
48	modulation by organism of defense response of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
49	modulation by organism of immune response of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
50	modulation by organism of innate immunity in other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
51	modulation by symbiont of host defense response	6	1.90E-06	1.15E-04	7097, 7098, 7100
52	modulation by symbiont of host immune response	6	1.90E-06	1.15E-04	7097, 7098, 7100
53	modulation by symbiont of host innate immunity	6	1.90E-06	1.15E-04	7097, 7098, 7100
54	pathogen-associated molecular pattern dependent induction by organism of innate immunity of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
55	pathogen-associated molecular pattern dependent induction by symbiont of host innate immunity	6	1.90E-06	1.15E-04	7097, 7098, 7100
56	pathogen-associated molecular pattern dependent modulation by organism of innate immunity in other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
57	pathogen-associated molecular pattern dependent modulation by symbiont of host innate immunity	6	1.90E-06	1.15E-04	7097, 7098, 7100
58	positive regulation by organism of defense response of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
59	positive regulation by organism of immune response of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
60	positive regulation by organism of innate immunity in other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
61	positive regulation by symbiont of host defense response	6	1.90E-06	1.15E-04	7097, 7098, 7100
62	positive regulation by symbiont of host immune response	6	1.90E-06	1.15E-04	7097, 7098, 7100
63	positive regulation by symbiont of host innate immunity	6	1.90E-06	1.15E-04	7097, 7098, 7100
64	response to host immune response	6	1.90E-06	1.15E-04	7097, 7098, 7100
65	response to immune response of other organism involved in symbiotic interaction	6	1.90E-06	1.15E-04	7097, 7098, 7100
66	immune effector process	210	1.91E-06	1.15E-04	11326, 3600, 604, 7097, 7098, 710, 712, 713, 714, 718
67	immunoglobulin mediated immune response	70	2.99E-06	1.77E-04	604, 710, 712, 713, 714, 718
68	B cell mediated immunity	71	3.90E-06	2.25E-04	604, 710, 712, 713, 714, 718
69	activation of plasma proteins involved in acute inflammatory response	40	4.94E-06	2.81E-04	11326, 710, 712, 713, 714, 718
70	regulation of interleukin-6 production	42	5.19E-06	2.91E-04	4615, 7097, 7098
71	positive regulation of interleukin-8 production	10	5.38E-06	2.98E-04	7097, 7098, 7100
72	regulation of transcription regulator activity	146	5.78E-06	3.16E-04	23411, 3399, 6188, 7097, 7098
73	regulation of immune system process	424	5.94E-06	3.20E-04	11326, 1997, 3600, 4860, 604, 7097, 7098, 710, 7100, 712, 713, 714, 718, 719, 7423, 8399
74	macrophage activation involved in immune response	10	6.04E-06	3.22E-04	7097, 7098, 7100
75	regulation of transcription factor activity	145	6.66E-06	3.46E-04	23411, 3399, 6188, 7097, 7098
76	antigen processing and presentation of peptide or polysaccharide antigen via MHC class II	13	6.67E-06	3.46E-04	3108, 3109, 3122
77	modification by symbiont of host morphology or physiology	9	6.88E-06	3.53E-04	7097, 7098, 7100
78	modification of morphology or physiology of other organism involved in symbiotic interaction	21	7.22E-06	3.65E-04	358, 7097, 7098, 7100

79	regulation of innate immune response	61	7.69E-06	3.85E-04	7097, 7098, 710, 7100
80	regulation of vascular endothelial growth factor receptor signaling pathway	16	1.09E-05	5.30E-04	4734, 7423
81	positive regulation of angiogenesis	43	1.15E-05	5.54E-04	285, 358, 7132, 718, 719, 7423
82	symbiosis, encompassing mutualism through parasitism	64	1.17E-05	5.57E-04	358, 4734, 6993, 7097, 7098, 7100
83	positive regulation of type I interferon production	15	1.18E-05	5.58E-04	7097, 7098
84	microglial cell activation involved in immune response	7	1.25E-05	5.81E-04	7097, 7098, 7100
85	positive regulation of interleukin-12 production	12	1.26E-05	5.81E-04	7097, 7098
86	regulation of defense response	156	1.29E-05	5.90E-04	3600, 4615, 604, 7097, 7098, 710, 7100, 7132, 718
87	cellular response to UV	6	1.31E-05	5.92E-04	358, 4734
88	adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains	114	1.47E-05	6.58E-04	604, 710, 712, 713, 714, 718
89	positive regulation of multicellular organismal process	279	1.53E-05	6.76E-04	1373, 358, 4615, 60675, 7097, 7098, 7100, 718, 719, 929, 9388
90	cytokine production involved in immune response	22	1.60E-05	6.82E-04	604, 7097
91	response to defenses of other organism involved in symbiotic interaction	8	1.62E-05	6.82E-04	7097, 7098, 7100
92	response to host	8	1.62E-05	6.82E-04	7097, 7098, 7100
93	response to host defenses	8	1.62E-05	6.82E-04	7097, 7098, 7100
94	adaptive immune response	116	1.63E-05	6.82E-04	604, 710, 712, 713, 714, 718
95	antigen processing and presentation	50	1.69E-05	7.04E-04	3108, 3109, 3122, 563
96	translation	394	2.07E-05	8.51E-04	11224, 1937, 1978, 1981, 2197, 25873, 283, 3315, 6135, 6154, 6173, 6176, 6188, 6193, 6207, 6222, 6232, 92399
97	vascular endothelial growth factor receptor signaling pathway	22	2.21E-05	8.99E-04	4734, 7423
98	regulation of intracellular protein kinase cascade	328	2.33E-05	9.38E-04	2697, 2874, 3059, 3965, 4615, 6275, 6283, 6398, 7098, 7105, 7132, 7423
99	regulation of type I interferon production	19	2.57E-05	1.03E-03	7097, 7098
100	immune response-activating signal transduction	72	2.62E-05	1.04E-03	1997, 7097, 7098, 7100, 719
101	acute inflammatory response	100	2.87E-05	1.10E-03	1050, 11326, 12, 710, 712, 713, 714, 718, 9332
102	cellular response to light stimulus	7	2.87E-05	1.10E-03	358, 4734
103	immune response-regulating signaling pathway	80	3.19E-05	1.20E-03	1997, 7097, 7098, 7100, 719
104	detection of biotic stimulus	20	3.38E-05	1.27E-03	23643, 7097, 7098
105	type I interferon production	21	3.84E-05	1.42E-03	7097, 7098
106	regulation of cytokine production involved in immune response	19	3.96E-05	1.46E-03	604, 7097
107	interferon-beta production	17	4.07E-05	1.47E-03	7097, 7098
108	regulation of interferon-beta production	17	4.07E-05	1.47E-03	7097, 7098
109	positive regulation of interferon-beta production	13	4.10E-05	1.47E-03	7097, 7098
110	I-kappaB phosphorylation	11	5.28E-05	1.86E-03	7097, 7098
111	positive regulation of MAPKKK cascade	87	8.00E-05	0.003	7098, 7423
112	anti-apoptosis	228	8.38E-05	0.003	1410, 23411, 25816, 26574, 3315, 4615, 57099, 60675, 7423, 81542, 8870, 9531
113	regulation of humoral immune response	13	8.59E-05	0.003	710, 718
114	negative regulation of cytokine production	42	8.86E-05	0.003	11326, 604, 7097
115	intracellular protein kinase cascade	583	8.90E-05	0.003	1410, 1950, 23643, 2697, 2874, 3059, 3965, 4615, 60675, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132, 7423, 9459
116	signal transmission via phosphorylation event	583	8.90E-05	0.003	1410, 1950, 23643, 2697, 2874, 3059, 3965, 4615, 60675, 6275, 6283, 6398, 7097, 7098, 7100, 7105, 7132, 7423, 9459
117	positive regulation of NF-kappaB import into nucleus	15	9.03E-05	0.003	7097, 7098
118	regulation of immune effector process	103	9.09E-05	0.003	3600, 604, 7097, 710, 718
119	negative regulation of transcription factor activity	61	9.53E-05	0.003	23411, 3399, 6188
120	negative regulation of transcription regulator activity	61	9.53E-05	0.003	23411, 3399, 6188
121	regulation of angiogenesis	87	9.73E-05	0.003	285, 358, 7132, 718, 719, 7423
122	interferon-beta biosynthetic process	7	1.01E-04	0.003	7098
123	positive regulation of interferon-beta biosynthetic process	7	1.01E-04	0.003	7098
124	regulation of interferon-beta biosynthetic process	7	1.01E-04	0.003	7098
125	regulation of lipid transport	40	1.05E-04	0.003	706, 8399, 9388
126	lymphocyte mediated immunity	108	1.06E-04	0.003	604, 710, 712, 713, 714, 718
127	cellular response to radiation	9	1.07E-04	0.003	358, 4734

