DISSERTATION

NOVEL TRANSCRIPTOMIC MECHANISMS OF BRAIN AGING

Submitted by

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ABSTRACT

NOVEL TRANSCRIPTOMIC MECHANISMS OF BRAIN AGING

As the world ages, the incidence of age-related diseases like dementia is expected to increase. Brain aging is characterized by declines in cognitive function that may develop into mild cognitive impairment, which increases the risk for dementia. In fact, age is the primary risk factor for late-onset Alzheimer's disease, which is the most common age-related dementia. The adverse cellular and molecular processes that underlie cognitive decline with aging in the brain are known collectively as the "hallmarks of brain aging." Advances in next-generation sequencing (e.g., transcriptomics/RNA-seq) have made it possible to investigate age- and disease-related changes in the brain at the broad gene expression level, and to identify potential therapeutic targets. With the support of my committee and mentoring team, I completed three studies using transcriptomics that characterize novel mechanisms that underlie brain aging. My findings include: (1) doxorubicin chemotherapy accelerates brain aging at the gene expression level, (2) apigenin nutraceutical supplementation targets age-related inflammation in the brain and rescues cognitive impairment in old mice, and (3) epigenetic dysregulation of transposable elements (remnants of viral infection in the genome) with aging contributes to age-related inflammation in Alzheimer's disease. Together, my work provides insight into transcripts and cellular/molecular pathways that are modifiable and may be therapeutic targets to delay or prevent consequences of brain aging.

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DEDICATION

For my dad, who cultivated my love of knowledge since before I could even read but didn't get to see me reach this milestone, and for all the amazing women in my life who have shown me what girls can do.

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CHAPTER 1: HALLMARKS AND MECHANISMS OF BRAIN AGING

1. INTRODUCTION

The world's population is aging, and the incidence of age-related diseases, including dementia, is expected to markedly increase[1,2]. Organismal aging is characterized by a collection of cellular/molecular processes known as the hallmarks of aging[3], and in general these occur in brain aging as well, with several important exceptions[4]. Brain aging is characterized by declines in cognitive function (primarily memory and learning, attention and processing speed, and executive function)[5]. In some people, these declines may develop into mild cognitive impairment (MCI), which increases the risk for dementia and neurodegenerative diseases like Alzheimer's disease (AD). These age- and disease-related cognitive declines are caused in part by the specific adverse biological events in the brain known as the "hallmarks of brain aging."

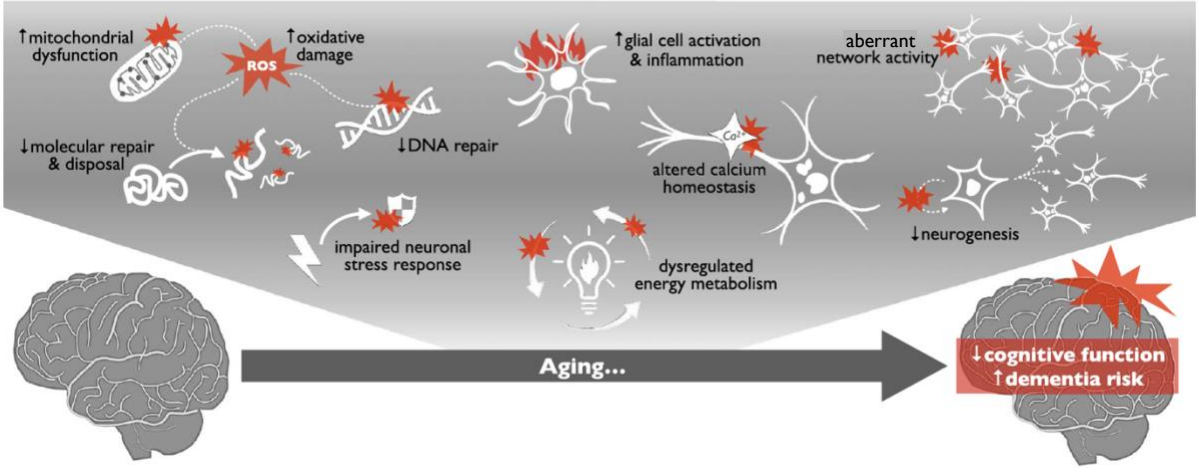

Figure 1.1. Aging is the primary risk factor for the development of cognitive dysfunction, mild cognitive impairment (MCI), and dementia. Numerous, established biological "hallmarks of brain aging" (shown above) contribute to age-related reductions in cognitive function and increased neurodegeneration and dementia risk with aging (from Wahl *et al*., 2021).

Although the hallmarks of brain aging mirror the systemic hallmarks of aging, they also include several brain-specific phenomena (illustrated in Fig. 1.1). Importantly, the hallmarks of brain aging are necessarily involved, and often more pronounced, in most neurodegenerative diseases, including AD. Thus, brain aging and neurodegeneration may be part of a continuum, and a combination of hallmarks/risk factors may determine whether dementia or disease develops[2]. This review will discuss the hallmarks of brain aging and use them as a lens through which to explore novel transcriptomic mechanisms of brain aging and potential therapeutics.

2. THE HALLMARKS OF BRAIN AGING

2.1 Dysregulated energy metabolism

Dysregulated energy metabolism is considered an underlying contributor to all hallmarks of brain aging. The brain accounts for ~20% of human resting energy expenditure, and neurons are highly sensitive to disrupted nutrient regulation[6]. During aging, fasting glucose levels increase as cells become less effective at importing glucose in response to insulin (i.e., insulin resistance). Elevated fasting glucose is linked with accelerated brain aging, poorer cognitive function, and dementia[7]. In fact, high blood glucose is a cellular stressor and can be especially cytotoxic in neurons[8-10]. Interestingly, animal models of chronic hyperglycemia show not only alterations in neuronal size/morphology, but also impaired cognitive function as measured by spatial memory[11,12]. Brain function is particularly sensitive to the adverse effects of insulin resistance as glucose is a key energy source for neurons, and this can be compounded by metabolic and cardiovascular changes with aging (e.g., dyslipidemia, and increased triglycerides and low-density lipoprotein cholesterol). In addition to its important peripheral role, insulin signaling in the brain is crucial for neuronal survival, synaptic function/plasticity, and cognition[13-15]. Furthermore, impaired peripheral and brain insulin signaling is linked with cognitive decline in AD animal models and patients[16-18].

2.2 Mitochondrial dysfunction and oxidative damage accumulation

Numerous studies have shown that mitochondrial dysfunction, especially in neurons, increases with brain aging[4]. Mitochondria are centrally important in the brain, where they play key roles in calcium homeostasis and neuronal metabolism. Dysfunctional mitochondria also are centrally implicated in neurodegenerative diseases, including Parkinson's disease and AD, in which they play a key role in the accumulation of cellular damage. Oxidative damage accumulates with aging because of oxidative stress, defined as an imbalance between antioxidant defenses (e.g., glutathione, superoxide dismutase) and reactive oxygen species (ROS) production[3]. Mitochondrial dysfunction with aging is a major source of ROS due to inefficiency of the electron transport chain, which leads to reduced adenosine triphosphate (ATP) generation and increased electron leakage that can produce ROS. ROS can damage biomolecules like proteins, lipids, and DNA – all of which can impair cellular function directly (because damaged cellular components do not function properly) or indirectly (because damaged biomolecules may interfere with function in other cellular compartments). ROS can also damage cellular structures, including mitochondria and even mitochondrial DNA (mtDNA). Neurons are especially sensitive to ROS because of their high, oxidizable lipid content and rate of oxidative metabolism[19], and these overall declines in mitochondrial function and efficiency are exacerbated by reductions in disposal of damaged/dysfunctional mitochondria (mitophagy) and mitochondrial biogenesis mediated by proliferator-activated receptor gamma coactivator alpha (PGC1-α)[20], especially in neurons.

Age-related mitochondrial dysfunction is also an important contributor to other hallmarks of brain aging such as inflammation. ROS-induced mitochondrial damage can lead to mtDNA in the cytosol that can stimulate inflammation by activating anti-viral signaling pathways like cGAS (cyclic GMP-AMP synthase)-STING[21]. Additionally, anomalies in mtDNA transcription can generate immunogenic products such as mitochondrial double-stranded RNA (dsRNA)

that is recognized by the mitochondrial membrane-bound dsRNA sensor, mitochondrial antiviral-signaling protein (MAVS) [22]. These data are consistent with the idea that mitochondrial dysfunction is a key player in brain aging and can further contribute to other hallmarks of brain aging like chronic inflammation.

2.3 Reduced molecular repair and disposal

A primary reason for mitochondrial dysfunction and the accumulation of oxidative damage during aging is reduced activity/efficacy of cellular repair and disposal systems. There is a wealth of literature showing systemic, age-related reductions in both autophagic-lysosomal degradation of proteins and organelles, and proteasomal activity to eliminate old/damaged proteins. These systems are critical in neurons, which are postmitotic and must maintain their functional capacity throughout the lifespan. The buildup of undegraded, damaged, and/or aggregated proteins due to reduced autophagy and proteasome activity is central to the pathology of many neurodegenerative diseases (e.g., amyloid beta plaques in AD, alpha synuclein deposits in Parkinson's disease) [2,23].

2.4 Dysregulated calcium homeostasis

Cellular control of important ions and minerals becomes dysregulated in most aged tissues, and impaired calcium (Ca^{2+}) homeostasis with aging is a particular problem in neurons. Ca^{2+} plays a role in numerous brain functions, including neurotransmission, neuronal excitability, synaptic plasticity, and long-term memory consolidation. The concentration of Ca^{2+} also modulates gene expression, as Ca^{2+} influx through N-methyl-D-aspartate (NMDA) receptors can activate transcription factors like cyclic AMP response element-binding protein (CREB) and PGC-1α. With aging, neuronal Ca^{2+} homeostasis becomes impaired due to increased Ca^{2+} influx and dysregulation of intracellular Ca^{2+} modulators like mitochondria. This leads to

altered gene expression and neuronal function that are linked with cognitive decline and can also contribute directly to neuronal death via Ca^{2+}-driven excitotoxicity[4].

2.5 Impaired adaptive cellular stress response

All cells activate stress resistance networks (e.g., antioxidant and anti-inflammatory pathways) in response to stressors. In the brain, these adaptive responses are important in settings of electrochemical, ionic, and even psychological stress. With aging, however, most cells become markedly less effective at mounting adaptive stress responses[3]. In neurons, this is partly due to age-related decreases in the expression/activity of stress sensors like AMP protein kinase (AMPK) and sirtuin 1 (SIRT1), and protective neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). These proteins stimulate gene expression that promote antioxidant defenses, healthy mitochondrial function, and Ca^{2+} handling[4].

2.6 Aberrant neuronal network activity

The brain relies on communication among billions of neurons. Optimal function of neuronal networks requires balanced activity of glutamatergic (excitatory) neurons and GABAergic (inhibitory) interneurons. However, during aging, activity within these circuits (largely white matter communication via myelinated axons) is dysregulated, which can result in hyperexcitability and excitotoxic damage. This may lead to degeneration of fiber systems involved in decision making and learning/memory[4,24].

2.7 Glial cell activation and inflammation

Neuroinflammation is characterized by increased numbers of immune-activated, proinflammatory astrocytes and microglia that secrete neurotoxic cytokines[24]. This glial cell activation is linked with brain aging, MCI, and most neurodegenerative diseases. In fact,

markers of neuroinflammation and related neurodegeneration (e.g., glial fibrillary acidic protein (GFAP) and neurofilament light chain (NfL)) have even been shown to increase with aging in cognitively unimpaired people[2].

Importantly, peripheral inflammation has also been linked with brain structural abnormalities, reduced cognitive function, and greater dementia risk. As an example, recent studies demonstrate that elevated levels of C-reactive protein (CRP; a common clinical marker of inflammation) are associated with reduced brain white matter integrity in older adults[25], and epidemiological data show that systemic inflammation may precede neurodegenerative diseases by decades[26]. These data are consistent with evidence showing that age-related neurodegenerative diseases like AD can "begin" 20+ years before clinical manifestations or diagnosis.

2.8 Impaired DNA repair

The accumulation of DNA damage is an important hallmark of aging, and it plays a causal role in several premature aging syndromes. DNA damage is also intricately linked with brain aging, cognitive decline, and neurodegeneration[4]. Genomic damage that accumulates with aging can contribute to cellular senescence (in which cells cease dividing and begin to produce proinflammatory molecules) and apoptosis (programmed cell death), leading to functional decline of organs/systems (including the brain) and reduced longevity[27]. Many factors contribute to DNA damage with aging including UV radiation, replication errors, or ROS[3]. DNA lesions, which often manifest as double-stranded DNA breaks, occur often and randomly throughout the genome over the lifespan, and the ability to repair this damage decreases with aging[28]. These lesions initiate a DNA damage response (DDR) that temporarily pauses the cell cycle for DNA repair mechanisms and chronic DDR activation can lead to a permanent halting or change in cell fate such as apoptosis or senescence (also

a hallmark of brain aging) [29]. The ability to repair genomic damage is essential in neurons throughout the lifespan as they are unable to divide to dilute the deleterious effects of dysfunctional cells or replace damaged cells.

2.9 Impaired neurogenesis and stem cell exhaustion

Aging is associated with reduced cell renewal and organ tissue/repair[27]. While most neurons do not proliferate, the dentate gyrus of the hippocampus, subventricular zone of the lateral ventricle, and the olfactory bulbs all contain small populations of neural stem cells[30]. As is the case with most stem cells, the ability of these stem cells to self-renew and generate new progenitors (i.e., neurogenesis) declines with aging, which may account for some of the structural declines (e.g., loss of hippocampal volume and overall tissue mass, enlarged ventricles) observed in the aging brain[4].