128	type I interferon biosynthetic process	10	1.12E-04	0.003	7098
129	tumor necrosis factor superfamily cytokine production	36	1.19E-04	0.003	7097, 7098, 929
130	regulation of DNA binding	165	1.19E-04	0.003	23411, 3399, 6188, 7097, 7098
131	blood vessel morphogenesis	276	1.30E-04	0.004	1950, 2697, 283, 285, 358, 60675, 7132, 718, 719, 7423
132	positive regulation of protein transport	83	1.43E-04	0.004	1950, 283, 7097, 7098
133	positive regulation of innate immune response	50	1.48E-04	0.004	7097, 7098, 7100
134	negative regulation of immune effector process	18	1.57E-04	0.004	604, 710
135	regulation of production of molecular mediator of immune response	41	2.06E-04	0.006	604, 7097
136	response to lipopolysaccharide	122	2.14E-04	0.006	1373, 23643, 3587, 7079, 7097, 7132, 929
137	leukocyte mediated immunity	133	2.22E-04	0.006	604, 710, 712, 713, 714, 718
138	myeloid leukocyte cytokine production	9	2.23E-04	0.006	604, 7097
139	translational initiation	60	2.34E-04	0.006	1978, 1981, 3315, 6188, 6193
140	negative regulation of DNA binding	69	2.44E-04	0.006	23411, 3399, 6188
141	angiogenesis	234	2.45E-04	0.006	1950, 283, 285, 358, 60675, 7132, 718, 719, 7423
142	regulation of tumor necrosis factor production	33	2.57E-04	0.007	7097, 7098, 929
143	tumor necrosis factor production	33	2.57E-04	0.007	7097, 7098, 929
144	response to mercury ion	9	2.60E-04	0.007	358, 5224
145	positive regulation of intracellular transport	46	2.64E-04	0.007	1950, 4734, 7097, 7098
146	prostaglandin metabolic process	25	2.73E-04	0.007	27306, 6916, 7132
147	prostanoid metabolic process	25	2.73E-04	0.007	27306, 6916, 7132
148	response to exogenous dsRNA	11	2.82E-04	0.007	7098
149	wound healing	215	3.28E-04	0.008	2162, 2697, 3587, 3588, 4814, 710, 7423
150	response to virus	153	3.49E-04	0.009	10346, 10410, 10581, 1937, 3315, 3440, 3600, 3665, 3669, 51191, 7098, 8519
151	defense response to Gram-positive bacterium	29	3.51E-04	0.009	7097
152	regulation of binding	209	3.55E-04	0.009	23411, 3399, 6188, 7097, 7098
153	notochord development	7	3.92E-04	0.009	3399
154	response to organic substance	886	4.14E-04	0.01	10049, 10076, 1050, 11080, 1373, 1410, 1978, 2052, 23643, 2697, 283, 285, 3059, 3315, 3399, 358, 3587, 4615, 58191, 6402, 7079, 7097, 7098, 7100, 713, 7132, 7913, 871, 900, 929
155	positive regulation of phosphorylation	135	4.28E-04	0.01	1950, 283, 3059, 7423
156	regulation of gene-specific transcription	220	4.29E-04	0.01	1050, 23411, 2874, 7097, 7098
157	detection of molecule of bacterial origin	8	4.72E-04	0.011	23643, 7097
158	regulation of stress-activated protein kinase signaling cascade	90	4.74E-04	0.011	2874, 7098
159	NF-kappaB import into nucleus	26	4.81E-04	0.011	7097, 7098
160	regulation of NF-kappaB import into nucleus	26	4.81E-04	0.011	7097, 7098
161	positive regulation of defense response	85	5.03E-04	0.012	7097, 7098, 7100, 7132, 718
162	response to protein stimulus	115	5.26E-04	0.012	10049, 11080, 285, 3315, 3399, 7913, 871
163	negative regulation of binding	84	5.48E-04	0.012	23411, 3399, 6188
164	positive regulation of transmembrane transport	26	5.90E-04	0.013	7097, 7098
165	regulation of localization	726	6.27E-04	0.014	10076, 10488, 11037, 1410, 1950, 23411, 2697, 283, 285, 358, 4734, 58191, 5867, 604, 706, 7097, 7098, 718, 719, 7423, 8399, 84876, 91624, 9388
166	prostaglandin biosynthetic process	16	6.29E-04	0.014	27306, 6916
167	prostanoid biosynthetic process	16	6.29E-04	0.014	27306, 6916
168	protein maturation	116	6.30E-04	0.014	11326, 710, 712, 713, 714, 718, 871, 9049
169	chemokine production	26	6.32E-04	0.014	7097, 7098
170	regulation of cell fate commitment	9	6.61E-04	0.014	22943
171	regulation of cell fate specification	9	6.61E-04	0.014	22943
172	regulation of cellular response to stress	138	6.76E-04	0.015	23411, 2874, 6188, 7098
173	interferon-alpha production	9	7.27E-04	0.015	7098
174	regulation of interferon-alpha production	9	7.27E-04	0.015	7098
175	positive regulation of chemokine production	16	7.36E-04	0.016	7097, 7098
176	myeloid cell activation involved in immune response	27	7.80E-04	0.016	7097, 7098, 7100
177	cellular response to molecule of bacterial origin	28	8.45E-04	0.018	7097, 929
178	stress-activated protein kinase signaling cascade	122	9.49E-04	0.02	1410, 2874, 7098, 9459

179	blood vessel development	324	9.91E-04	0.02	1950, 2697, 283, 285, 358, 60675, 7132, 718, 719, 7423
180	regeneration	86	9.98E-04	0.021	10076, 1050, 1462, 2697, 285, 4814
181	positive regulation of transcription factor import into nucleus	20	1.05E-03	0.021	7097, 7098
182	regulation of JNK cascade	81	1.06E-03	0.022	2874, 7098
183	negative regulation of smooth muscle cell proliferation	13	1.08E-03	0.022	283, 3600
184	positive regulation of transport	276	1.12E-03	0.023	10488, 1950, 283, 358, 4734, 7097, 7098, 718, 8399, 84876, 9388
185	regulation of transport	514	1.17E-03	0.023	10488, 11037, 1410, 1950, 23411, 2697, 283, 358, 4734, 5867, 706, 7097, 7098, 718, 8399, 84876, 9388
186	response to pH	13	1.17E-03	0.023	2697, 84329
187	gene-specific transcription from RNA polymerase II promoter	161	1.21E-03	0.024	1050, 23411, 2874, 7097, 7098
188	regulation of gene-specific transcription from RNA polymerase II promoter	161	1.21E-03	0.024	1050, 23411, 2874, 7097, 7098
189	positive regulation of cytokine biosynthetic process	53	1.35E-03	0.026	7097, 7098
190	icosanoid metabolic process	51	1.35E-03	0.026	241, 27306, 6916, 7132, 8399
191	positive regulation of phosphate metabolic process	138	1.44E-03	0.028	1950, 283, 3059, 7423
192	positive regulation of phosphorus metabolic process	138	1.44E-03	0.028	1950, 283, 3059, 7423
193	negative regulation of immune system process	90	1.45E-03	0.028	11326, 1997, 604, 710
194	regulation of interferon-gamma production	34	1.51E-03	0.028	7098
195	microglial cell activation	10	1.54E-03	0.029	7097, 7098, 7100
196	positive regulation of chemokine biosynthetic process	8	1.59E-03	0.029	7098
197	positive regulation of protein import into nucleus	25	1.64E-03	0.03	7097, 7098
198	response to unfolded protein	65	1.68E-03	0.03	10049, 11080, 3315, 871
199	chemokine biosynthetic process	12	1.70E-03	0.03	7098
200	chemokine metabolic process	12	1.70E-03	0.03	7098
201	regulation of B cell apoptosis	8	1.70E-03	0.03	604
202	negative regulation of cell death	402	1.71E-03	0.03	1410, 23411, 25816, 26574, 3315, 358, 4615, 57099, 604, 60675, 7423, 81542, 8870, 900, 9531
203	cellular response to xenobiotic stimulus	27	1.71E-03	0.03	6283, 9049
204	xenobiotic metabolic process	27	1.71E-03	0.03	6283, 9049
205	interferon-gamma production	35	1.75E-03	0.031	7098
206	response to xenobiotic stimulus	31	1.80E-03	0.032	6283, 9049
207	cellular defense response	51	1.80E-03	0.032	10219, 23643, 3823, 4688, 7305
208	response to fungus	22	1.82E-03	0.032	6283, 7097
209	negative regulation of apoptosis	391	1.83E-03	0.032	1410, 23411, 25816, 26574, 3315, 358, 4615, 57099, 604, 60675, 7423, 81542, 8870, 900, 9531
210	S phase of mitotic cell cycle	25	1.85E-03	0.032	5422, 5424, 604, 8099, 84967
211	regulation of mononuclear cell proliferation	93	1.85E-03	0.032	11326, 3600, 4860, 604
212	regulation of lymphocyte proliferation	92	1.88E-03	0.032	11326, 3600, 4860, 604
213	positive regulation of nucleocytoplasmic transport	32	1.92E-03	0.033	4734, 7097, 7098
214	negative regulation of programmed cell death	396	1.93E-03	0.033	1410, 23411, 25816, 26574, 3315, 358, 4615, 57099, 604, 60675, 7423, 81542, 8870, 900, 9531
215	regulation of leukocyte proliferation	94	1.95E-03	0.033	11326, 3600, 4860, 604
216	negative regulation of lipid transport	15	1.96E-03	0.033	
217	negative regulation of multi-organism process	18	1.96E-03	0.033	7097
218	cell migration	455	1.97E-03	0.033	10076, 10488, 128954, 1462, 2191, 2697, 283, 285, 4478, 58191, 7097, 719, 7423, 91624
219	regulation of digestive system process	14	1.99E-03	0.033	358
220	regulation of G2/M transition of mitotic cell cycle	9	2.00E-03	0.033	900
221	cellular response to abiotic stimulus	13	2.02E-03	0.033	358, 4734
222	JNK cascade	109	2.03E-03	0.033	2874, 7098, 9459
223	macrophage activation	27	2.05E-03	0.034	7097, 7098, 7100, 8399
224	response to peptidoglycan	11	2.09E-03	0.034	7097
225	unsaturated fatty acid metabolic process	54	2.16E-03	0.035	241, 27306, 6916, 7132, 8399
226	detection of external stimulus	71	2.17E-03	0.035	56925, 7097, 7098
227	lymphocyte proliferation	122	2.40E-03	0.038	11326, 3600, 4860, 604
228	mononuclear cell proliferation	124	2.42E-03	0.038	11326, 3600, 4860, 604
229	regulation of cholesterol transport	23	2.43E-03	0.038	706, 9388
230	regulation of sterol transport	23	2.43E-03	0.038	706, 9388

231	negative regulation of response to stimulus	122	2.47E-03	0.038	23411, 285, 2874, 604, 6188, 710
232	vasculature development	334	2.47E-03	0.038	1950, 2697, 283, 285, 358, 60675, 7132, 718, 719, 7423
233	interaction with host	39	0.003	0.039	4734, 6993, 7097, 7098, 7100
234	cytokine biosynthetic process	89	0.003	0.04	7097, 7098
235	cytokine metabolic process	90	0.003	0.04	7097, 7098
236	response to glucocorticoid stimulus	97	0.003	0.04	10076, 1050, 1373, 358, 713
237	cell activation involved in immune response	55	0.003	0.041	604, 7097, 7098, 7100
238	leukocyte activation involved in immune response	55	0.003	0.041	604, 7097, 7098, 7100
239	locomotion	599	0.003	0.041	10076, 10488, 128954, 1462, 2191, 2697, 283, 285, 2919, 4478, 4734, 5355, 58191, 6036, 60675, 7097, 719, 7423, 91624
240	positive regulation of production of molecular mediator of immune response	15	0.003	0.041	7097
241	regulation of transcription in response to stress	6	0.003	0.041	4734
242	regulation of transcription from RNA polymerase II promoter	745	0.003	0.043	100125288, 1050, 1997, 23411, 26574, 2874, 29128, 3399, 3660, 3665, 4734, 57658, 5932, 604, 7097, 7098, 7132, 7913, 79366
243	positive regulation of lipid transport	20	0.003	0.043	8399, 9388
244	regulation of protein secretion	63	0.003	0.043	1950, 283
245	response to external stimulus	645	0.003	0.043	10488, 1050, 1373, 1509, 23411, 285, 2919, 358, 4615, 5355, 56925, 58191, 6036, 604, 60675, 7097, 7098, 710, 7132, 718, 719, 7423, 9388
246	leukocyte proliferation	126	0.003	0.043	11326, 3600, 4860, 604
247	leukocyte activation	336	0.003	0.043	11326, 3600, 4860, 604, 7097, 7098, 7100, 81542, 8399
248	ribosomal large subunit biogenesis	11	0.003	0.043	6135, 6154
249	cellular response to lipoteichoic acid	6	0.003	0.043	7097, 929
250	response to lipoteichoic acid	6	0.003	0.043	7097, 929
251	positive regulation of intracellular protein transport	43	0.003	0.047	1950, 7097, 7098
252	positive regulation of ERK1 and ERK2 cascade	34	0.003	0.047	7423
253	lymphocyte chemotaxis	9	0.003	0.047	58191
254	protein maturation by peptide bond cleavage	81	0.003	0.047	11326, 710, 712, 713, 714, 718
255	response to corticosteroid stimulus	104	0.003	0.047	10076, 1050, 1373, 358, 713
256	response to dsRNA	27	0.003	0.048	7098
257	peptidyl-tyrosine modification	112	0.003	0.049	1950, 3059, 7423
258	RNA catabolic process	74	0.004	0.049	1763, 3669, 6036, 6039, 87178
259	tetrahydrobiopterin biosynthetic process	6	0.004	0.049	6697
260	cellular response to inorganic substance	11	0.004	0.05	241, 358
261	cellular response to metal ion	11	0.004	0.05	241, 358
262	cellular response to stress	677	0.004	0.05	1410, 1462, 1509, 1763, 23411, 26574, 27244, 2874, 29128, 358, 3978, 4438, 4734, 5422, 5424, 5932, 604, 6188, 7098, 79677, 83932, 900, 9459
263	positive regulation of transcription regulator activity	88	0.004	0.05	7097, 7098
264	positive regulation of NF-kappaB transcription factor activity	58	0.004	0.051	7097, 7098
265	negative regulation of lymphocyte apoptosis	9	0.004	0.051	604
266	response to heat	62	0.004	0.052	11080, 1410, 3315, 929
267	regulation of epidermis development	21	0.004	0.053	23764
268	regulation of protein transport	145	0.004	0.053	1950, 23411, 283, 7097, 7098
269	positive regulation of cellular component movement	138	0.004	0.053	10488, 58191, 604, 7097, 719, 7423
270	positive regulation of transcription factor activity	87	0.004	0.055	7097, 7098
271	establishment of mitotic spindle orientation	7	0.004	0.057	6993
272	establishment of spindle orientation	7	0.004	0.057	6993
273	cell activation	381	0.005	0.059	11326, 3600, 4860, 604, 7097, 7098, 7100, 81542, 8399
274	response to abiotic stimulus	393	0.005	0.06	11080, 1410, 2697, 2766, 285, 3315, 358, 4734, 5424, 56925, 7098, 84329, 929
275	receptor-mediated endocytosis	73	0.005	0.061	10268, 4734, 58191
276	regulation of inflammatory response	87	0.005	0.061	4615, 604, 7097, 7098, 710, 7132, 718
277	pancreatic juice secretion	8	0.005	0.061	358
278	mitotic cell cycle	480	0.005	0.061	10459, 1950, 1978, 246184, 26574, 5422, 5424, 54443, 54930, 55145, 5696, 604, 6993, 8099, 81930, 84967, 900, 90293
279	cell cycle phase	509	0.005	0.061	10459, 1950, 1978, 246184, 4438, 5422, 5424, 54443, 54930, 604, 6993, 8099, 81930, 84967, 900, 90293
280	regulation of cytokine biosynthetic process	79	0.005	0.062	7097, 7098