2.10 Other: Cellular senescence and telomere attrition

Cellular senescence is initiated by acute and chronic cell damage, and senescent cells accumulate with aging in multiple tissues[31]. Senescence contributes to cellular events that may be important during aging and disease, including morphological changes, proliferation arrest, telomere dysfunction, inflammation/immune activation, and DNA damage. Senescent cells remain metabolically active after they have stopped dividing and develop a pro-inflammatory, “senescence-associated secretory phenotype” (SASP), which is known to contribute to peripheral and neuroinflammation[32,33]. One plausible reason for increased senescent cells and SASP with aging is telomere shortening. Telomeres are repetitive DNA sequences that cap the ends of chromosomes and protect DNA from degradation during replication[27]. The shortening of telomeres during aging contributes to genomic material loss and can impair overall cellular function, leading to replicative senescence or apoptosis[34]. Telomere shortening and cellular senescence are both established hallmarks of aging in

peripheral tissues. Their role in brain aging is less clear, but telomere maintenance, which contributes to the production of neuronal stem cells, may protect against neurodegenerative diseases[4,27].

3. BRAIN AGING THROUGH A TRANSCRIPTOMIC LENS

Because the hallmarks of brain aging are so highly interconnected, it can be difficult to understand their contributions to brain aging and disease overall using standard laboratory methods. However, recent technological advances in next-generation sequencing (e.g., RNA-seq/transcriptomics) and bioinformatics technologies have allowed for the identification of novel mechanisms of brain aging and even potential therapies[35-38]. By profiling all (or most) potential analytes within a sample (e.g., DNA, RNA, protein), "omics" make it possible to broadly profile multiple processes of aging that may be involved in age- and disease-related phenotypes. With a more complete understanding of how different biomolecules change with aging and age-related disease, we may be able to identify therapeutic targets.

In this dissertation, I employed transcriptomics to investigate the three distinct projects related to the hallmarks of brain aging. The emphases of these projects, as described below, following projects will be outlined below: 1) external factors that may accelerate brain aging, 2) potential therapeutics that may protect brain structure and function during aging, and 3) endogenous sources of neuroinflammation that may be key drivers of brain aging.

3.1 Chemotherapy-induced cognitive impairment as a model of premature brain aging

Cancer is the second leading cause of death in the United States, and >30% of cancer patients receive chemotherapy[39]. Doxorubicin (Doxo) is a chemotherapy drug often prescribed for many common forms of cancer, but it is associated with many adverse side effects, including chemotherapy-induced cognitive impairment (CICI)[40-43]. Many

consequences of peripheral damage and dysfunction caused by Doxo are related to the peripheral, and brain-specific, hallmarks of aging (e.g., mitochondrial dysfunction, oxidative damage, impaired calcium homeostasis)[44,45]. In fact, childhood cancer survivors who received chemotherapy treatment develop age-associated conditions like cardiovascular disease earlier than their age-matched counterparts, suggesting that Doxo treatment may induce premature cellular aging[46].

Memory loss is a primary element of CICI, and these decrements are driven by adverse changes in the hippocampus[42,47]. Declines in working memory do not usually occur in healthy adults prior to middle-age or later in life (i.e., aged 65 or older)[5]; however, Doxo treatment in young adult cancer patients can cause working memory impairments like that observed in chronological aging[5,42]. In this way, CICI may be a model of premature brain aging[48]. Growing evidence illustrates the transcriptome changes underlying cognitive decline in normal, chronological aging[49], but the transcriptomic influence of Doxo on the hippocampus, and how this relates to brain aging are poorly understood. Furthermore, potential mechanisms that have linked the adverse effects of Doxo with the hallmarks of brain aging (e.g., mitochondrial dysfunction) have not been studied at the broad gene expression level. Importantly, the adverse effects of Doxo are thought to be driven by its impact on mitochondrial function, as the drug accumulates in mitochondria and causes oxidative stress, which may result in cell damage and/or death[50,51]. However, the effect of Doxo on transcriptome differences related to mitochondrial damage have not been characterized in the brain specifically. Therefore, the purpose of the first manuscript in this dissertation was to investigate the potential “age-accelerating” effects of Doxo on the mouse hippocampal transcriptome and cognitive function, and to test the efficacy of the mitochondrion-targeted antioxidant, MitoQ, as a candidate therapy for preventing these effects.

My primary finding was that, when given peripherally to young mice, Doxo accumulates in the brain, causes aging-like changes in hippocampal gene expression related to altered neuronal function like those seen in mouse and human brain aging, and increases fear/anxiety behavior (often reported in human cancer patients)[35,37,52,53]. I also found that while MitoQ may partially prevent adverse changes to the brain at the gene expression level, it may not protect against adverse behavioral changes. These data suggest a potential biological link between Doxo and CICI that warrants further investigation. Collectively, these observations are consistent with the idea that Doxo causes cognitive and behavioral changes similar to those that occur with age, and that this may be due to brain-specific, pro-aging biological effects of the drug.

3.2 Apigenin as a potential anti-brain aging therapeutic

As described above, neuronal dysfunction and neuroinflammation are major hallmarks of brain aging that contribute to reduced cognitive function[54-56]. However, there is a lack of safe and accessible treatments that protect or improve brain health with aging[57,58]. Nutraceuticals (bioactive plant compounds) have the potential to target cellular/molecular processes that contribute to brain aging and could protect brain and cognitive function in advanced age. One such nutraceutical is apigenin, a flavone found in various plants including chamomile, parsley, and celery. Apigenin has been reported to inhibit multiple hallmarks of aging, including cellular senescence, mitochondrial dysfunction, oxidative stress, and impaired proteostasis *in vitro* and *in vivo* in peripheral tissues[59-62], as well as in rodent models of neurodegeneration[63]. However, the underlying transcriptomic mechanisms of pure apigenin treatment on hallmarks of brain aging have not been comprehensively studied. To address this gap in the literature, I performed brain transcriptomics on old and young mice treated with apigenin, and I conducted *in vitro* studies using human astrocytes treated with apigenin

to elucidate the mechanisms associated with apigenin-mediated learning and memory improvements.

My findings from this study were that apigenin may improve cognitive function by modulating age-associated gene expression related to innate immune activation and inflammation, and that non-neuronal cells like astrocytes are key drivers of these changes. In complementary experiments, I found that astrocytes that were aged *in vitro* but treated with apigenin had reduced markers of inflammation and cellular senescence (which is linked with pro-inflammatory signaling) compared to untreated cells. These data suggest potential "anti-aging" effects of apigenin in the brain, perhaps by reducing hallmarks of brain aging including neuroinflammation**.**

3.3. Transposable elements as a source of neuroinflammation in brain aging and AD

Age-associated neuroinflammation is an important driver of sporadic (i.e., not genetic/familial) AD, but the underlying processes are incompletely understood. While transcriptomic studies of inflammation are increasingly common, most have focused on coding genes that are related to aging and disease. However, the human genome consists of approximately 45% non-coding, repetitive DNA sequences known as transposable elements (TEs). TEs have been historically neglected in RNA-seq studies as "junk DNA" [64], but they are a topic of increasing interest in aging research, particularly because TE transcripts can form immunogenic dsRNA and cDNA[65-69]. We, and others, have shown that TE transcript expression increases during aging and AD development, and there is evidence supporting the idea that TE expression may be both a marker and mechanism of brain aging including the observations that: a) TE transcript expression is elevated with, and can predict, age[66]; b) TE transcript burden can be reduced with classic healthy aging interventions (e.g., calorie restriction, rapamycin, exercise)[36]; and c) accumulation of TE transcripts is

associated with key hallmarks of brain aging (e.g., inflammation, genomic instability and DNA damage, cellular senescence) and age-related disease[70]. Importantly, TE dysregulation occurs in all tissues with aging and our group has previously shown that targeting TE activity can improve brain aging phenotypes. Therefore, understanding mechanisms of peripheral TE dysregulation with advancing age may provide crucial insight on TE dysregulation in the brain.

One potential explanation for TE transcript elevation with aging and AD is declining epigenetic control (e.g., reduced chromatin and DNA methylation) of TE-rich regions of the genome, allowing transcription of previously inaccessible DNA[71,72]. Of note, reduced epigenetic maintenance is an established feature of both peripheral and brain aging, as well as genome instability (including impaired DNA repair). However, no previous studies have used next-generation sequencing to characterize both DNA methylation *and* chromatin accessibility with age and AD to interrogate TE transcript dysregulation. Therefore, I performed whole genome bisulfite (methylation) and transposase accessible chromatin sequencing alongside total RNA-seq to comprehensively profile TE transcript expression and epigenetic control in blood samples from human patients who were cognitively asymptomatic or diagnosed with MCI/AD. My primary findings were that a) most TEs with increased transcription can also be traced to epigenetically dysregulated (i.e., hypomethylated and/or chromatin-accessible) regions of the genome; b) 22 of these dysregulated TE transcripts were particularly dysregulated with both aging and disease; and c) epigenetically TE transcripts with age and disease can predict age, cognitive function, and circulating markers of inflammation and neurodegeneration. Together, these data suggest that age- and AD-associated declines in epigenetic control contribute to TE transcript dysregulation and may underlie systemic inflammation that can lead to AD.

4. SUMMARY AND CONCLUSION

Brain aging is characterized by a collection of adverse cellular/molecular processes that mirror the peripheral/systemic hallmarks of aging including dysregulated energy metabolism, oxidative damage, glial cell-mediated inflammation, mitochondrial dysfunction, impaired cellular waste disposal, dysregulated neuronal calcium signaling, impaired genome repair, impaired stress response, cellular senescence, telomere depletion, stem cell exhaustion, and impaired neuronal network signaling. The above hallmarks can be interrogated with transcriptomics and are sensitive to both age-accelerating and age-protective influences, as well as diseases of aging like AD.

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CHAPTER 2: ACCELERATED AGING OF THE BRAIN TRANSCRIPTOME BY THE COMMON CHEMOTHERAPEUTIC DOXORUBICIN[1]

1. INTRODUCTION

Doxorubicin (Doxo) is a chemotherapy drug often prescribed for many common forms of cancer. One unfortunate side effect of Doxo is a phenomenon referred to as chemotherapy-induced cognitive impairment (CICI)[1-3]. Memory loss is a key component of CICI with Doxo and is driven largely by adverse changes in the hippocampus (a critical center for memory formation)[3-5]. Healthy adults typically do not experience reduced working memory until middle-age or late-life[6]. However, young adults that receive Doxo treatment often experience a reduction in working memory similar to that observed in chronological aging[3,6]. Thus, it has been suggested that CICI could be a model of accelerated brain aging[7].

There is evidence for transcriptome changes underlying cognitive dysfunction in chronological aging[8], but the influence of Doxo on the hippocampal transcriptome, and how this relates to brain aging, remains unknown. Furthermore, potential mechanisms that have been linked with the adverse effects of Doxo (e.g., mitochondrial dysfunction) and related therapies have not been studied at the broad gene expression level in the brain. Importantly, the adverse effects of Doxo are thought to be driven by its impact on mitochondrial function, as the drug accumulates in mitochondria and causes oxidative stress, which may result in cell damage and/or death[9,10]. Therefore, the purpose of this pilot investigation was to examine the influence and potential "aging-like" effects of Doxo on the hippocampal

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transcriptome and cognitive function in mice, and to test the efficacy of a candidate therapy (MitoQ, a mitochondrion-targeted antioxidant) for preventing these effects.

2. MATERIALS AND METHODS

2.1. Animals

Male C57BL6/J mice were purchased from The Jackson Laboratories at 3 months of age and acclimatized for 4 weeks prior to beginning the study. Mice were housed in standard cages on a 12:12 h light:dark cycle and allowed ad-libitum access to standard rodent chow (Harlan 7917) and water. Mice were randomly assigned to receive Doxo (n=10; 10 mg/kg suspended in sterile PBS, intraperitoneal injection; R&D Systems) or Sham (n=4; 150 µL sterile PBS, intraperitoneal injection). In C57BL6/J mice, this age is equivalent to ~25 years of age in humans[11], which falls within the adolescent and young adult age range for cancer (15–39 years of age), a common age range for diagnosis of lymphoma and leukemia[12,13] and treatment with Doxo[13]. Moreover, 10 mg/kg Doxo is roughly equivalent to the typical dose for chemotherapy in this population. Doxo and Sham solutions were freshly prepared prior to injection. One cohort of mice (n=4) was also supplemented with the mitochondrial-targeted antioxidant MitoQ ([Doxo + MitoQ], 250 µM in drinking water, light-protected) for 4 weeks, as previously described[14] immediately following Doxo administration. Mice were euthanized at 5 months of age via isoflurane anesthesia and cardiac exsanguination. Brains were rapidly removed and hippocampi were dissected, then immediately flash-frozen in liquid nitrogen, and stored at −80 ◦C.

2.2. RNA sequencing and gene expression analyses

RNA-seq and gene expression analyses were performed on a subset of mice via standard methods as previously described[15]. Briefly, frozen hippocampi (n=3 per group) were transferred into Trizol (ThermoFisher) and rigorously vortexed. RNA was isolated with an

RNA-specific spin column kit (Direct-Zol, Zymo Research) and treated with DNase to remove genomic DNA. Poly(A)-selected libraries were generated using sera-mag magnetic oligo dT beads (ThermoFisher) and Illumina TruSeq kits. All libraries were sequenced on an Illumina NovaSEQ 6000 platform to produce >40 M 150-bp paired-end FASTQ reads per sample. Reads were trimmed and filtered with the fastp program[16], then aligned to the mm10 *Mus musculus* genome using the STAR aligner[17]. Gene counts were generated using LiBiNorm[18], differential gene expression was analyzed with DESeq2[19], and differentially expressed genes were analyzed for gene ontology (GO) enrichment using rank-ordered lists in the GOrilla algorithm[20] and g:Profiler program[21]. The most specific GO biological processes were identified as terminal nodes using GO.db for R[22]. Raw data were deposited at the Gene Expression Omnibus (accession GSE178812), and raw fastq files on mouse brain aging were accessed via the NCBI Sequence Read Archive (accession SRP078611).

2.3. Behavioral analyses

All tests were performed in a sound-proof room designated for cognitive/behavioral analyses, and all equipment was wiped down with 80% ethanol between trials. Mice were acclimatized to the testing room for ≥2 h before the start of each test and removed from testing if they exhibited any signs of stress. Fear and anxiety responses were measured using the open field test as previously described[23]. Open field tests were performed once for each mouse at the end of each treatment period. Briefly, mice were placed into the center of a white opaque plexiglass box (L: 40.5 cm; W: 40.5 cm; H 30.5 cm) and allowed to move freely for 5 min. Animal motion, distance traveled, and time in the center area (20 cm × 20 cm) were tracked with AnyMaze software (Stoelting). Novel object recognition (NOR) tests were performed as previously described[24] in the same plexiglass box to assess working memory with a 4-day sequence (one 5-min trial per day). Trial 1 included an open field test to observe animal movement without objects. In trials 2 and 3, mice were exposed to two

identical, non-toxic, and odorless objects (~5 cm from each wall). In the final trial, one of the identical objects was replaced with a randomly chosen novel object of roughly the same size. Recognition index (RI) was calculated as the ratio of time spent exploring the novel object over total object exploration time. Mice were not included in the analysis if they did not reach 5-min total exploration time.

2.4. Hippocampal accumulation of Doxo

Hippocampi were collected and flash frozen at 1- and 4-hour post-Doxo or sham injection (n=3 per group/time). Doxo was extracted in ice-cold lysis buffer (5:3:2 MeOH:MeCN:water), and samples were homogenized with glass beads (NextAdvance) in a Bullet Blender for 5 min at 4 ◦C, then vortexed for 30 min at 4 ◦C and clarified via centrifugation. Supernatants were immediately analyzed on a ThermoFisher Vanquish UHPLC and Thermo Q Exactive Mass Spectrometer using a Kinetex C18 column (Phenomenex) as previously described[25]. The mass spectrometer was operated in t-SIM with MS1 acquisition at 70,000 resolution, AGC of 3e6, and maximum injection time of 400 ms. Peaks were filtered to include the m/z for the Doxo [M + H]+ ion at (544.18), and spectral matching was performed against Doxo HCl external standard (Cayman Chemical) at 5 µM. Raw files were converted to mzXML, and Doxo peaks were integrated using Maven (Princeton) as described previously described[25].

2.5. Statistics

All statistical tests, number of mice, and p-values are indicated in figure legends. Data were analyzed and presented using GraphPad Prism software. Behavioral data were not normally distributed, so a Mann-Whitney U test was used to assess NOR and open field behavioral differences between groups. Differentially expressed genes were detected using Deseq2

software[19], and gene expression/GO overlap significance was calculated using GeneOverlap program[26].

3. RESULTS

3.1. Transcriptome changes in response to Doxo and/or MitoQ

First, to characterize the effects of Doxo on the brain transcriptome, we performed RNA-seq on the hippocampus, the brain region most involved in learning/memory. We found that Doxo caused modest but significant changes in gene expression, increasing ~30 transcripts and reducing ~70 (Fig. 2.1A). However, Doxo-treated mice that were also supplemented with MitoQ appeared to have a partially "rescued" hippocampal transcriptome, with fewer gene expression differences vs. controls (Fig. 2.1B-C), and genes modulated by Doxo but preserved with MitoQ were related to neuronal and mitochondrial health/function (e. g., Slc36a2, Lars2).

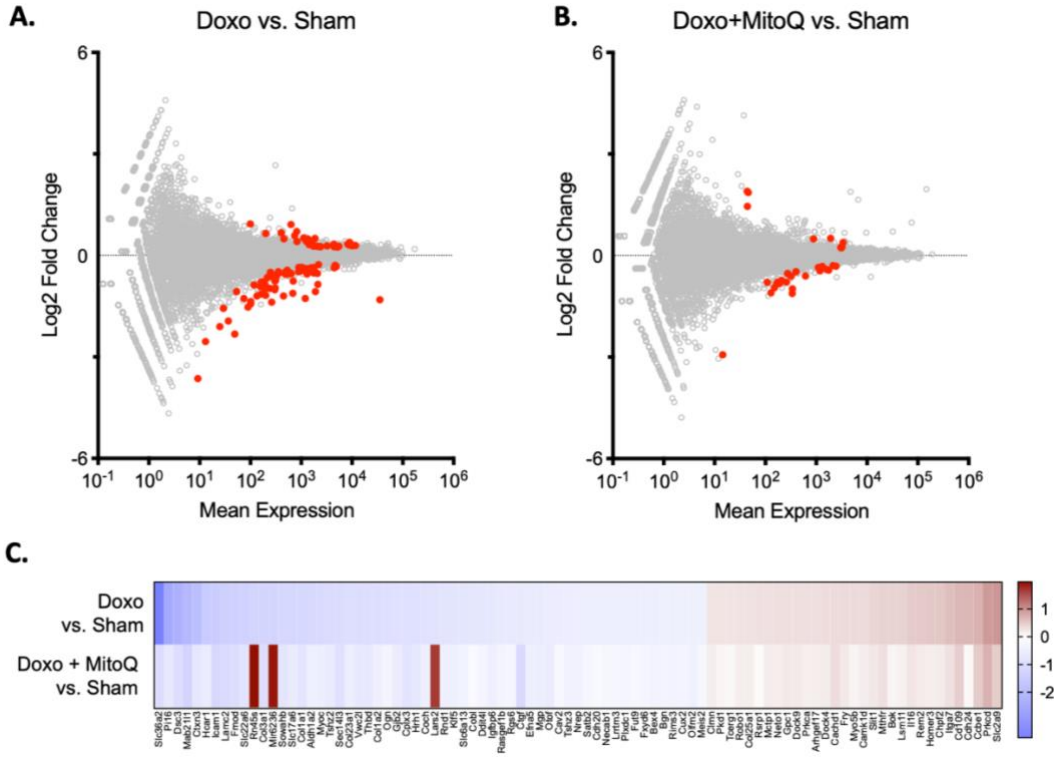

Figure 2.1. Doxorubicin causes gene expression changes in the brain; these are partially rescued by MitoQ. A) Doxorubicin (Doxo)-treated mice compared to controls (n=3

per group, red points p<0.005, FDR<0.2). Note 31 significantly increased transcripts and 72 significantly decreased transcripts; **B)** Doxo+ MitoQ-treated mice compared to sham controls (n=3 per group). Note 9 significantly increased transcripts and 22 significantly decreased transcripts; **C)** Heat map showing log2Fold changes of top genes modulated by Doxo and/or MitoQ. Note general reversal of Doxo-induced expression patterns with MitoQ.

Because global gene expression changes in the hippocampus were modest, we next performed GO analyses to identify more general biological processes (transcriptional modules) altered by Doxo and MitoQ. The top GO modules altered by Doxo alone were enriched for processes related to neuronal function/health and behavior (Fig. 2.2A, next page). In contrast, the top biological processes altered by Doxo + MitoQ treatment were fewer (indicating fewer differences vs. sham controls) and less enriched for neuron-related GO terms (Fig. 2.2B). These observations suggest that Doxo may modulate neuronal function and/or behavior-related transcriptional networks, and that this may be partially inhibited by MitoQ. Interestingly, when we compared Doxo-induced brain transcriptome changes to age-related changes in published transcriptome data on both mice and humans[27,28], we found clear similarities in modules related to neuronal/synaptic function and neuron development (Fig. 2.2C). GO modules common to aging but not Doxo treatment were mainly related to protein homeostasis and/or generic cellular development processes.

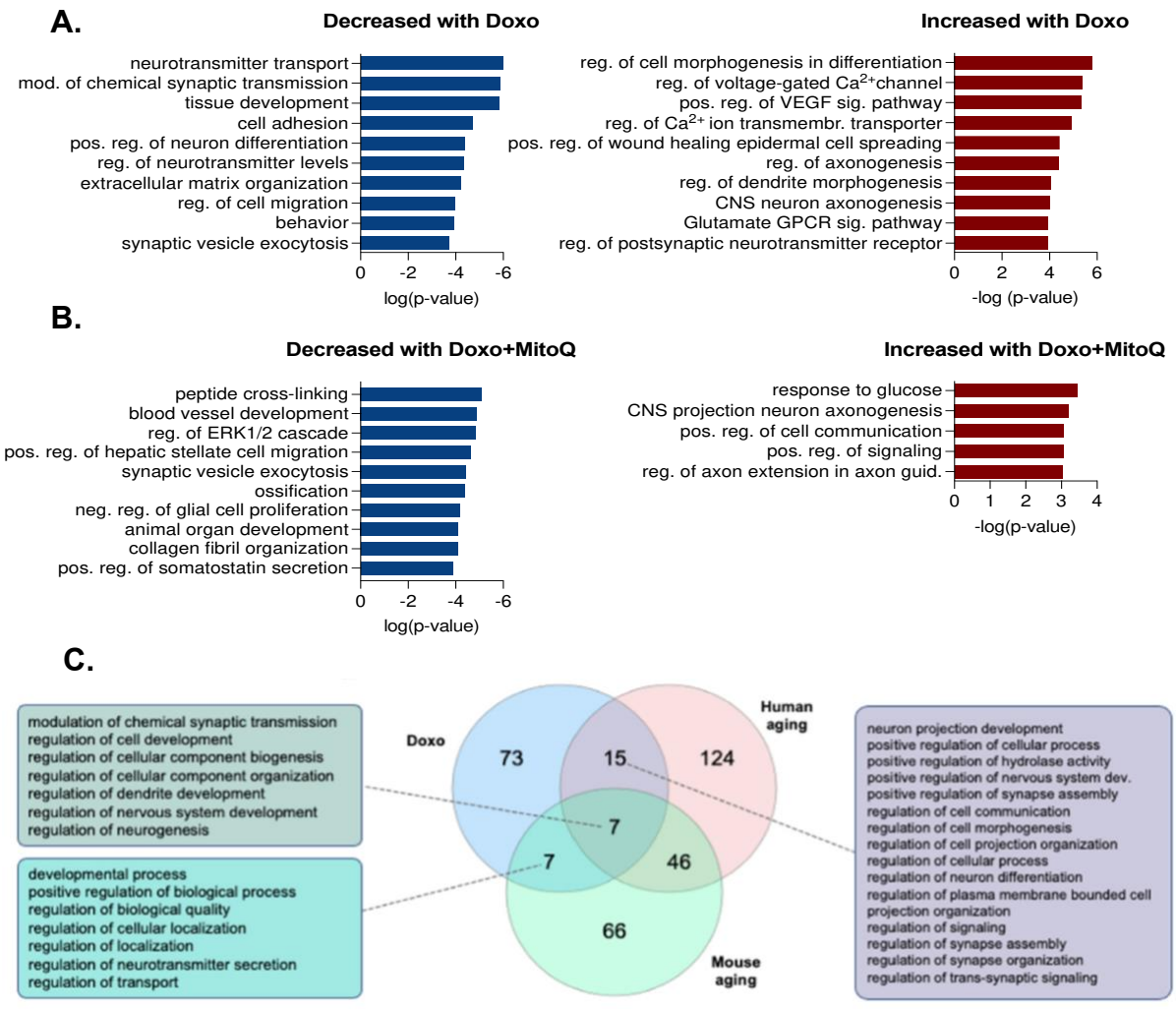

Figure 2.2. Biological processes modulated by Doxorubicin and/or MitoQ; similarity to age-related transcriptome changes. A) Top transcriptional modules (terminal GO terms) modulated by Doxorubicin (Doxo) include processes related to neuronal function and behavior; **B)** Neuronal function and behavior-related transcriptional modules modulated by Doxo are fewer and less significant in Doxo + MitoQ-treated mice; **C)** Transcriptional modules altered with Doxo treatment and brain aging (in mice and humans) are enriched for neuronal function/synaptic signaling-related GO terms. Mouse and human aging data from (Gruner *et al*., 2016; Dillman *et al*., 2017).

Because CICI with Doxo appeared to mimic the overall neuron-related transcriptome effects of brain aging, we further examined similarities among the specific biological processes with increased or decreased expression in additional RNA-seq datasets on the brains of old mice[29] and old humans from our recently published data[30]. Of the processes that decreased

the most with Doxo treatment, we found that ~60% of those same processes were decreased in mouse and human brain aging (Fig. 2.3A, next page). These processes included learning and memory, neuronal structure, and chemical synaptic transmission, all of which are impaired with brain aging[31]. Additionally, ~30% of the remaining biological processes most decreased with Doxo were similar to those reduced in the brains of old humans, including neurotransmitter secretion, neuron regeneration, and responses to cellular stress.