281	protein processing	106	0.005	0.064	11326, 710, 712, 713, 714, 718
282	regulation of gastrulation	9	0.005	0.067	
283	positive regulation of cytokine production involved in immune response	11	0.005	0.067	7097
284	regulation of lymphocyte apoptosis	15	0.006	0.067	604
285	regulation of interleukin-8 production	21	0.006	0.067	7097, 7098, 7100
286	regulation of response to external stimulus	187	0.006	0.069	10488, 285, 4615, 604, 7097, 7098, 710, 7132, 718, 719, 7423
287	interferon-gamma biosynthetic process	14	0.006	0.069	7098
288	peptidyl-tyrosine phosphorylation	110	0.006	0.069	1950, 3059, 7423
289	complement activation, alternative pathway	14	0.006	0.069	11326, 718
290	regulation of cell activation	189	0.006	0.07	11326, 3600, 4860, 604, 8399
291	positive regulation of interferon-gamma biosynthetic process	11	0.006	0.07	7098
292	positive regulation of protein secretion	45	0.006	0.071	283
293	regulation of multi-organism process	52	0.006	0.072	3600, 7097
294	production of molecular mediator of immune response	64	0.006	0.072	604, 7097
295	interphase of mitotic cell cycle	137	0.006	0.072	1978, 5422, 5424, 604, 8099, 84967, 900
296	rRNA transcription	18	0.006	0.072	283
297	positive regulation of humoral immune response	8	0.006	0.072	718
298	transcription from RNA polymerase II promoter	900	0.006	0.073	100125288, 1050, 1997, 23411, 23764, 26574, 2874, 29128, 29777, 3399, 3660, 3665, 4734, 57658, 5932, 604, 7097, 7098, 7132, 7913, 79366
299	interleukin-8 production	23	0.007	0.075	7097, 7098, 7100
300	cell cycle process	662	0.007	0.076	10459, 1950, 1978, 246184, 27244, 4438, 5422, 5424, 54443, 54930, 5696, 604, 6993, 8099, 81930, 84967, 900, 90293
301	regulation of establishment of protein localization	153	0.007	0.076	1950, 23411, 283, 7097, 7098
302	mesenchyme development	81	0.007	0.077	1592, 6275
303	regulation of leukocyte migration	40	0.007	0.077	7097, 719, 7423
304	myeloid leukocyte activation	71	0.007	0.077	7097, 7098, 7100, 8399
305	superoxide anion generation	14	0.007	0.079	26574, 4688
306	regulation of chemokine production	24	0.007	0.081	7097, 7098
307	regulation of leukocyte mediated immunity	59	0.007	0.082	604, 718
308	tetrahydrobiopterin metabolic process	7	0.007	0.083	6697
309	acute-phase response	44	0.008	0.083	1050, 12, 9332
310	regulation of chemokine biosynthetic process	11	0.008	0.083	7098
311	regulation of leukocyte activation	177	0.008	0.083	11326, 3600, 4860, 604, 8399
312	regulation of B cell mediated immunity	24	0.008	0.083	604, 718
313	regulation of immunoglobulin mediated immune response	24	0.008	0.083	604, 718
314	protein secretion	94	0.008	0.084	1950, 283, 4860
315	positive regulation of cell migration	129	0.008	0.084	10488, 58191, 7097, 719, 7423
316	defense response to virus	41	0.008	0.084	3600, 7098
317	tissue regeneration	30	0.008	0.084	2697, 4814
318	regulation of body fluid levels	200	0.008	0.084	187, 2162, 358, 3587, 3588, 710
319	positive regulation of leukocyte migration	31	0.008	0.084	7097, 719, 7423
320	positive regulation of inflammatory response	43	0.008	0.084	7097, 7098, 7132, 718
321	interspecies interaction between organisms	335	0.008	0.085	10346, 10488, 1050, 1981, 200186, 23411, 358, 3665, 4734, 5422, 5696, 6993, 7097, 7098, 7100, 7132
322	cell motility	484	0.008	0.085	10076, 10488, 128954, 1462, 2191, 2697, 283, 285, 4478, 58191, 7097, 719, 7423, 91624
323	localization of cell	484	0.008	0.085	10076, 10488, 128954, 1462, 2191, 2697, 283, 285, 4478, 58191, 7097, 719, 7423, 91624
324	cellular response to lipopolysaccharide	25	0.008	0.085	929
325	regulation of nucleocytoplasmic transport	84	0.008	0.085	1950, 23411, 4734, 7097, 7098
326	positive regulation of mononuclear cell proliferation	65	0.008	0.086	3600, 4860, 604
327	icosanoid biosynthetic process	36	0.008	0.086	241, 27306, 6916
328	T cell proliferation	86	0.008	0.086	11326, 3600, 4860
329	regulation of protein import into nucleus	67	0.008	0.086	1950, 23411, 7097, 7098
330	positive regulation of lymphocyte proliferation	64	0.008	0.086	3600, 4860, 604
331	reproductive process	791	0.009	0.087	100125288, 10149, 10488, 23411, 23764, 283, 285, 4438, 4734, 51314, 5224, 604, 60675, 6993, 7079, 7913
332	regulation of adaptive immune response based on	51	0.009	0.088	604, 718

	somatic recombination of immune receptors built from immunoglobulin superfamily domains				
333	positive regulation of leukocyte proliferation	66	0.009	0.088	3600, 4860, 604
334	reproduction	793	0.009	0.089	100125288, 10149, 10488, 23411, 23764, 283, 285, 4438, 4734, 51314, 5224, 604, 60675, 6993, 7079, 7913
335	interphase	146	0.009	0.091	1978, 5422, 5424, 604, 8099, 84967, 900
336	establishment of tissue polarity	12	0.009	0.091	57216
337	positive regulation of immune effector process	46	0.009	0.091	7097, 718
338	negative regulation of RNA metabolic process	412	0.009	0.091	100125288, 10049, 1050, 23411, 2874, 3399, 3660, 3665, 4734, 604, 6207, 84232
339	transepithelial transport	10	0.01	0.093	358
340	cellular response to hypoxia	6	0.01	0.093	358
341	cellular response to oxygen levels	6	0.01	0.093	358
342	renal water transport	5	0.01	0.093	358
343	regulation of phospholipase A2 activity	6	0.01	0.094	283
344	regulation of cell proliferation	834	0.01	0.095	10076, 10488, 1050, 11326, 138151, 1950, 2014, 23411, 2697, 27244, 283, 2919, 3059, 358, 3600, 4860, 563, 604, 6282, 648, 7423, 8519
345	phospholipid catabolic process	21	0.01	0.095	9388
346	response to oxygen levels	155	0.01	0.097	1410, 283, 285, 358, 51167, 54541, 7097, 9124
347	positive regulation of gene-specific transcription	145	0.01	0.097	1050, 7097, 7098
348	B cell apoptosis	11	0.01	0.098	604

Supplemental Table 4.4. Among genes with lower expression in Alzheimer's, several molecular functions were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	neuropeptide hormone activity	21	5.46E-07	1.89E-04	2922, 5368, 7425
2	cation transmembrane transporter activity	525	1.09E-05	0.001	155066, 482, 6529
3	inorganic cation transmembrane transporter activity	203	5.44E-05	0.005	155066, 482, 6529
4	porin activity	6	6.50E-05	0.005	10452
5	calmodulin-dependent protein kinase activity	20	1.14E-04	0.008	
6	ion transmembrane transporter activity	683	1.34E-04	0.009	10452, 155066, 482, 6529
7	MAP kinase tyrosine/serine/threonine phosphatase activity	12	1.43E-04	0.009	1846, 1848
8	MAP kinase phosphatase activity	13	1.74E-04	0.009	1846, 1848
9	monovalent inorganic cation transmembrane transporter activity	160	2.38E-04	0.012	155066, 482, 6529
10	nucleoside-triphosphate diphosphatase activity	6	4.97E-04	0.021	3704, 79077
11	hydrogen ion transmembrane transporter activity	83	0.001	0.023	155066
12	NADH dehydrogenase (quinone) activity	38	0.001	0.044	4704
13	NADH dehydrogenase (ubiquinone) activity	38	0.001	0.044	4704
14	NADH dehydrogenase activity	38	0.001	0.044	4704
15	transmembrane transporter activity	859	0.002	0.055	10452, 11182, 155066, 21, 22, 482, 6529
16	cis-trans isomerase activity	36	0.002	0.068	2954, 9360
17	substrate-specific transporter activity	916	0.004	0.099	10452, 11182, 155066, 3049, 482, 6529
18	SNARE binding	29	0.004	0.113	
19	mannosyltransferase activity	15	0.005	0.12	10585, 79087, 80235
20	phosphotyrosine binding	10	0.005	0.12	53
21	oxygen transporter activity	8	0.005	0.123	3049
22	ATPase activity	330	0.005	0.123	155066, 21, 22, 482, 57680
23	RNA polymerase II carboxy-terminal domain kinase activity	13	0.006	0.127	
24	sodium:potassium-exchanging ATPase activity	10	0.007	0.138	482
25	calcium channel regulator activity	13	0.007	0.138	9379
26	ATPase activity, coupled	266	0.007	0.138	155066, 21, 22, 482, 57680
27	channel regulator activity	53	0.007	0.14	57730, 9379
28	gated channel activity	296	0.008	0.141	10452
29	ATPase activity, coupled to transmembrane movement of ions	67	0.008	0.141	155066, 482
30	ATPase activity, coupled to transmembrane movement of substances	100	0.009	0.146	155066, 21, 22, 482
31	oxidoreductase activity, acting on the aldehyde or oxo group of donors	32	0.009	0.146	220, 223
32	aldehyde dehydrogenase (NAD) activity	9	0.009	0.147	220, 223
33	voltage-gated channel activity	185	0.011	0.157	10452
34	voltage-gated ion channel activity	185	0.011	0.157	10452
35	O-methyltransferase activity	12	0.011	0.157	23070
36	oxidoreductase activity	648	0.011	0.157	10157, 220, 223, 242, 2954, 4704, 728294, 7923
37	hydrolase activity, acting on acid anhydrides	742	0.012	0.157	155066, 21, 22, 3704, 4636, 482, 57680, 585, 79077, 79132
38	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	738	0.012	0.157	155066, 21, 22, 3704, 4636, 482, 57680, 585, 79077, 79132
39	calmodulin binding	140	0.012	0.157	
40	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	102	0.012	0.159	155066, 21, 22, 482
41	GABA receptor activity	22	0.013	0.159	
42	P-P-bond-hydrolysis-driven transmembrane transporter activity	109	0.013	0.159	155066, 21, 22, 482
43	primary active transmembrane transporter activity	109	0.013	0.159	155066, 21, 22, 482
44	pyrophosphatase activity	735	0.013	0.16	155066, 21, 22, 3704, 4636, 482, 57680, 585, 79077, 79132
45	ATPase activity, coupled to movement of substances	101	0.013	0.161	155066, 21, 22, 482
46	RNA methyltransferase activity	27	0.014	0.164	23070
47	active transmembrane transporter activity	324	0.014	0.165	155066, 21, 22, 482, 6529
48	aminoacyl-tRNA ligase activity	46	0.016	0.171	54938, 80222
49	ligase activity, forming aminoacyl-tRNA and related compounds	46	0.016	0.171	54938, 80222
50	ligase activity, forming carbon-oxygen bonds	46	0.016	0.171	54938, 80222
51	ion channel activity	366	0.017	0.178	10452
52	S-adenosylmethionine-dependent methyltransferase activity	90	0.018	0.182	23070, 6839
53	nucleoside-triphosphatase activity	706	0.019	0.192	155066, 21, 22, 4636, 482, 57680, 585, 79132
54	potassium-transporting ATPase activity	12	0.02	0.193	482