When performing GO analyses on the genes with the most increased expression in Doxo, and those most elevated with mouse and human brain aging, we discovered an overlap between biological processes related to DNA damage and repair, neurodegenerative processes (e.g., amyloid beta, a key peptide prone to aggregation during human brain aging), and synapse organization (Fig. 2.3B). There was a smaller, but highly relevant subset of GO modules associated with genes with increased expression in Doxo mouse brain *and* associated with human brain aging only (i.e., these specific processes were not shared with mouse brain aging). The processes were related to neuronal death, stress response, and protein homeostasis (or proteostasis), all of which are established phenomena of brain aging across multiple models. These data are consistent with established hallmarks of cellular aging[31,32] and further support the idea that chemotherapy in young mice may recapitulate transcriptome changes that occur during normal brain aging.

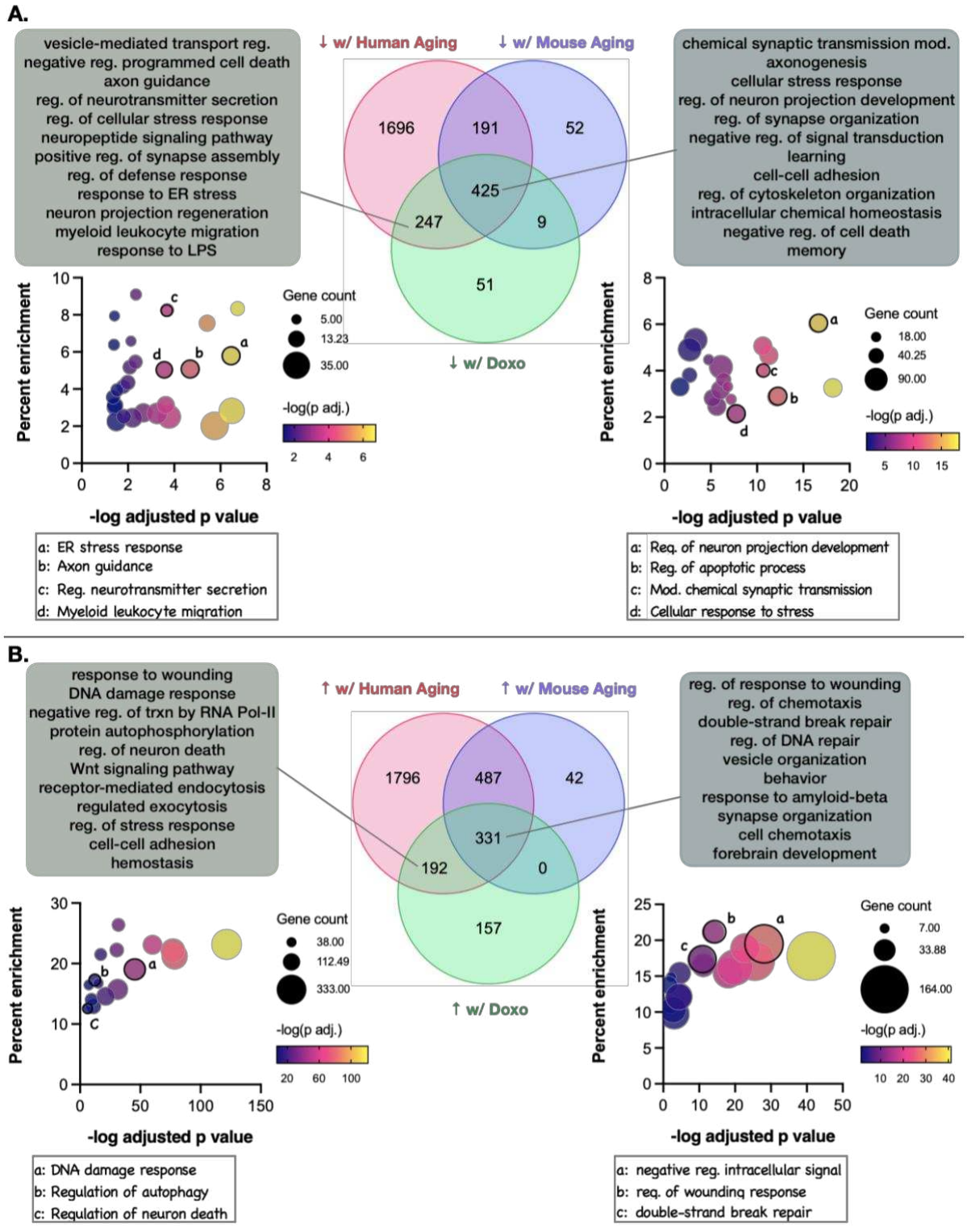

Figure 2.3. Further detail: Doxorubicin-induced transcriptome changes mimic those seen in mouse and human brain aging. A) Venn diagram showing the intersection of transcriptional modules related to genes that decreased in expression the most with Doxorubicin (Doxo, $p<0.05$), aging in mice ($p<0.5$), and aging in humans ($p<0.05$). **Left:** significant processes (transcriptome signatures) that decreased the most with Doxo and human brain aging only and bubble plot showing significance and enrichment values for

those processes with Doxo treatment. **Right:** processes that decreased with Doxo, human aging, and mouse aging, including a bubble plot of significance and enrichment for those processes in Doxo mice; **B)** Venn diagram showing transcriptome modules related to the genes that increased the most with Doxo ($p<0.05$), mouse ($p<0.5$), and human ($p<0.05$) aging. **Left:** processes and bubble plot showing individual significance and enrichment in human brains alone and Doxo. **Right:** a similar list of processes showing those that increased the most in Doxo, as well as mouse and human brain aging, accompanied by a bubble plot of significance and enrichment values.

3.2. Behavioral changes in response to Doxo and/or MitoQ

Next, we asked if Doxo-induced transcriptome changes are linked with behavioral/cognitive differences. Because chemotherapy is associated with "aging-like" cognitive dysfunction in humans, we first assessed learning/memory with the NOR test. Although Doxo did not impact NOR performance significantly, we did observe a strong trend towards lower total exploration time (Fig. 2.4A) and NOR score as quantified by the recognition index (Fig. 2.4B).

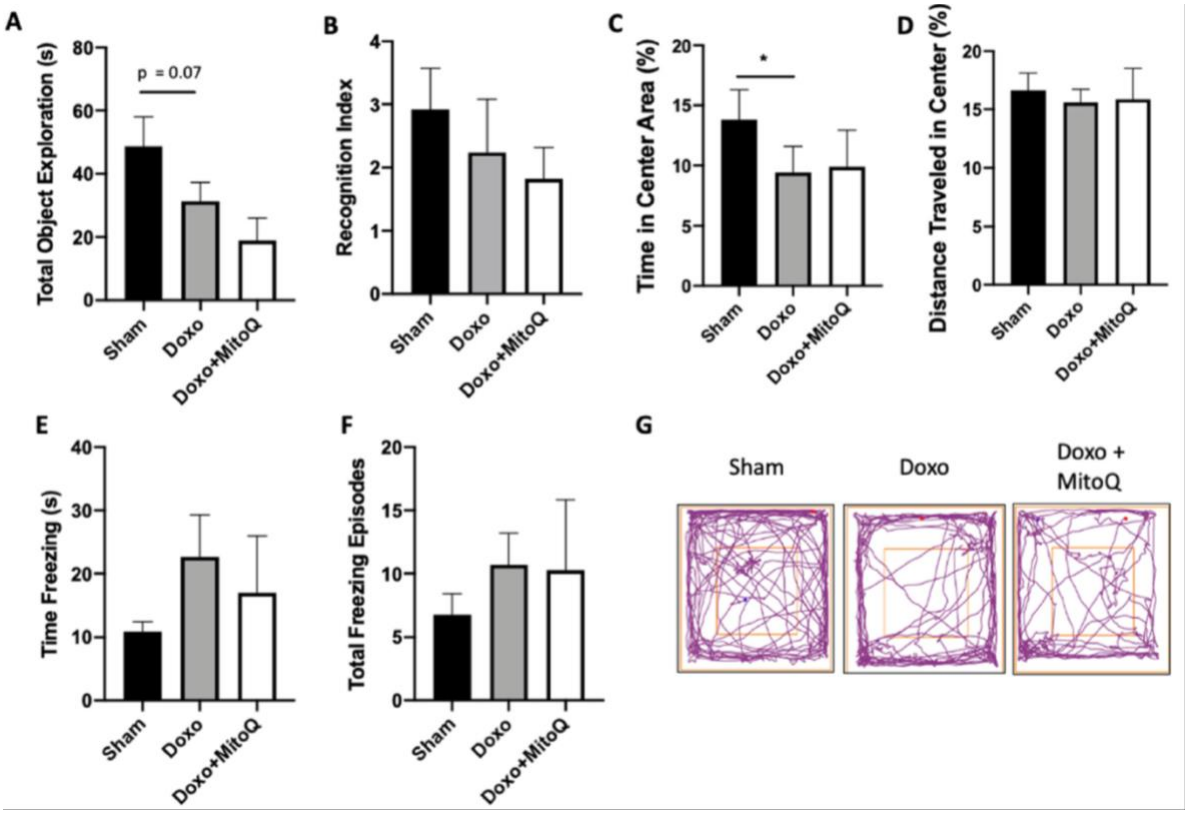

Figure 2.4. Doxorubicin causes marked behavioral changes that are not rescued by MitoQ. A) Total exploration time and object engagement during the novel object recognition

test in mice treated with or without doxorubicin (Doxo) or MitoQ. **B)** Novel object recognition index (ratio of time exploring novel object vs. total object exploration time). **C)** Percentage of time spent in arena center during open field test. **D)** Percentage of the total distance traveled in open field arena center. **E)** Total time spent freezing during open field test. **F)** Total number of freezing episodes during the open field test. **G)** Representative open field activity traces. * $p<0.05$ vs sham, n=4-10/group.

These differences in NOR performance may have been due to marked behavioral changes (e.g., increased fear/anxiety) in Doxo-treated mice, as open field analyses showed that these animals spent significantly less time in the arena center (Fig. 2.4C-D). Indeed, mice treated with Doxo tended to explore less and spend more time freezing (Fig. 2.4E-G). MitoQ did not prevent these adverse cognitive/behavioral effects of Doxo, despite its protective effects on the transcriptome. Thus, these data suggest that Doxo-induced gene expression differences may translate into related behavioral changes, but the biological effects of MitoQ may not be sufficient to prevent this.

Finally, we aimed to determine if Doxo might be directly affecting the brain, versus having strictly systemic/indirect influences on transcriptomic changes and cognitive function. To do so, in a secondary cohort of mice we assessed hippocampal abundance of Doxo via mass spectrometry at 1 and 4 h post-administration of sham or Doxo (time points at which Doxo has been previously shown to accumulate in tissues)[33,34]. We found an ~2.5- fold increase ($p = 0.17$) in hippocampal Doxo accumulation 4 h post- injection with a relative magnitude similar to that reported in other studies showing Doxo accumulation in the brain[33], whereas no differences were observed at the 1-h time point, or with sham injections (Fig. 2.5).

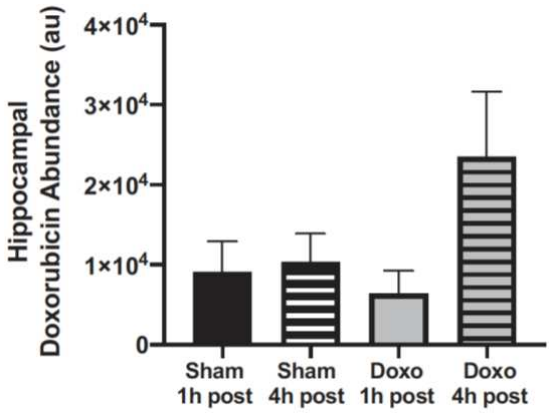

Figure 2.5. Doxorubicin crosses the blood brain barrier. Mass spectrometry data showing the hippocampal abundance of Doxorubicin (Doxo) 1 and 4 hours following intraperitoneal administration of sham (sterile saline) or Doxo (10 mg/kg in sterile saline). n=3 per group.

4. DISCUSSION

Our primary finding is that Doxo causes hippocampal gene expression changes related to altered neuronal function and behavior and increases fear/anxiety behavior in young mice. We also show that Doxo-induced transcriptome changes are similar to select age-related gene expression differences in the brain (particularly those related to neuronal/synaptic function), and that MitoQ supplementation may partially prevent these adverse changes to the brain transcriptome but may not protect against adverse behavioral changes. These findings are biomedically relevant, because CICI is a frequently reported side effect of Doxo/chemotherapy[3], but its underlying biological mechanisms and potential therapeutics are poorly understood. Finally, consistent with previous reports[33], we show that Doxo may directly affect the hippocampus, as small amounts appear to accumulate there following administration of the compound.

Despite the well-known adverse effects of Doxo on cognitive function, few studies have investigated the transcriptome changes it causes, and none have looked specifically at the brain. Most have focused on tissues like the heart (e.g., cardiomyocytes) and peripheral

blood cells (e.g., white blood cells). These reports show that Doxo causes increases in reactive oxygen species, necrosis/apoptosis of healthy cells (e.g., Doxo-induced cardiotoxicity), impaired calcium homeostasis, myelosuppression (reduced blood cell production), and mitochondrial dysfunction[35-38]. Here, we document transcriptome alterations (including altered neuronal/synaptic function signaling modules) in the brain, and specifically the hippocampus/memory center. Our data may provide insight into mechanisms underlying CICI, which is less frequently studied at the biological level, in part because Doxo and other chemotherapeutic agents are not thought to cross the blood-brain barrier. Indeed, the transcriptome changes we observed could indicate that small amounts of Doxo or its metabolites may reach the brain, as our mass spectrometry data and others' suggest[33], or that peripheral effects of Doxo may contribute to gene expression changes in the central nervous system. In either case, we also show that MitoQ may prevent these changes at the biological level, which may provide mechanistic insight on CICI. Of note, Doxo is used to treat multiple forms of cancer that are common among older adults (e.g., breast and prostate cancers), and its effects have the potential to be more pronounced in this less-resilient population[39]. Thus, the combination of advanced age and Doxo could be a "double hit" to cognitive function in older adults. Future studies are needed to determine: 1) the additive effects of Doxo and aging on the brain transcriptome and the relationship with cognitive function; 2) the indirect/systemic vs. direct effects of Doxo on the brain transcriptome and associated changes in cognitive function; 3) the potential for greater protection with longer MitoQ treatment (which may be indicated based on other studies on the compound[40]) and/or other therapeutic approaches; and 4) specific mechanisms of Doxo toxicity in the brain, such as DNA damage, which has been documented in other cell types[41].

The behavioral changes we observed with Doxo (increased fear, lack of exploration) are consistent with data on CICI in humans, as cancer patients often report anxiety and

depression alongside cognitive impairments[42]. Previous studies have documented similarly increased fear/anxiety and impaired spatial cognition in rodents treated with Doxo[43-45]. Our behavioral data confirm these findings, and our transcriptome data add to the literature by providing evidence of biological changes in the brain that could explain such behavioral differences (e.g., altered neurotransmitter transport, synaptic transmission, etc.). These data suggest a potential biological link between Doxo and CICI that warrants further investigation. Future studies could determine if similar effects are observed with the addition of other anti-neoplastic drugs (Doxo is often co-administered with cyclophosphamide), and/or if different treatment paradigms produce similar biological/behavioral effects (mice in our study were treated with a single injection of Doxo, unlike the repeated exposures used in cancer treatment regimens). Others may also be interested in studying sex-specific effects of these treatments. We conservatively chose to study male mice, as Doxo- induced changes in cognitive function may be more severe in females (reasoning that if we observed differences in male mice, it would provide rationale to study both sexes in follow-up studies). If combined with experiments aimed at identifying mechanisms of Doxo-induced changes in the brain as described above, such studies could more clearly define the role of Doxo and/or other chemotherapeutic agents in CICI.

Could Doxo chemotherapy cause a form of premature aging? Interestingly, epidemiological data show that chemotherapy survivors may have an increased risk for numerous diseases of aging such as cardiovascular disease and dementia/neurodegeneration[46-48]. Furthermore, many adverse cellular effects of Doxo resemble the “hallmarks of aging” (e.g., DNA damage, oxidative stress, mitochondrial dysfunction)[32,49]. We also found a clear overlap among biological processes that change with Doxo treatment and those associated with aging in the brain. Most of these were related to neuronal/synaptic function, which is reportedly the primary collection of processes that changes with aging in the human brain transcriptome[28].

Moreover, we note that behavioral changes seen with aging resemble those caused by Doxo in our study, as older animals typically explore less, and exhibit altered fear/anxiety behaviors[50]. Taken together, these observations are consistent with the idea that Doxo causes cognitive/behavioral changes similar to those that occur with age, and that this may be due to brain-specific, "aging-like" biological effects of the drug. These findings may serve as a basis for investigating mechanisms of CICI through the lens of aging research, which could provide insight into new treatments for chemotherapy/cancer survivors.

5. CONCLUSION

Changes to the brain transcriptome evoked by Doxo chemotherapy (CICI) may be: 1) similar to key transcriptome changes observed with chronological brain aging in both mice and humans; and 2) associated with behavioral changes consistent with those observed in aging. Supplementation with the mitochondria-targeted antioxidant, MitoQ, may preserve biological processes in the brain that are altered with Doxo, and future studies using these approaches could provide insight into the potential mechanisms underlying CICI.

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CHAPTER 3: PROTECTIVE EFFECTS OF APIGENIN ON THE BRAIN TRANSCRIPTOME WITH AGING[2]

1. INTRODUCTION

Aging is the primary risk factor for many chronic diseases[1]. Brain aging specifically is associated with declines in cognitive function that increase the risk of developing mild cognitive impairment and dementia[2]. Declining neuronal function and neuroinflammation (driven by glial cells like astrocytes) are major hallmarks of brain aging that contribute to reduced cognitive function[3-7]. However, despite the established role of these key processes in brain aging and disease[3], safe and accessible treatments for improving brain health with aging are lacking[5,8].

Certain bioactive plant compounds, sometimes referred to as "nutraceuticals," have the potential to target cellular/molecular processes that contribute to brain aging and, perhaps, protect the brain and cognitive function in advanced age. One such nutraceutical is apigenin, a flavone found in various plants including chamomile, parsley, and celery. Apigenin has been reported to inhibit multiple hallmarks of aging, including cellular senescence, mitochondrial dysfunction, oxidative stress, and impaired proteostasis *in vitro* and *in vivo* in peripheral tissues[9-12], as well as in rodent models of neurodegeneration[13,14]. However, although select apigenin derivatives have been shown to improve cognitive function with aging in mice[15], the underlying mechanisms, including the effects of apigenin on key drivers of brain aging, have not been comprehensively studied. Therefore, to provide broad insight into apigenin's potential mechanisms of action in the brain, we performed a study in which we treated young and old wildtype mice with apigenin in drinking water, measured resulting

[2] Currently in revision at *Mechanisms of Ageing & Development*

changes in learning/memory, and examined associated mechanisms via brain transcriptomics and complementary *in vitro* studies in primary human astrocytes.

2. MATERIALS AND METHODS

2.1 Animal Husbandry and Apigenin Treatment

Male (for initial proof of concept) C57BL/6N mice were obtained from the National Institute on Aging rodent colony (maintained by Charles River, Wilmington, MA) at 4 and 25 months of age and acclimatized for at least 4 weeks prior to beginning the study. Mice were singly housed in standard cages on a 12:12 hour light:dark cycle and allowed *ad libitum* access to an irradiated, fixed, and open rodent chow (Inotiv/Envigo 7917) and drinking water (Boulder, Colorado municipal tap water that underwent reverse osmosis and chlorination). Young (6 months) and old (27 months) mice were treated with control (0.2% carboxymethylcellulose [Sigma-Aldrich]) or apigenin (0.5 mg/mL [Fisher Scientific] in 0.2% carboxymethylcellulose) in drinking water for 6 weeks as previously described[16]. After cognitive-behavioral testing (described below), mice were euthanized via isoflurane anesthesia and cardiac exsanguination. Brains were rapidly removed, immediately flash-frozen in liquid nitrogen, and stored at -80°C.

2.2 Behavioral analyses

All tests were performed in a sound-proof room designated for cognitive/behavioral analyses, and all equipment was cleaned with 80% ethanol between trials. Mice were allowed 2 hours to acclimatize to the testing room before the start of each test. Because the influence of apigenin on cognitive function has been reported previously[15,17-20], we performed a simple series of brief, established tests to confirm its effects. Cognitive function was assessed using the novel object recognition test (NOR, an index of learning and recognition memory). NOR testing was performed as previously described[21,22] to assess working

memory with a 4-day sequence (one 5-min trial per day). Briefly: animals were placed in the center of a white opaque plexiglass box (L: 40.5 cm; W: 40.5 cm; H 30.5 cm) and allowed to move freely for 5 min. Trial 1 included an open field test to observe animal movement and anxiety/freezing behaviors in the same plexiglass box without objects as previously described[23]. In trials 2 and 3, mice were exposed to two identical, non-toxic, and odorless objects (~5 cm from each wall). In the final trial, one of the identical objects was replaced with a randomly chosen novel object of roughly the same size. Open field data were quantified and analyzed with AnyMaze (Stoelting, USA). Recognition index was calculated as the ratio of time spent exploring the novel object divided by total object exploration time. Discrimination ratio was calculated as the difference between the time spent with the novel object minus time spent with the old object, divided by the total exploration time. Mice were not included in the analysis if they did not reach 10 seconds total exploration time as previously described[24].

2.3 RNA sequencing and gene expression analyses

To capture as many effects and potential mechanisms of action for apigenin in the brain as possible, transcriptomic (RNA-seq) and gene expression analyses were performed on a subset of mice via standard methods as previously described[22,25]. Briefly, frozen samples (n=4-5 per group) were transferred into Trizol (ThermoFisher), and ceramic beads were added for homogenization in a Bullet Blender (NextAdvance) at maximum speed for 5 minutes. RNA was isolated with an RNA-specific spin column kit (Direct-Zol, Zymo Research) and treated with DNase to remove genomic DNA. Poly(A)-selected libraries were generated using sera-mag magnetic oligo dT beads (ThermoFisher) and Illumina TruSeq kits. All libraries were sequenced on an Illumina NovaSEQ 6000 platform to produce >40 M 151-bp paired-end FASTQ reads per sample. Reads were trimmed and filtered with the fastp program[26], then aligned to the mm10 *Mus musculus* genome using the STAR aligner[27].

Gene counts were generated using STAR quantMode (--outFilterScoreMinOverLread 0.3 --outFilterMatchNminOverLread 0.3 to account for RNA quality and increase unique reads), differential gene expression was analyzed with DESeq2[28]. Differentially expressed genes ($p<0.01$) were analyzed for gene ontology (GO) enrichment using the g:Profiler program[29]. Because whole brains were used, to isolate cell-specific signatures in bulk sequencing data, RNA-seq data were deconvolved as described elsewhere[30]. Briefly, single-cell RNA-seq (scRNA-seq) data on mouse whole cortex and hippocampus (SMART-seq, 2019; 10x-SMART-seq taxonomy, 2020) were downloaded from the Allen Brain Map portal[31] and a cortical subset was generated. Of this subset, the following major cell populations were retained for analysis: astrocytes, endothelial cells, interneurons (which connect and regulate sensory and motor neuron interactions), microglia, oligodendrocytes, and mural cells (which include pericytes and vascular smooth muscle cells). Normalized DESeq2 gene counts for old vs. young controls and old apigenin-treated vs. old control comparisons were filtered by significance ($p<0.05$) and then sorted by log2 fold change (L2FC), generating two gene sets for each comparison ($L2FC > 0$ and $L2FC<0$). Of these, the top ~150 were selected for scaling according to the scRNA-seq dataset. Next, the normalized expression of these same genes in the scRNA-seq dataset was obtained and used for dimensional reduction via principal component analysis to obtain cell-type specific signatures in the bulk RNA-seq comparisons that mapped to major cell types specified in the scRNA-seq subset.

2.4 Cell culture

In vitro studies were performed to evaluate the effects of apigenin in a cell culture model of glial cell aging using primary human astrocytes (ScienCell). Cells were cultured in poly-L-lysine coated CellTreat 12-well plates and maintained with human astrocyte media (ScienCell) as previously described[32,33]. To model aging in astrocytes, “young” fetal astrocytes were repeatedly subcultured for 10-12 passages to generate “aging-like”

astrocytes that were no longer dividing to reach confluency. These cells were treated with 25 μM apigenin[34] in media for 24 hours. Media was aspirated and cells were rinsed with sterile 1X calcium- and magnesium-free DPBS, then stored at -80°C for downstream analyses.

2.5 Western blot analyses

Frozen primary astrocytes were lysed in ice-cold RIPA buffer containing 100 mM KCl, 40 mM Tris HCl, 10 mM Tris base, 5 mM $MgCl_2$, 1 mM EDTA, and 1 mM ATP (pH 7.5), phoSTOP phosphatase inhibitor, cOmplete protease inhibitor, and sodium orthovanadate (ThermoFisher). Protein concentration was determined using a BCA kit (ThermoFisher/Pierce). 10 μg of protein per sample was separated by electrophoresis (Bio-Rad Criterion system) and transferred to nitrocellulose membranes (Trans-Blot Turbo, Bio-Rad), then blocked in Tris-buffered saline + 5% milk. Primary antibodies included phosphorylated nuclear factor kappa B (p-NFκB, 1:1000 dilution, ABclonal), cyclin-dependent kinase inhibitor 2A ($p16^{INK4A}$, 1:1000, Cell Signaling), phosphorylated H2A histone family member x (pH2a.x, 1:1000, Cell Signaling), and interferon gamma (IFNγ, 1:1000, ABclonal). GAPDH (1:2000, Novus Biologicals) was used for normalizing protein expression on all blots. Membranes were incubated in primary antibody overnight at 4°C, followed by HRP-conjugated secondary antibodies (1:1000; Cell Signaling) for 1 h at room temperature and then 1 min in ECL reagent (ThermoFisher/Pierce) for imaging on a ProteinSimple Alpha Imager. Western blot images were analyzed using Fiji[35].

2.6 Statistics

All statistical tests, numbers of mice, and p-values are indicated in figure legends. Data were analyzed and presented using GraphPad Prism software. One-way ANOVA with Tukey's *post-hoc* testing was used to assess NOR and open field behavioral differences among groups. Time spent immobile during the open field test was assessed using a Kruskal-Wallis

test with Dunn's *post-hoc* tests because the data were not normally distributed. Differentially expressed genes were detected using DESeq2 as described above, and p-value outputs from the g:Profiler program were used for GO analyses.