55	glutamate receptor activity	29	0.021	0.198	
56	syntaxin-1 binding	8	0.022	0.202	
57	cation:amino acid symporter activity	11	0.023	0.21	6529
58	2 iron, 2 sulfur cluster binding	17	0.024	0.214	
59	cation channel activity	263	0.024	0.214	
60	substrate-specific channel activity	376	0.025	0.224	10452
61	ARF GTPase activator activity	26	0.026	0.224	
62	syntaxin binding	23	0.031	0.258	
63	aldehyde dehydrogenase [NAD(P)+] activity	5	0.032	0.262	220
64	calcium channel activity	78	0.032	0.263	
65	antibiotic transporter activity	7	0.033	0.265	
66	metal ion transmembrane transporter activity	125	0.034	0.272	482, 6529
67	passive transmembrane transporter activity	393	0.036	0.273	10452
68	peptidyl-prolyl cis-trans isomerase activity	34	0.036	0.273	9360
69	oxidoreductase activity, acting on the aldehyde or oxo group of donors, disulfide as acceptor	8	0.036	0.273	
70	oxidoreductase activity, acting on NADH or NADPH, quinone or similar compound as acceptor	44	0.036	0.273	4704
71	channel activity	392	0.039	0.285	10452
72	oxidoreductase activity, acting on sulfur group of donors, oxygen as acceptor	6	0.04	0.287	
73	hormone activity	96	0.04	0.287	2922, 5368, 7425
74	tetracycline transporter activity	6	0.041	0.292	
75	E-box binding	7	0.041	0.292	7291
76	sodium ion transmembrane transporter activity	75	0.042	0.295	482, 6529
77	oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor	22	0.043	0.296	220, 223
78	beta-tubulin binding	19	0.043	0.299	2010, 585
79	fructose binding	6	0.048	0.324	

Supplemental Table 4.5. Among genes with higher expression in Alzheimer's, several molecular functions were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	structural constituent of ribosome	149	1.33E-10	0	11224, 2197, 25873, 6135, 6154, 6173, 6176, 6188, 6193, 6207, 6222, 6232
2	pancreatic ribonuclease activity	10	7.58E-08	0	283, 6036, 6039
3	glycosaminoglycan binding	148	2.16E-06	0.0005	1462, 26577, 283, 4057, 6402, 7097, 7423, 79625, 929, 9388
4	endoribonuclease activity, producing 3'-phosphomonoesters	13	2.64E-06	0.0005	283, 6036, 6039
5	RAGE receptor binding	7	4.03E-06	0.0007	6275, 6283
6	pattern binding	163	9.22E-06	0.0014	1462, 26577, 283, 4057, 6402, 7097, 7423, 79625, 929, 9388
7	carbohydrate binding	339	1.31E-05	0.0015	10219, 1462, 26577, 283, 3823, 3965, 4057, 6402, 7097, 7423, 79625, 929, 9388, 9936
8	peptidoglycan binding	9	4.14E-05	0.0043	7097, 929
9	endonuclease activity, active with either ribo- or deoxyribonucleic acids and producing 3'-phosphomonoesters	18	5.51E-05	0.0048	283, 6036, 6039
10	coreceptor activity	19	0.0001	0.0089	10268, 23643
11	cytokine binding	113	0.0002	0.0093	1436, 2532, 3587, 3588, 5355, 7132
12	heparin binding	110	0.0002	0.0117	26577, 283, 4057, 6402, 7423, 79625, 9388
13	transcription regulator activity	923	0.0003	0.0138	100125288, 10049, 10346, 10488, 1050, 10848, 138151, 165, 1997, 23411, 27005, 2874, 29128, 29777, 3399, 3660, 3665, 55145, 57658, 604, 7764, 7913, 79366, 84232, 9049, 9124
14	lipopolysaccharide binding	13	0.0004	0.0167	23643, 7097, 929
15	rRNA binding	24	0.0004	0.0168	283, 6135, 6222
16	cytokine receptor binding	179	0.0006	0.024	10488, 2919, 3440, 3600, 4615, 58191, 7100, 7423
17	ribonuclease activity	58	0.0022	0.0683	283, 3669, 563, 6036, 6039, 87178
18	vascular endothelial growth factor receptor binding	6	0.0022	0.0685	7423

19	structural molecule activity	570	0.0024	0.0707	1101, 11224, 1410, 2197, 24146, 25873, 4478, 4604, 6135, 6154, 6173, 6176, 6188, 6193, 6207, 6222, 6232, 81493, 81578, 84617, 9499
20	cyclic nucleotide-dependent protein kinase activity	9	0.0032	0.0915	5613
21	water transmembrane transporter activity	12	0.0033	0.0915	358
22	phosphate binding	13	0.0039	0.1026	10797, 4860
23	microtubule plus-end binding	10	0.0046	0.1153	81930
24	cytokine receptor activity	56	0.0051	0.1203	3587, 3588
25	metalloexopeptidase activity	37	0.0059	0.1283	10269, 10404, 165
26	death receptor activity	13	0.0066	0.1377	7132
27	monocarboxylic acid binding	57	0.007	0.1377	1592, 241, 563, 6646, 91452
28	glycosphingolipid binding	6	0.0072	0.1379	6402
29	monosaccharide binding	42	0.0076	0.1402	3965
30	proline-rich region binding	9	0.008	0.1408	4734
31	sphingolipid binding	8	0.0085	0.1464	6402
32	metallocarboxypeptidase activity	24	0.0088	0.1464	10404, 165
33	I-SMAD binding	10	0.009	0.1464	
34	actin binding	315	0.0094	0.1474	170954, 283, 4478, 4542, 4604, 54443, 81930, 822, 91624, 9499
35	transcription repressor activity	336	0.0104	0.1569	100125288, 10049, 10346, 10848, 138151, 165, 1997, 23411, 2874, 3399, 604, 7764
36	ubiquitin binding	35	0.0106	0.1569	3315, 4734, 81930
37	anion binding	16	0.0109	0.1569	10797, 4860
38	IgG binding	6	0.0114	0.1569	
39	voltage-gated chloride channel activity	15	0.0115	0.1569	1192, 1193
40	low-density lipoprotein receptor activity	11	0.012	0.1584	58191
41	protein complex binding	238	0.0123	0.1592	1050, 1373, 2857, 3059, 3384, 7045, 7132
42	cAMP-dependent protein kinase activity	7	0.0133	0.1602	5613
43	carboxypeptidase activity	36	0.0144	0.1662	10404, 165, 642
44	GDP binding	26	0.0152	0.171	2669, 5867
45	small conjugating protein binding	36	0.0153	0.171	3315, 4734, 81930
46	interleukin-1 receptor binding	11	0.0159	0.171	7100
47	metalloendopeptidase inhibitor activity	10	0.0165	0.171	56925, 7079
48	metalloenzyme inhibitor activity	10	0.0165	0.171	56925, 7079
49	metalloenzyme regulator activity	10	0.0165	0.171	56925, 7079
50	intramolecular oxidoreductase activity	40	0.0165	0.171	10130, 27306, 6916
51	sugar binding	173	0.0185	0.187	10219, 1462, 3823, 3965, 6402, 9936
52	endoribonuclease activity	39	0.0187	0.1871	283, 6036, 6039
53	exopeptidase activity	78	0.0205	0.1975	10269, 10404, 165, 2191, 642
54	ATPase regulator activity	7	0.0205	0.1975	10049
55	water channel activity	11	0.0208	0.1978	358
56	cyclic nucleotide-gated ion channel activity	6	0.0238	0.2144	358
57	intracellular cyclic nucleotide activated cation channel activity	6	0.0238	0.2144	358
58	scavenger receptor activity	43	0.0255	0.2238	58191, 9332
59	RNA polymerase II transcription factor activity	236	0.0261	0.2238	1050, 29128, 29777, 3660, 3665, 7913
60	regulatory region DNA binding	148	0.0263	0.2238	1050, 57658, 7764
61	transcription regulatory region DNA binding	148	0.0263	0.2238	1050, 57658, 7764
62	interferon-alpha/beta receptor binding	8	0.0306	0.2581	3440
63	collagen binding	38	0.0314	0.2613	871
64	peptidase inhibitor activity	141	0.032	0.2619	12, 25816, 56925, 6590, 7079, 710, 718, 871
65	endopeptidase inhibitor activity	133	0.0341	0.272	12, 25816, 56925, 6590, 7079, 710, 718, 871
66	tumor necrosis factor receptor activity	11	0.0355	0.2733	7132
67	alcohol transmembrane transporter activity	7	0.0365	0.2747	358
68	polyol transmembrane transporter activity	7	0.0365	0.2747	358
69	transcription cofactor activity	358	0.0367	0.2747	10346, 10488, 10848, 165, 23411, 27005, 2874, 29777, 3399, 57658, 9049, 9124
70	RNA polymerase binding	6	0.0387	0.2849	4734
71	phospholipase inhibitor activity	12	0.0389	0.2849	
72	growth factor binding	111	0.0394	0.2862	3487, 3587, 3588, 4052
73	sequence-specific DNA binding	582	0.0423	0.295	100125288, 10488, 1050, 1997, 23764, 55145, 57658, 604, 7764
74	titin binding	9	0.0464	0.3166	4604
75	tumor necrosis factor binding	12	0.0466	0.3166	7132
76	cytokine activity	188	0.0487	0.3268	2919, 3440, 3600, 58191, 6398
77	lipoprotein receptor activity	15	0.0499	0.3321	58191