3. RESULTS

3.1 Apigenin may improve cognitive function in old mice by mediating inflammatory and innate immune gene expression

We observed age- and apigenin-related differences in behavior, consistent with what others have reported in experimental models of neurodegeneration using isolated apigenin derivatives[15,17-20]. For example, in open field testing, fear and anxiety responses were greater in old vs. young mice, as indicated by less distance traveled (Fig. 3.1A) and more time spent immobile (Fig. 3.1B) throughout the duration of the test. During the NOR test, old mice displayed reduced learning/memory, as measured by recognition and discrimination indices (Fig. 3.1C-D).

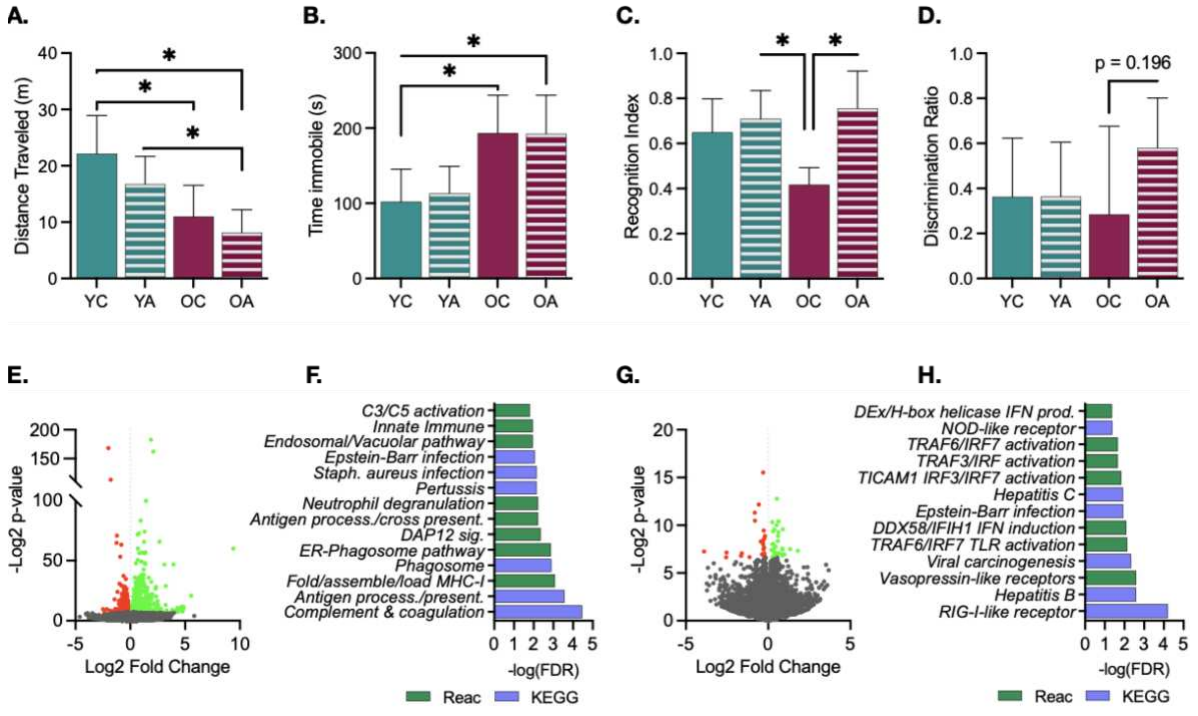

Figure 3.1. Apigenin improves learning/memory in old mice and modulates transcriptomic signatures of inflammation/immune activation. A-D) Cognitive-

behavioral data on young and old mice treated with apigenin in drinking water for 6 weeks (YA and OA), as well as vehicle-treated young and old controls (YC and OC). **A)** Total distance traveled in meters during open field testing; **B)** time spent immobile during open field testing; **C)** Recognition index in NOR testing (*i.e.*, time spent exploring novel object over total exploration time); **D)** Discrimination ratio in NOR testing (*i.e.*, the difference between the time spent with the novel object minus time spent with the familiar object, divided by the total exploration time). Cognitive-behavioral data are means ± SD; n=8-12/group; * $p<0.05$. **E-H)** Brain RNA-seq data on young and old apigenin-treated mice. **E)** Volcano plot showing gene expression differences in OC vs. YC, n=4-5/group, red/green points = $p<0.01$; **F)** Top biological processes/pathways related to gene expression differences in OC vs. YC, FDR<0.05; **G)** Volcano plot showing gene expression differences in OA vs. OC, n=4-5/group, red/green points $p<0.01$; **H)** Top significant pathways related to OA vs. OC, FDR<0.05. Pathway terms simplified for presentation; full list provided in supplement.

Although apigenin treatment did not decrease fear and anxiety in old mice (Fig. 3.1A-B), it did increase recognition index in old mice vs. untreated controls (Fig. 3.1C), and it also tended to improve discrimination ratio (Fig. 3.1D, $p = 0.196$ vs. controls), a measure of recognition memory sensitivity[36].

Although several studies have shown that apigenin treatment can ameliorate similar cognitive and behavioral impairments in mouse models of induced/premature aging and aged wild-type mice, none have examined its effects on the brain transcriptome. Therefore, we next performed RNA-seq and differential gene expression analyses on brain tissue from young and old apigenin-treated mice and controls. In old vs. young control animals, we found numerous differentially expressed genes at $p<0.01$ and FDR<0.1 (Fig. 3.1E). Pathway analyses indicated that the top biological processes associated with these genes/transcripts were related to immune responses, inflammation, and cytokine regulation (Fig. 3.1F). In apigenin-treated old animals vs. old controls, gene expression differences were less robust (consistent with ours and others' reports on pharmacological treatments in old mice), but we found ~50 genes that were differentially expressed at $p<0.01$ with modest FDR values (Fig. 3.1G). These gene/transcript differences were associated with biological processes related to development, behavior, and antiviral responses (Fig. 3.1H). Both behavioral and

transcriptome differences seen with apigenin may be due to an accumulation of apigenin in the brain tissue of treated animals (Fig. S3.1).

3.2 Apigenin reverses age-associated gene expression changes related to immune activation

Given our transcriptome findings above, to provide further insight into apigenin's potential mechanisms of action, we next examined patterns among the top increased and decreased genes/transcripts with aging (i.e., in old vs. young controls) and those same genes in old apigenin-treated mice vs. old controls. Of the genes/transcripts increased most with aging (FDR<0.1), many trended in the opposite direction (i.e., decreased expression) when comparing old apigenin-treated mice to old controls (Fig. 3.2A). Moreover, the top upregulated genes with aging that were downregulated in old apigenin-treated mice were associated with pathways reflecting innate immune function (e.g., complement and coagulation cascades, immune cell activity) and pathogen immune responses (Fig. 3.2B).

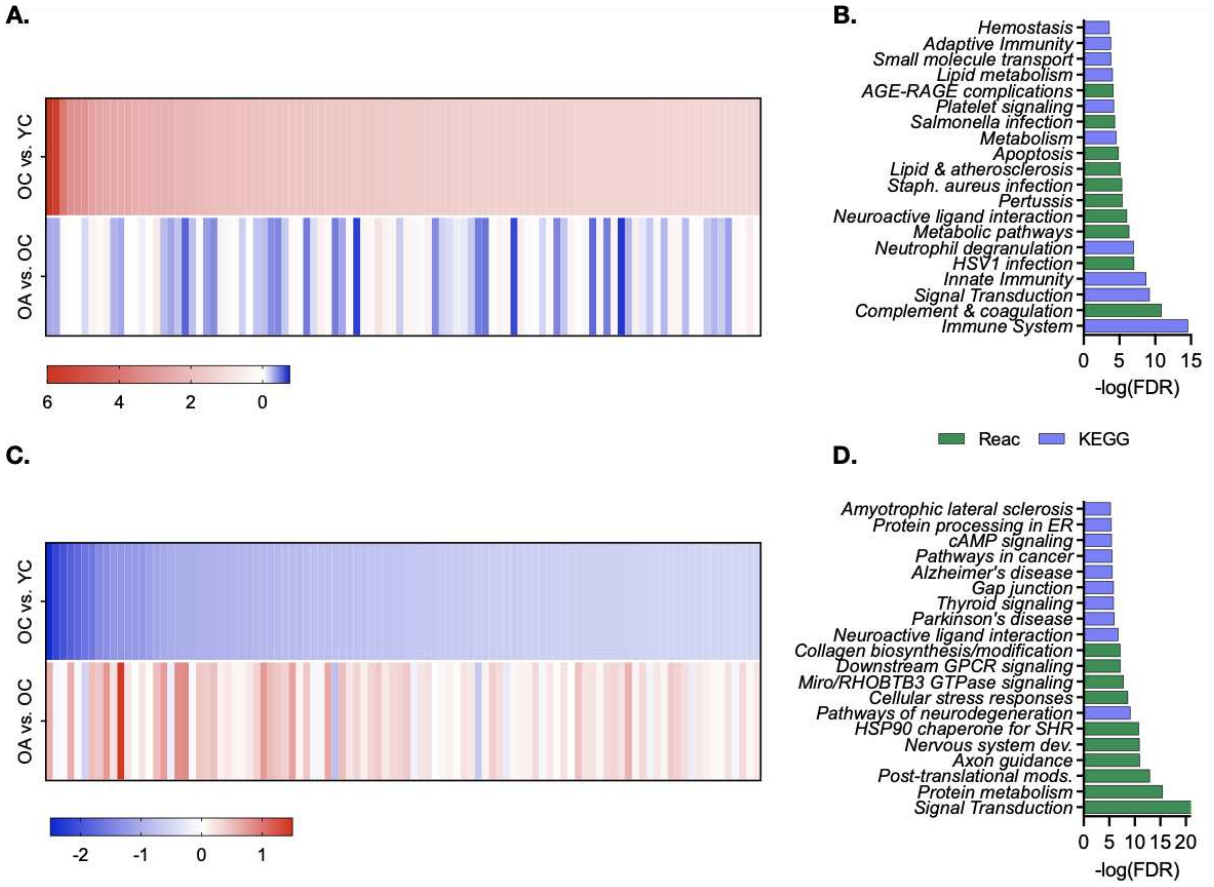

Figure 3.2. Apigenin reverses gene expression signatures related to inflammation and immune activation in the aging mouse brain. A) Log2 fold changes of genes with the greatest increase in expression in old control (OC) vs. young control (YC) mice and the same genes in old apigenin-treated mice (OA) vs. OC (top 100 genes shown); **B)** Top 20 significant KEGG and Reactome pathways (FDR<0.05) related to the most increased genes/transcripts in OC vs. YC that were also decreased in OA vs. OC; **C)** Log2 fold changes of genes with the greatest decrease in expression in OC vs. YC and those same genes in OA vs. OC (top 100 genes shown); **D)** biological processes/pathways (FDR<0.05) related to the most decreased genes/transcripts in OC vs. YC that were also increased in OA vs. OC. Pathway terms simplified for presentation, full list provided in supplement.

Similarly, of the top genes/transcripts with reduced expression with aging, many were increased in terms of relative expression in old apigenin-treated mice vs. old controls (Fig. 3.2C). These genes were related to pathways relevant to neurological function and diseases (e.g., axon guidance, Alzheimer's disease), cellular maintenance (e.g., protein metabolism, collagen biosynthesis/modification), and several brain-relevant homeostatic signaling

pathways like cyclic-AMP signaling (Fig. 3.2D). Collectively, these observations suggest that apigenin may modulate neuronal health and, perhaps even more strongly, immune function and inflammatory gene expression in the aging brain, which is consistent with reports on apigenin in other tissues/cell types[16,37-39].

3.3 Brain transcriptome changes induced by apigenin are modulated by non-neuronal cells.

To determine which cell types might contribute most to the differences in inflammatory/immune transcriptome signatures that we observed with aging and apigenin treatment, we deconvolved our bulk brain RNA-seq data using a single cell (sc)RNA-seq dataset on mouse cortex and a standardized workflow[30]. Principle component analyses of our deconvolved data showed a significant representation of neurons, as expected, but glial and vascular cells (i.e., non-neuronal cell populations) contributed most to the gene expression differences we observed with aging (Fig. 3.3A) and apigenin treatment (Fig. 3.3B), as reflected by greater variability along principal component axes (note axis scales). Among the transcripts that contributed to these deconvolved data, many were also associated with astrocytes in old mice [4] and were genes that reversed in terms of expression in (i.e., in Fig. 3.2A,C), such as C4b (involved in astrocyte clearance of microglial debris[40]), Gpr17 (abundant in cortical glia, associated with negative regulation of inflammation and neuronal recovery following injury[41]), and Sox11 (essential for neurogenesis and regeneration[42,43]). These findings are in line with the established contribution of non-neuronal cells, especially glial cells (e.g., astrocytes, microglia), to neuroinflammation[44,45], and with our observation that the main transcriptome signatures of aging and apigenin treatment in mice were associated with inflammatory signaling.