Supplemental Table 4.6. Among genes with lower expression in Alzheimer's, the binding motifs of several transcription factors were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	NRSF_01	418	0	0.022	10307, 23130, 26505, 401190, 57574, 627
2	NMYC_01	330	0.001	0.049	1846, 7291, 7425
3	CREB_01	283	0.001	0.049	148304, 1846, 9360
4	CREBP1CJUN_01	287	0.002	0.095	124044, 23070, 7425, 8364, 8366
5	MEIS1BHOXA9_02	231	0.004	0.13	1408, 627, 79680, 9360
6	AREB6_03	248	0.004	0.13	124044, 155066, 83694
7	CREB_02	137	0.005	0.13	140597, 63917, 8364, 8366
8	CREBP1_Q2	357	0.005	0.13	54984, 81576, 8364, 8366, 9360
9	NFY_01	22	0.01	0.225	
10	AREB6_01	161	0.011	0.225	23130, 5368, 9379
11	ROAZ_01	326	0.024	0.384	124808, 4330, 91851
12	RFX1_02	420	0.028	0.384	124044, 145407, 1848, 29070
13	TAXCREB_02	267	0.03	0.384	115548, 79132
14	ATF_01	394	0.03	0.384	8364, 8366, 9360
15	SREBP1_01	338	0.031	0.384	140597, 26995, 57795, 627
16	MEIS1_01	101	0.042	0.484	10307
17	LYF1_01	99	0.045	0.495	23130, 242

Supplemental Table 4.7. Among genes with higher expression in Alzheimer's, the binding motifs of several transcription factors were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	NFKAPPAB_01	214	0.0020	0.0951	11170, 1462, 24146, 27005, 2919, 3384, 56658, 56833, 57216, 57658, 58191, 81493, 84876
2	NKX22_01	219	0.0108	0.2252	285, 29128, 4734, 5422, 57633, 6646, 85450
3	OCT_C	267	0.0146	0.2704	10488, 1997, 25816, 4734, 55843, 56131, 58526, 6036, 712, 9531
4	PBX1_01	54	0.0300	0.3837	5166, 8334, 91947
5	HOXA3_01	183	0.0318	0.3837	2197, 4038, 648, 84173, 85450
6	CREL_01	274	0.0490	0.5000	1462, 23764, 2919, 55273, 56833, 57216, 57658, 58191, 84876

Supplemental Table 4.8. Among genes with higher expression in Alzheimer's, the binding sites of several microRNAs were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	mir-506	893	0.023	0.831	10269, 1050, 10848, 1462, 23593, 26511, 29128, 5355, 55227, 57658, 58526, 604, 64089, 754, 79710
2	mir-124	890	0.035	0.831	10269, 1050, 10848, 1462, 23593, 26511, 29128, 5355, 55227, 57658, 58526, 604, 64089, 754, 79710
3	mir-433	163	0.045	0.831	256435, 284370, 91947

Supplemental Table 4.9. Among genes with lower expression in Alzheimer's, the binding sites of several microRNAs were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	mir-129-5p	292	0.004	0.592	114034, 124808, 23251, 9379
2	mir-185	118	0.014	0.831	81606
3	mir-328	73	0.028	0.831	4330, 8364, 8366

Supplemental Table 4.10. Among genes with higher expression in Alzheimer's, several cytogenic bands were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	7p13-p12	7	8.12E-05	0.023	10268, 5224
2	17p13	30	1.85E-04	0.036	2874, 58191, 6154
3	4q25	26	2.08E-04	0.036	1950
4	10p12.31	5	3.68E-04	0.046	219681
5	1q25	21	4.45E-04	0.048	4688, 6646
6	1q23.2	7	1.09E-03	0.086	54935, 56833
7	1p36.3-p36.2	10	1.97E-03	0.122	
8	1p21.3	7	2.41E-03	0.129	51375
9	17q11.2-q12	9	2.50E-03	0.129	26574
10	5q31-q33	5	3.31E-03	0.143	
11	2p11.2	33	3.67E-03	0.152	822, 84173
12	22q11.1	5	0.005	0.19	128954
13	9q22	13	0.005	0.193	4814
14	Xp21.1	13	0.005	0.193	8406
15	1q21	78	0.006	0.193	6232, 6275, 6282, 6283
16	1q23	16	0.008	0.227	
17	8q11	6	0.009	0.251	
18	12q	12	0.012	0.301	
19	9q33.2	15	0.012	0.301	92399
20	3p26.2	6	0.014	0.329	57633
21	1p31-p22	5	0.014	0.334	22802
22	7p14	7	0.024	0.464	358
23	6q24	8	0.025	0.478	23593
24	14q21	7	0.026	0.48	57161
25	5q14.3	8	0.028	0.49	1462
26	12q24.11	24	0.033	0.541	338773, 84329
27	3q24	12	0.034	0.546	
28	2p16.3	7	0.038	0.604	11037
29	1p31.1	28	0.043	0.629	11080, 256435, 91624
30	6p22.1	42	0.043	0.629	29777
31	19q13.3	68	0.045	0.636	2014, 2828, 29998, 5424
32	15q22.1	6	0.049	0.661	79811

Supplemental Table 4.11. Among genes with lower expression in Alzheimer's, several cytogenic bands were enriched.

Rank	Name	#Genes	P-Value	FDR	SigGenes
1	16p13.3	169	1.81E-06	0.002	10573, 124093, 1877, 21, 3049
2	10p11.2	6	7.64E-06	0.003	7587
3	8p23	6	3.45E-04	0.046	54984
4	12p12.3	25	9.67E-04	0.086	8364, 8366
5	8p21.3	21	1.03E-03	0.086	10361
6	15q25.1	11	1.81E-03	0.122	23251
7	2q21.1	15	1.86E-03	0.122	653275
8	7q31.3	11	2.61E-03	0.129	10157
9	2p22.2	6	2.70E-03	0.129	10153
10	7p14.3	8	2.83E-03	0.129	6100
11	7p11.2	8	6.16E-03	0.203	
12	14q32.2	11	6.31E-03	0.203	57596
13	8p23.3	5	6.66E-03	0.206	9172
14	19q13.42	46	7.39E-03	0.221	
15	10q23.32	5	0.008	0.227	
16	9q34.11	42	0.011	0.286	26995
17	20q11.2-q12	6	0.016	0.354	4826
18	17p11.2	59	0.018	0.4	125170, 254272, 388341
19	2p23.1	6	0.018	0.4	81606
20	2q37.3	43	0.019	0.402	151176, 728294
21	2p25.3	10	0.019	0.402	
22	14q32	16	0.021	0.416	
23	5p15.33	14	0.024	0.471	65980
24	17q23	7	0.027	0.49	762
25	10q24.31	9	0.029	0.509	
26	1p36.33	31	0.031	0.53	9636
27	16q24.3	34	0.032	0.536	124044, 58189
28	9q34	44	0.039	0.611	11182
29	12q23.3	17	0.042	0.629	121053
30	6p21.33	24	0.044	0.629	8364, 8366
31	19p13.3	183	0.044	0.632	5657, 566, 84717
32	14q11.2-q12	8	0.047	0.647	
33	14q32.33	26	0.05	0.661	