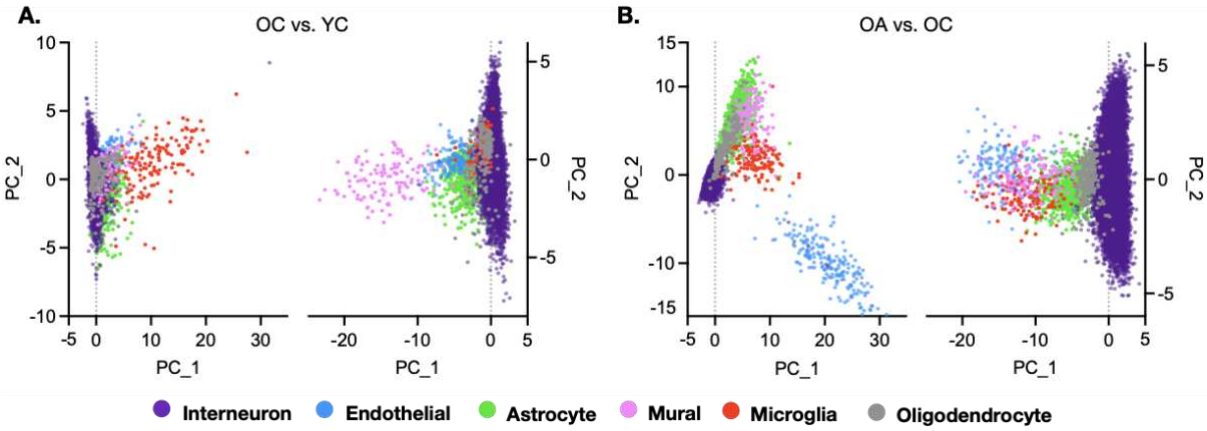

Figure 3.3. Non-neuronal and glial cells contribute strongly to gene expression changes with aging and apigenin treatment. A) Principal component analyses of the top genes that increased **(left)** and decreased **(right)** in expression in old vs. young control mice. **B)** Principal component analyses of the top ~150 genes that increased **(left)** and decreased **(right)** in old apigenin-treated mice vs. old controls. All data based on deconvolved bulk RNA-seq data from the same mice in Figures 1-2; n=4-5/group. Endothelial = endothelial cells from cerebral blood vessels, mural = vascular smooth muscle and pericytes.

3.4 Apigenin reduces senescence and inflammatory markers in an *in vitro* model of astrocyte aging

Finally, given our findings above and because growing evidence implicates astrocytes specifically in age-related neuroinflammation[33,46-49], we investigated the effects of apigenin in an *in vitro* model of glial cell aging. We treated primary fetal "young" and aged human astrocytes with apigenin for 24 hours. In aging-like vs. young control cells, western blot analyses showed increased expression of proteins related to cellular aging/senescence (cyclin-dependent kinase inhibitor 2A [p16/p16^{INK4a}][50], phosphorylated H2A histone family member X [pH2AX][51], Fig. 3.4B-C) and inflammation (phosphorylated NFκB [pNFκB][52], interferon gamma [IFNγ][53], Fig. 3.4D-E), confirming an aging-like, pro-inflammatory phenotype. However, in aged cells treated with apigenin, levels of these senescence and inflammatory markers were similar to those in young cells, and the expression of IFNγ, a

pro-inflammatory cytokine that increases with aging and is implicated in neuroinflammation, was reduced vs. untreated aged cells.

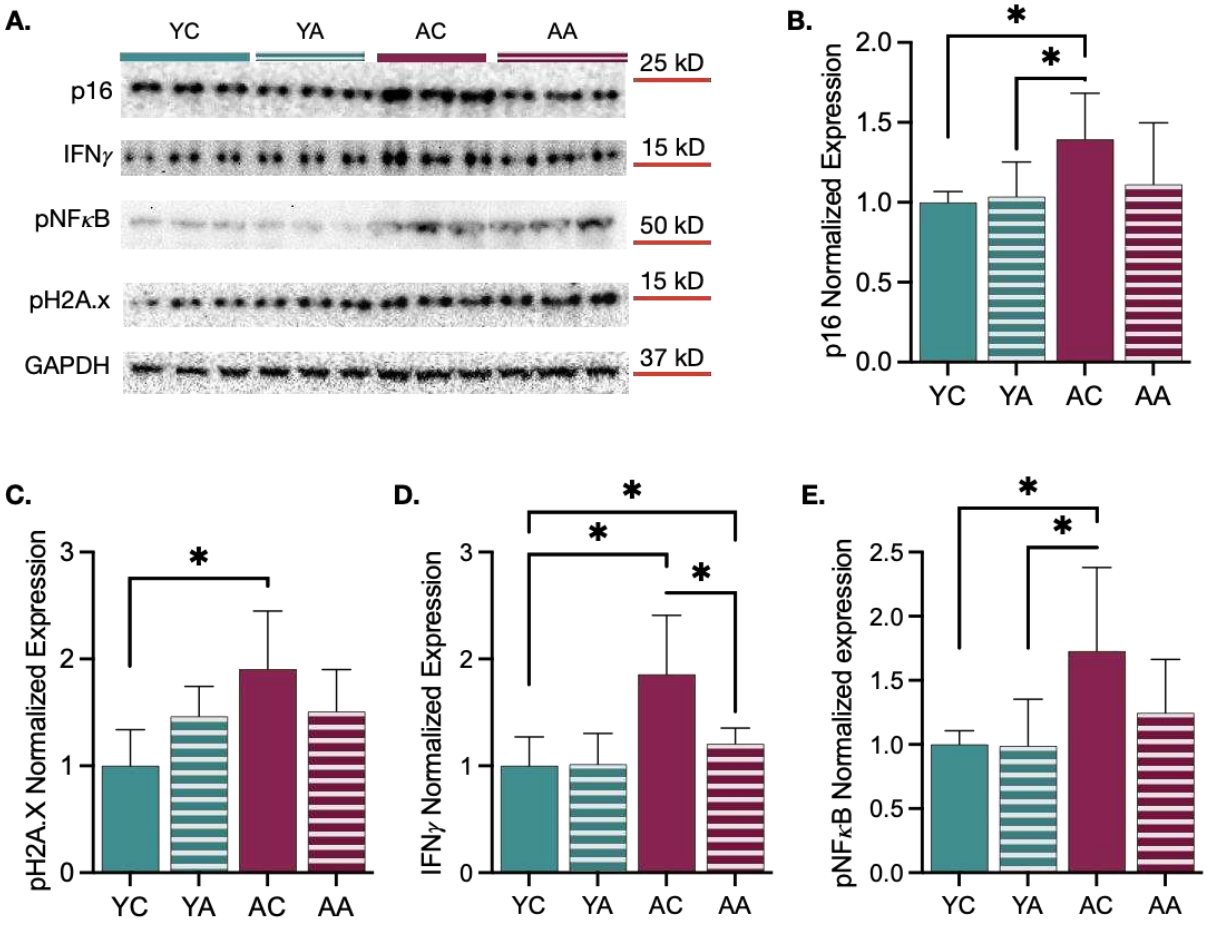

Figure 3.4. Apigenin reduces markers of senescence and inflammation in aging-like primary human astrocytes. A) Representative immunoblots for markers of senescence and inflammation. **B-C)** Relative levels of the cellular senescence markers **B)** p16 and **C)** phosphorylated H2ax in fetal/young and aging-like (replicatively aged) astrocytes treated with or without 25 μM apigenin for 24 h. **D-E)** Relative levels of the innate immune activation/inflammatory markers **D)** interferon gamma (IFNγ) and **E)** phosphorylated NFκB in the same cells. YC = Young control; YA = Young apigenin; AC = Aged control; AA = Aged apigenin. Data are means ± SD, normalized to GAPDH and YC; n=8-12/condition; * $p<0.05$.

4. DISCUSSION

Brain aging leads to declines in cognitive function that increase the risk for neurodegeneration. Growing evidence suggests that nutraceuticals such as apigenin could protect against brain aging/neurodegeneration, but the specific mechanisms by which

apigenin acts in the brain have not been studied. Our primary findings are that apigenin may improve cognitive function by modulating gene expression related to innate immune activation and inflammation in the aging mouse brain, and that these effects are likely driven by non-neuronal cells including astrocytes (a key glial cell type that contributes to age-associated neuroinflammation). In support of this idea, we found that treating astrocytes aged *in vitro* with apigenin reduced markers of inflammation and cellular senescence (which is linked with pro-inflammatory signaling). These data suggest potential "anti-aging" effects of apigenin in the brain, perhaps by reducing inflammation, a key hallmark of aging[54].

Neuroinflammation is a central mechanism of brain aging that is reflected by changes in the transcriptome[4,47,55,56], but it may be modified by brain-protective, anti-aging interventions, including nutraceuticals. For example, others have shown that supplementation with compounds like nicotinamide mononucleotide (an emerging anti-aging therapeutic that occurs naturally in certain fruits and vegetables) and ellagic acid (a polyphenol found in pomegranate) reduces inflammatory transcriptome signatures and cytokines in the brains of old mice[57,58]. Acute treatment with curcumin, a biologically active polyphenol found in turmeric, has also been shown to blunt lipopolysaccharide-induced inflammation in mouse brains[59], and α-Bisabolol (found in plants like sage and chamomile) is reported to reduce multiple proinflammatory cytokines in the brains of rotenone-injected rats[60].

Previous studies have shown that apigenin has similar neuroprotective[61] and anti-inflammatory[62,63] properties, and that it can protect against inflammation-associated cognitive impairment in experimental mouse models[17,19,20]. However, none have used transcriptomics to determine how apigenin modulates biological processes in the aging, non-transgenic mouse brain. Here, we show that apigenin may protect the aging brain from neuroinflammation by altering gene expression related to immune signaling, as well as

neuronal function and cellular homeostasis. We observed broad transcriptome differences in old vs. young control animals, and pathway analyses of these differences indicated the top biological processes associated with brain aging were related to innate immune activity and inflammation, consistent with what we[64,65] and others[66,67] have reported. In old mice treated with apigenin, however, most genes/transcripts that increased or decreased with aging trended in the opposite direction (i.e., were reversed in terms of relative expression). Furthermore, nearly all transcripts increased with aging but decreased with apigenin were involved in pathways related to innate and adaptive immune function and inflammation, whereas transcripts that decreased with aging but increased with apigenin were related to neuronal function, cell maintenance, and homeostatic signaling. These data agree with previous studies showing that apigenin favorably modulates several hallmarks of aging including inflammation[68], cellular senescence[10,11,39], and mitochondrial dysfunction (e.g., oxidative stress)[12,16,69], as well as processes that contribute to age-associated neurodegeneration[38,70-72]. Our findings are also consistent with evidence in other experimental models (e.g., of Parkinson's disease, infection, and brain injury) showing that apigenin may moderate neuroinflammation and immune signaling in the brain[13,18,37].

In line with the concept that neuroinflammation contributes to cognitive dysfunction with aging[44], we observed elevated fear and anxiety in old mice during behavioral testing, as well as evidence of reduced learning/memory (i.e., lower scores in NOR testing). However, the anti-inflammatory transcriptomic effects of apigenin were associated with improved recognition index (learning/memory) in treated old mice vs. controls. Importantly, whereas the majority of apigenin studies have been based on injection treatments (i.e., intraperitoneal, intravenous, subcutaneous[13-15,63,68]), we observed these effects in response to apigenin administered in drinking water. As such, our findings are an important addition to the literature because they suggest apigenin maintains its bioactivity even after the

digestion/absorption process (which is not true for many nutraceuticals). Additionally, many prior studies on apigenin have been based on treatment with isolated apigenin conjugates (e.g., apigenin-7-glycoside, luteolin[73,74]) rather than pure apigenin. Thus, our data showing that dietary administration of unmetabolized apigenin exerts biologically relevant effects on the brain transcriptome and cognitive function could suggest an easier route to clinical translation. In this context, future studies are needed to determine if specific apigenin metabolites accumulate in the brain when the compound is administered whole, and/or whether different preparations (e.g., in liposomes) may make it more bioavailable.

The precise causes of age-associated neuroinflammation are not fully understood, but current evidence suggests a central role for non-neuronal cells[75]. In support of this idea, we found that the gene expression changes mediated by aging and apigenin treatment were likely driven by non-neuronal cells including astrocytes, microglia, endothelial cells, and mural cells, as demonstrated by greater gene expression variability in deconvolution analyses of our RNA-seq data. These findings are in line with other reports of apigenin inhibiting glial cell-mediated inflammation in transgenic models of astrocyte-driven neuroinflammation[76]. To extend on data like these in the context of aging, we tested apigenin's effects in an *in vitro* model of glial/astrocyte aging. We found that apigenin may reduce inflammation associated with DNA damage and cellular senescence (hallmarks of aging that are also implicated in neuroinflammation) in these cells, consistent with previous work in other tissues[11,39]. However, ours is the first study to link apigenin with astrocyte-associated inflammation in the context of brain cell aging. We note that replicatively-aged astrocytes *in vitro* like those used here may not fully recapitulate the changes seen with biological aging *in vivo*. Also, while we focused on mechanisms of action for apigenin in the brain, it was administered orally in the present study and could have had systemic effects that contributed to the observed improvements in behavior and cognitive function. For

example, we have previously shown that apigenin can reduce vascular inflammation associated with aging[16], and current literature supports the idea that vascular inflammation can negatively impact cognitive function with advancing age[77,78]. As such, future studies are needed to confirm our findings (e.g., in primary astrocytes from older animals and/or humans) and characterize potential systemic interaction effects apigenin treatment, as these data will be important for assessing the translatability of apigenin as a novel anti-neuroinflammatory treatment. Finally, in addition to these potential future directions, future studies could include more comprehensive cognitive testing (e.g., Barnes maze, Morris water maze) in old mice treated with apigenin to determine the full extent of apigenin's potential effects on different domains of cognitive function. Also, as our present findings were based on bulk brain RNA-seq analyses, future work could investigate gene expression in more specific brain regions that are affected significantly by neuroinflammation with aging such as the hippocampus[79,80], nucleus accumbens[81], and entorhinal cortex[81], as these may benefit the most from apigenin treatment.