CHAPTER V

Conclusions and Future Directions

OVERALL SUMMARY

The goals of this dissertation are to understand (1) the regulation of homocysteine (Hcy), a one-carbon metabolite, by the heavy metal lead (Pb) and (2) the role of DNA methylation and gene expression in late onset Alzheimer's disease (LOAD), a spontaneous neurodegenerative disease. First, in the Normative Aging Study cohort, it is demonstrated that plasma total Hcy is regulated by Pb exposure and dietary availability of vitamin B6, vitamin B12, and folate. LOAD is associated with changes in circulating Hcy, which is involved with methyl-group substrate availability for DNA methylation. Second, modest, widespread differences in DNA methylation were observed in the frontal cortex of LOAD subjects vs. controls. A follow-up study suggests that these DNA methylation marks may have functional gene expression implications. Gene expression and DNA methylation values at an individual gene were validated in additional samples. This dissertation provides the foundation for further work on the environmental influences on Hcy and epigenetics in LOAD.

SUMMARY OF HOMOCYSTEINE (HCY) AND LEAD (PB) EXPOSURE

Hcy is a thiol-containing intermediate in the one-carbon metabolism cycle. It is involved in folate metabolism and corresponding nucleotide synthesis, methionine metabolism and corresponding methylation of DNA, RNA, and proteins, and finally glutathione (antioxidant) synthesis (Selhub 1999). Elevations in homcysteine are associated with cardiovascular disease (CVD) (Wald et al. 2002) and neurodegeneration (Mattson and Shea 2003), including AD (Seshadri et al. 2002). This dissertation chapter shows that recent lead exposure (measured in blood) is cross-sectionally associated with Hcy levels (8.14% increase in Hcy with 3ug/dl IQR increase in blood Pb). This association was modified by vitamin B6, vitamin B12, and folate dietary factors as determined through stratified analysis. Cumulative exposure to lead (measured in tibia) was also associated with homocysteine, but this relationship disappeared after blood lead was included in the model, suggesting that blood lead mediates the association between bone lead and homocysteine.

Strengths and Weaknesses

The current study is strengthened by the use of repeated measures of Hcy and blood Pb as well as repeated measures in Hcy with baseline bone Pb. This allows us to look at longitudinal changes in Hcy with Pb exposure, not simply cross-sectional associations. This study is the first to examine the Pb-Hcy relationship while examining plausible dietary interactions, namely folate and vitamins B6 and B12.

Previous research has looked at the cross-sectional association between lead exposure and homocysteine (Chia et al. 2007; Schafer et al. 2005). Other studies have observed changes in homocysteine with diet over time (Clarke and Armitage 2000). One paper has combined lead exposure and diet in the study of homocysteine (Yakub and Iqbal 2010). This study is the first (that we are aware of) to include repeated measures of lead exposure, Hcy, and diet. It is

strengthened by multiple measures of lead exposure (tibia, patella, and blood) and of dietary status (plasma levels and food frequency questionnaire levels).

This study had limited power to detect interactions, due in part to missing nutrient data. In addition, results using food frequency questionnaires (FFQ) have been debated in the literature largely due to the poor correlation between FFQ and repeated dietary recall (Byers 2001; Kristal et al. 2005). In our study the consistent associations observed with concurrent measures of plasma nutrients validate the use of FFQ.

Scope and Implications of the Work

The results of this longitudinal study suggest a strong link between homocysteine and lead exposure. This dissertation did not address the downstream consequences of the association. Toxicology studies are needed to decipher the molecular mechanisms linking homocysteine and lead exposure. Potentially, Pb and Hcy may work through a common mechanism of binding and disrupting available sulfur-containing proteins (Krumdieck and Prince 2000; Needleman 2004), which may suggest synergistic or additive toxicity.

Based on the research in this dissertation, behavioral interventions to reduce blood Pb and improve dietary intake of vitamins B6 and B12 and folate will have a protective effect by lowering Hcy. Multiple randomized control trials have attempted to reduce circulating Hcy levels in people who are already ill by using dietary interventions (Mei et al. 2010). These studies have been successful in reducing circulating Hcy, but did not produce the hypothesized health benefits, specifically reductions in coronary heart disease, stroke, cardiovascular events, or all-cause mortality. Perhaps taking a public health approach with interventions prior to disease onset are needed to prevent incident cases.

SUMMARY OF DNA METHYLATION IN AD

The second part of the dissertation observed throughout the genome moderate changes in DNA methylation that are associated with LOAD in the frontal cortex (Bakulski et al. 2012). This study was the largest genome-wide DNA methylation investigation of the LOAD brain to date. Using 12 matched-pair LOAD case and control frontal cortex samples, we quantitatively determined DNA methylation at 27,578 CpG sites. Across 948 CpG sites that were statistically different between LOAD cases and controls after adjusting for age and sex, the mean methylation difference was 2.9%. A CpG site in the promoter for the gene Transmembrane Protein 59 (*TMEM59*) was 7.3% hypomethylated in LOAD cases. This gene is involved in post-translational modification of Amyloid Precursor Protein (*APP*), and thus β-amyloid plaque formation. We validated the DNA methylation findings using a second DNA methylation detection platform and 13 additional matched pairs. DNA methylation was associated with *TMEM59* mRNA gene expression, but not with the quantity of the full-length protein. This study suggests that DNA methylation may be involved in LOAD, but future research is needed to determine the extent.

Strengths and Weaknesses

A major strength of this research was the use of well pathologically characterized human tissue from a brain region involved in LOAD. The majority of epigenetic epidemiology studies test blood DNA methylation (Foley et al. 2009). The association between circulating lymphocytes and brain epigenetics is unknown (though our research group has a funded NIEHS p30 pilot grant to investigate this question). Access to post-mortem human samples via the Michigan Alzheimer's Disease Center provided valuable information on the *in vivo* inaccessible tissue.

On the other hand, a potential weakness of this research is the use of tissue samples made up of complex mixtures of cell types. Changes in composite DNA methylation may be due to changes in the percent cell-types

(Houseman et al. 2012). The data presented in this paper could be interpreted as actual epigenetic changes with disease, as indicators of different cellular mixtures, or as markers of past environmental exposures.

At the time, the Illumina Infinium HumanMethylation27K BeadArray was the most comprehensive and cost efficient method available. This technology queries the quantitative DNA methylation levels of 27,578 CpG sites throughout the genome with approximately two tag CpG sites per gene. Previous LOAD-DNA methylation research was based on gene-specific DNA methylation founded in *a priori* assumptions about the disease. The Illumina array was the most comprehensive DNA methylation array available and our study provided the most un-biased assessment of the DNA methylome of LOAD.

Genomic technology is rapidly evolving. In retrospect, with newer array and next-generation sequencing technologies available, the Illumina 27K BeadArray represents a fraction of the potential DNA methylation information that is available today. Illumina's latest 450K array has more than fifteen times the number CpG sites and these sites have a less biased genomic distribution. The 27K array CpG sites were 92.0% in promoters and 72.5% in CpG islands. Potentially important non-genic regulation sites were largely not included in the model used in the dissertation. Studies suggest that CpG locations in gene deserts may be functionally important in the brain (Maunakea et al. 2010) and unfortunately they were unable to be captured in our study.

Scope and Implications of the Work

This research suggests DNA methylation may be involved in LOAD, but it is far from definitive. The methylation changes and sample size were modest, and results need to be confirmed in additional studies before mechanistic conclusions may be drawn. Several research groups have ongoing projects to investigate Alzheimer's disease and epigenetics. The results of those studies combined with our research will provide a larger body of evidence on the topic. In addition, the effect sizes observed were moderate and future work will want to

consider separating complex cell mixtures to identify changes in neurons as a priority. Further, the findings at *TMEM59* were validated through gene expression, but the full-length protein levels did not differ+ between LOAD cases and controls. The functional significance of these DNA methylation markers across the genome is presented in the next section of the dissertation.

SUMMARY OF GENE EXPRESSION AND DNA METHYLATION IN AD

The final section of the dissertation builds on the second experimental section. Using the same LOAD and neuropathologically confirmed controls as above, we observed correlations between DNA methylation differences and gene expression differences in the frontal cortex when comparing. Integrating genome-wide analyses of DNA methylation and gene expression allowed us to detect additional potential sites at low false discovery rates with LOAD associated epigenetic change. This research suggests that DNA methylation differences may have functional consequences, potentially relevant in disease.

Strengths and Weaknesses

The samples and the technologies used overlap between this dissertation chapter and the previous, therefore the strengths and weaknesses of the samples and the Illumina technology still apply. In addition, combining datasets from two companies (Illumina and Affymetrix) with multiple probesets per gene proved to be methodologically challenging. We followed a previously published pipeline to integrate the data (Sartor et al. 2011). This method proved to be successful in identifying genes for follow-up.

Scope and Implications of the Work

This research extends previous dissertation study to continue building case for DNA methylation in LOAD. Integrating gene expression and DNA methylation analyses yielded greater numbers of CpG sites for potential follow-up and mechanistic investigation. This study is an example of how DNA methylation

research can be enhanced when done alongside gene expression research. As studies increase the sample number of LOAD and control brains tested, this method is recommended.

FUTURE DIRECTIONS

This dissertation provides opportunities for rich potential areas of further research. Late-onset Alzheimer's disease is an enormous public health problem and the burden will continue to increase (International 2010). LOAD is traditionally understudied in the laboratory relative to EOAD, due to the availability of EOAD analogous animal models. Despite the public health potential, within the LOAD literature, environmental risk factors and potential mechanisms of environmental toxicants are further underrepresented. Future research topics, directly related to this dissertation, include testing for a human epidemiologic link between Pb exposure and LOAD, examination of the roles of additional metals in neurodegeneration, cell-type specificity in the epigenetic molecular epidemiology, modifications to histones, and further work on dietary factors and methylation. This is a broad field of research in which could potentially contribute to our understanding and prevention of an insidious disease.

Exposure to lead has negative neurological consequences in early life (Fewtrell et al. 2004; Grosse et al. 2002). It is also associated with cognitive decline in late life (Shih et al. 2007; Weisskopf et al. 2004; Wright et al. 2003), potentially through increased hippocampal gliosis (Weisskopf et al. 2007). Though a large body of research demonstrates Pb is associated with accelerated declines in cognition, the hypothesized causal association between lead exposure and LOAD, implied in this dissertation, has not been rigorously tested. Potential epidemiologic studies to test this link are challenged by the current clinical diagnoses of AD. Clinical diagnoses of AD from 30 ADRC's were

compared to post-mortem neuropathological diagnoses, with 71-87% sensitivity and 44-71% specificity (Beach et al. 2012). The ranges of clinical diagnosis of disease misclassification would require a large study size for environmental epidemiology research.

There are recent advances in neuroimaging (Matsuda and Imabayashi 2012) and circulating biomarkers (Chintamaneni and Bhaskar 2012; Doecke et al. 2012) that improve AD diagnosis and are available for research purposes. For example, the UM ADC utilizes positron emission tomography (PET) imaging methods to visualize β-amyloid in the brain and diagnose cases more accurately (Burke et al. 2011). Brain scan confirmed AD cases and controls could be measured for lead exposure at the UM Retrospective Lead Exposure Laboratory using the Cd[109] K-shell X-ray Fluorescence instrument. Alternatively, post-mortem studies with neuropathologically confirmed LOAD and control samples may be able to reconstruct past metals exposure history using teeth. Researchers have identified a layer of molar tissue with relatively low turnover that dates from early development that can be quantified for metals using laser-ablation ICP-MS (Hare et al. 2011). With method validation, a case-control approach may be able to accurately reconstruct past exposure history. Finally the Pb-LOAD argument could be tested with brain post-mortem lead mapping. Demonstrating that Pb is present at the site of pathology would strengthen the argument. Post-mortem soft tissue metal content can be mapped in slices using rapid-scanning x-ray fluorescence methods (Popescu et al. 2009). Grants that propose to study the Pb-induced epigenetic changes in LOAD will need to first demonstrate the Pb-LOAD relationship in humans.

Several metals beyond Pb have been implicated in AD with varying degrees of evidence, including aluminum (Frisardi et al. 2010; Shcherbatykh and Carpenter 2007), iron (Mandel et al. 2007), copper (Brown 2009; Shcherbatykh and Carpenter 2007), zinc (Shcherbatykh and Carpenter 2007), and mercury (Gerhardsson et al. 2008). Many metals work through shared mechanisms in the

body. For example, lead, cadmium, mercury, and nickle bind protein sulfhydryl groups and deplete glutathione, resulting in oxidative stress (Stohs and Bagchi 1995). Future LOAD research could expand to include rigorous epidemiologic inquiry of metals in addition to Pb, using similar methods proposed above for Pb. Post-mortem exposure studies utilizing ICP-MS methods are capable of measuring up to twelve metals simultaneously. The half-life of each of these metals varies in tissues such as bone and skin, thus post-mortem exposure assessment for metals besides Pb may not reflect exposure prior to disease onset. KXRF *in vivo* technologies are being developed for additional metals including cadmium and arsenic (personal communication, Linda Huiling Nie, Purdue University). *In vivo* metals epidemiology studies coupled with early stage disease diagnosis techniques may be an effective strategy to test metals exposure as a risk factor for LOAD.

Reports have proposed pesticides (Baldi et al. 2003; Santibanez et al. 2007), solvents (Kukull et al. 1995), electromagnetic field (Sobel et al. 1996), and particulate matter in air pollution (Calderon-Garciduenas et al. 2004) may be risk factors for LOAD. The links between these exposures and LOAD have not been yet been validated. These types of environmental exposure studies have been underrepresented in the AD literature, likely due to the challenges of retrospective exposure assessment in older adults. Retrospective exposure assessment for pesticides and solvents relies heavily on questionnaire data in the absence of effective biomarkers. Future work may test the association between additional exposures and LOAD onset using long-running large cohorts where exposure information has been collected over time.

In addition to the influence of toxicants on one-carbon metabolism and epigenetics, future research may look at the direct and indirect effects of diet on the system. Results from the lead and homocysteine research paper demonstrate that dietary B6, B9, and B12 levels modify the association between blood Pb and homocysteine, an important methyl-donor for DNA methylation.

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We have shown that DNA methylation change is associated with LOAD (Bakulski et al. 2012). Future research could test whether AD-associated DNA methylation changes are prevented with B-vitamin supplemented diet. The study of nutrient toxicant interactions is an emerging field in environmental health with potential for public health intervention.

To understand more precise mechanisms involved in LOAD pathogenesis, molecular epidemiology of epigenetics will need to refine the tissues and cell types studied. AD epigenetic epidemiology compared blood from AD cases and controls as a tissue of epidemiologic convenience (Bollati et al. 2011; Maes et al. 2007). Blood DNA methylation has not yet been tested as a biomarker of brain epigenetics. Whole brain region epigenetics is closer to the disease site, but it still represents a mixture of cell types that each likely have characteristic epigenetic profiles. Recent studies suggest that changes in circulating lymphocyte epigenetics represent shifting cell type proportions (Houseman et al. 2012). The DNA methylation differences observed in this dissertation in the human brain with LOAD may represent the understood shifting cell type proportions with disease. In future research, cell types will need to be separated from the tissue matrix. This is logistically challenging in frozen archived tissues where the cell membrane is often no longer intact. Ongoing work in the laboratory (particularly by 2nd year Ph.D. student, Zishaan Farooqui) seeks to separate nuclei of neurons from non-neurons. This allows for the study of epigenetics in neurons, and associations with environmental exposures, but it does not allow for RNA or protein functional validation. Further advancements may make the study of specific cell types more feasible.

The focus of the current research has been DNA methylation. However, recent research suggests that histones may be an important target for epigenetic modifications in the brain. Histone modifications play a role in memory formation

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(Levenson et al. 2004) and HDAC inhibitor treatment increases memory formation (Vecsey et al. 2007). Indeed, HDAC inhibitors have been proposed for human AD clinical trials (Kazantsev and Thompson 2008) . In the male rat brain, *App* mRNA expression is repressed by thyroid hormone (T3) sensitive histone modifications (Belakavadi et al. 2011). T3 Treatment decreases H3K4 methylation and H3 acetylation at the *App* promoter. This silences *App,* reversed with histone deacetylase (HDAC) and histone lysine demethylase inhibitor treatment (Belakavadi et al. 2011). Histone modifications are also environmentally sensitive (Mathers et al. 2010). Together, these observations suggest that future environmental molecular epidemiology research on AD should target histone modification.

CONCLUDING REMARKS

In summary, this dissertation has provided important molecular epidemiology insights to chronic disease. It has demonstrated moderate DNA methylation differences in the LOAD brain vs. controls that may have functional gene expression consequences. In addition, homocysteine may be an important target for lead exposure toxicity and may link lead exposure to chronic disease including CVD and neurodegenerative disease. This work has implications for prevention and potential homocysteine intervention through lowering blood lead levels. This research has also spurred multiple additional research questions at the intersection of Alzheimer's disease, one-carbon metabolism, epigenetics, and lead exposure.

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