5. CONCLUSION

Collectively, our findings are consistent with growing evidence in support of apigenin's neuroprotective effects and the idea that it can suppress inflammation-related gene and protein expression, perhaps especially in non-neuronal cells, and thereby improve cognitive function with aging. Future, more detailed investigations are needed, but our results provide further evidence that apigenin may be a potential nutraceutical treatment for age-related neuroinflammation and cognitive decline.

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CHAPTER 4: EPIGENETICALLY DYSREGULATED TRANSPOSABLE ELEMENTS ARE ASSOCIATED WITH AGE-RELATED COGNITIVE DECLINE AND ALZHEIMER'S DISEASE[3]

1. INTRODUCTION

Advancing age is the primary risk factor for late-onset Alzheimer's disease (AD), but how age increases AD risk is poorly understood. Brain aging itself involves declines in cognitive function, which may develop into mild cognitive impairment (MCI) that increases the risk of developing AD[1,2]. Brain aging and MCI are also characterized by similar/related adverse events that are further implicated in AD (e.g., the accumulation of tau and amyloid beta [Aβ]), and most AD cases are sporadic and age-related with no clear familial/genetic component[3]. As such, identifying and understanding mechanisms that underlie brain aging/MCI and may be involved in age-associated AD *per se* is an important research goal.

Neuroinflammation, characterized by inflammatory cytokine production and innate immune activation that can be neurotoxic, is a central mechanism of brain aging and AD, and it often precedes classic AD pathology including Aβ and tau aggregation[2,4]. Importantly, systemic inflammation, or "inflammaging", is also a key feature of aging in general, and it is reported to precede and/or occur in parallel with neuroinflammation[5-7]. While the precise mechanisms of age-associated inflammation/neuroinflammation are not fully understood, these observations suggest a potential common mechanism underlying these processes, and recent studies suggest that transposable elements (TEs) may play an important role in this context[8-12]. TEs represent nearly half of the human genome, consisting of non-coding,

[3] Currently in preparation for submission to *Geroscience*.

repetitive sequences that have accumulated throughout evolution. The main types of TEs include: 1) retrotransposons such as long terminal repeats (LTRs), long interspersed nuclear elements (LINEs), and short interspersed nuclear elements (SINEs); and 2) DNA transposons[13]. Retrotransposons are of particular interest because a small number of them (notably from the LINE1 family in humans) retain their ability to "copy and paste" into different regions of the genome via reverse transcription (RNA to DNA) and integration (DNA re-insertion into the genome) [13,14]. We and others have shown that TEs, including LINEs, are transcriptionally dysregulated (i.e., at the gene expression level) with aging and AD in multiple tissues such as brain and blood, suggesting that TE transcript dysregulation is a systemic feature of aging/disease[10,12]. This TE dysregulation could contribute to age/AD-related inflammation, because TE transcripts can form immunostimulatory double-stranded RNA (dsRNA) and/or complementary DNA (cDNA) [14-17]. Indeed, preclinical studies in rodents and other model organisms show that transcription of "active" (i.e., transposition competent) TEs is directly involved in the aging process through the activation of cellular inflammation[18-20]. There also is good evidence that dsRNA and cDNA are elevated in the brain with aging and neurodegenerative disorders including AD, progressive supranuclear palsy (a tauopathy), and in multiple transgenic tau pathology models[21-23]. Thus, the central role of inflammation in brain aging and AD is consistent with current evidence of age- and AD-related TE dysregulation and TE transcript-induced inflammation[20,24].

Because many TEs have retroviral origins, they are typically heavily chromatinized and methylated to suppress their transcription. However, increasing evidence supports the idea epigenetic changes (e.g., reduced chromatin and DNA methylation) may drive TE transcript dysregulation with aging and AD[25-28]. For example, transcriptional dysregulation of TEs undulates with age and disease, and there are distinct blood TE transcript signatures related to MCI/AD, in which select TE classes/families are reduced in MCI before an exaggerated

"storm" of TE dysregulation in AD[29]. As such, circulating TE transcripts may be a biomarker of age-related cognitive decline and could predict late-onset AD. However, the epigenetic mechanisms of age- and AD-related TE dysregulation have not been comprehensively characterized, and no prior studies have utilized both global chromatin and DNA methylation profiling to interrogate TE dysregulation throughout the genome with age and AD. Here, we used blood samples from a large group of human subjects to profile transcriptome differences, including in TE expression, with normal aging, MCI, and AD. Then, we selected samples from a carefully matched subset of subjects in which to characterize differences in chromatin accessibility and DNA methylation that might underlie particularly dysregulated TEs with aging, MCI, and AD. We found that the majority of TE transcripts with increased expression could also be traced to epigenetically dysregulated regions of the genome, and that a subset of these transcripts were related to cognitive function and important blood markers of neurodegeneration in the larger sample group.

2. MATERIALS AND METHODS

2.1 Participants

Samples and data for the present analyses were obtained from a study previously conducted at the University of Colorado Anschutz Medical Campus Alzheimer's and Cognition Center (CUACC). A total of 122 older adults (aged 53-87) from the following groups were selected from the database: healthy, asymptomatic older adults (AS, n=75), older adults with symptomatic MCI (n=34), and older adults with Alzheimer's dementia (n=13). All subjects participated in a battery of cognitive function testing, health history assessments, neurological and physical examinations, and informant interviews. To be included as AS, participants had to be community dwelling older adults with no diagnosis of MCI or dementia and no evidence of neurodegeneration based on a neurological exam. MCI was differentiated from MCI due to possible AD as previously described[30-32], and patients with

major psychiatric disorders, non-AD neurological conditions (e.g., Parkinson's disease), recent history of a focal brain lesion, substance abuse, significant medical illness or conditions that would interfere with cognitive testing were excluded. All participants/cases were evaluated and agreed on by a board-certified neuropsychologist, board-certified behavioral neurologist, and clinical research coordinator. All participants provided informed consent under a protocol approved by the University of Colorado Multiple Institutional Review Board (COMIRB) [33].

2.2 Cognitive assessments

The protocols used for all cognitive function tests are described in detail elsewhere[30,34,35]. Briefly, testing included the Montreal Cognitive Assessment (MoCA) [36], which is a major clinical tool for evaluating cognitive health/dementia status, and tests in the following subdomains of cognitive function as part of the Spanish English Neuropsychological Assessment Scales (SENAS): verbal episodic memory, spatial location memory (SpLoc), executive function, and semantic knowledge[30,35]. Verbal episodic memory was measured because of its importance for memory formation that is impacted in early AD[37], and verbal memory (VM) composite scores were determined by a multi-trial list-learning measure as previously described[30]. SpLoc composite scores were calculated based on the spatial localization scale, which assesses the ability to observe and replicate 2-dimensional spatial interactions[34]. Executive function scores were determined with working memory (visual and digit backward recall, list sorting), and fluency testing. Semantic composite scores were measured using a nonverbal picture association and verbal object naming tasks, as previously described[35].

2.3 Biomarker assessment

Blood samples were collected via standard venipuncture. After collection, whole blood was centrifuged at 22 °C for 15 minutes at 1500×g and plasma was removed. Isolated plasma was centrifuged at 4 °C for 10 minutes at 2200×g and stored at −80 °C for subsequent analyses. Biomarker/protein analysis of glial fibrillary acidic protein (GFAP) and canonical biomarkers of amyloid beta ($A\beta_{1-42}$, $A\beta_{1-40}$), neurodegeneration (neurofilament light chain [NfL] and total tau), and inflammation were quantified using the Quanterix ultra-sensitive single molecule array (SIMOA) immunoassay and SR-X Analyzer system with manufacturer-supplied antibody kits.

2.4 Nucleic acid isolation and sequencing

2.4.1 RNA-seq: RNA was recovered from frozen whole blood samples in PaxGene blood collection tubes using PaxGene-specific isolation kits (Qiagen), and total RNA-seq was performed as previously described[12]. Briefly, RNA libraries were generated using Tecan Globin/Ribo depletion kits and sequenced on an Illumina NovaSEQ 6000 platform to generate >40M 151-bp, paired-end reads per sample. Differential gene expression was analyzed using standard techniques as previously described[10-12,38,39]. Reads were trimmed and quality filtered with *fastp*[40] and aligned to the human genome (UCSC, hg38 *Homo sapiens*) using the STAR aligner[41], followed by gene and TE count generation with TEtranscripts[42]. Differentially expressed genes and TEs were then identified using DESeq2[43]. To identify gene and TE transcripts that correlated with markers of health, inflammation, neurodegeneration, and cognitive function, we performed a weighted gene correlation network analysis (WGCNA) [44] using normalized transcript counts for all samples, a minimum module size of 75, and a power threshold of 16. Prior to analysis, samples were clustered by Euclidean distance and samples with a distance greater than 1 million were

removed as outliers. Module member genes/transcripts were analyzed for gene ontology (GO) enrichment using the g:Profiler program[45].

2.4.2 Whole genome bisulfite sequencing (WGBS): To profile methylation differences, we generated WGBS data using genomic DNA from CryoStor-frozen white blood cells using the Zymo DNA Miniprep Plus kit. Samples with low concentrations or residual contamination from isolation were processed using the Zymo Clean & Concentrate Kit. For WGBS, genomic DNA was first subjected to bisulfite conversion. Libraries were made using the Swift Accel-NGS Methyl-Seq DNA library kit and sequenced at 60x coverage/sample on an Illumina NovaSEQ 6000 (151-bp, paired-end reads). WGBS reads were mapped using abismal[46], and the methylation analysis pipeline MethPipe[47] was used to remove duplicate reads, calculate methylation levels at individual sites, estimate bisulfite conversion rates, and identify hypo- and hyper-methylated regions of the genome (i.e., differentially methylated regions [DMRs]). The resulting list of DMRs (bed file) was then intersected with a gtf/bed file of a repetitive DNA sequence annotation of the hg38 human genome (obtained from repeatmasker.org) to identify the TEs located in these regions using Bedtools[48].

2.4.3 Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq): To characterize global chromatin accessibility, ATAC-seq libraries were prepared using the Active Motif ATAC-seq library kit as previously described[49,50]. Cryopreserved white blood cells were rapidly thawed in a 37 °C water bath for 2 minutes and gently mixed to resuspend. Intact nuclei were isolated (~75,000 cells per sample) and subjected to tagmentation to select transposase-bound DNA fragments (i.e., DNA in open chromatin regions). Tagmented DNA fragments were purified and then amplified via 10 cycles of polymerase chain reaction. Libraries were sequenced on an Illumina NovaSEQ 6000 to generate >50M 151-bp, paired-end reads per sample. ATAC-seq reads were trimmed and

filtered using *fastp,* and aligned to the human genome with Bowtie2[51]. MACS2 was used for ATAC peak identification to call differentially accessible peaks (FDR<0.01) [52]. Bedtools[48] was used as above for WGBS data to intersect ATAC peaks and TE origin loci (by comparing peaks to the RepeatMasker human genome annotation including TEs) to identify TEs that could be traced to open chromatin regions.

2.5 Statistical analyses

Clinical data were tested for normality using GraphPad Prism software. Normally distributed data were quantified using a one-way ANOVA followed by Tukey's multiple comparisons test. Group differences that were not normally distributed were assessed using the Kruskal-Wallis test and the two-step Benjamini, Krieger, and Yekutieli correction for multiple comparisons. Differential expression of genes and TE transcripts were quantified using DESeq2. WGCNA module and TE transcript differential expression heat maps were constructed using GraphPad Prism. Hierarchal clustering of TEs by z-transformed log2-fold change for these heatmaps was performed using Morpheus[53]. JMP Pro was used for regression analyses of TE transcript predictors of age, neurodegeneration, inflammation, and cognitive function. A forward stepwise regression model was used to determine the top 6 to 12 TE transcripts that predicted clinical measurements.

3. RESULTS

3.1 Overall subject characteristics

We found that all major anthropometric characteristics (e.g., education, body mass index, blood pressure) were similar among groups, although MCI subjects were slightly older than AS (Table 4.1). However, as expected, we found reduced global cognitive function, as reflected by lower total MoCA scores, in MCI, with further decline with AD when compared to both MCI and AS (Table 4.1, Fig. 4.1A).

Table 4.1. Subject characteristics for 75 healthy, asymptomatic older adults, 34 older adults with mild cognitive impairment, and 13 with Alzheimer's disease.

Condition	AS	MCI	AD
Age (years)	69 ± 6.6	74 ± 5.7	67 ± 9.06
Sex	24 M / 51 F	16 M / 18 F	7 M / 6 F
Education (years)	17 ± 2.2	16.7 ± 2.5	16 ± 2.3
Body mass index (kg/m²)	26.3 ± 3.7	28.3 ± 10.1	25.4 ± 3.9
MoCA total	26.8 ± 2.2	22.1 ± 2.8*	14.5 ± 5.8*†
Systolic BP (mmHg)	127 ± 12.7	133 ± 14.8	128 ± 14.4
Diastolic BP (mmHg)	72 ± 9.1	72 ± 9.6	70 ± 7.9

Data shown as mean ± SD, * p < 0.05 compared to AS, † p < 0.05 compared to MCI
AS: asymptomatic; MCI: mild cognitive impairment; AD: Alzheimer's disease; MoCA: Montreal Cognitive Assessment

In addition to impaired cognitive function with MCI and AD, we also observed increased serum levels of NfL (Fig. 4.1B) and GFAP (Fig. 4.1C), which are important clinical biomarkers of neurodegeneration and neuroinflammation, respectively. Thus, the groups in this study were largely similar with the exception of lower cognitive function and increased neurodegeneration biomarkers in MCI/AD, which is consistent with what others have reported as central features of age-associated cognitive decline and neurodegenerative diseases.

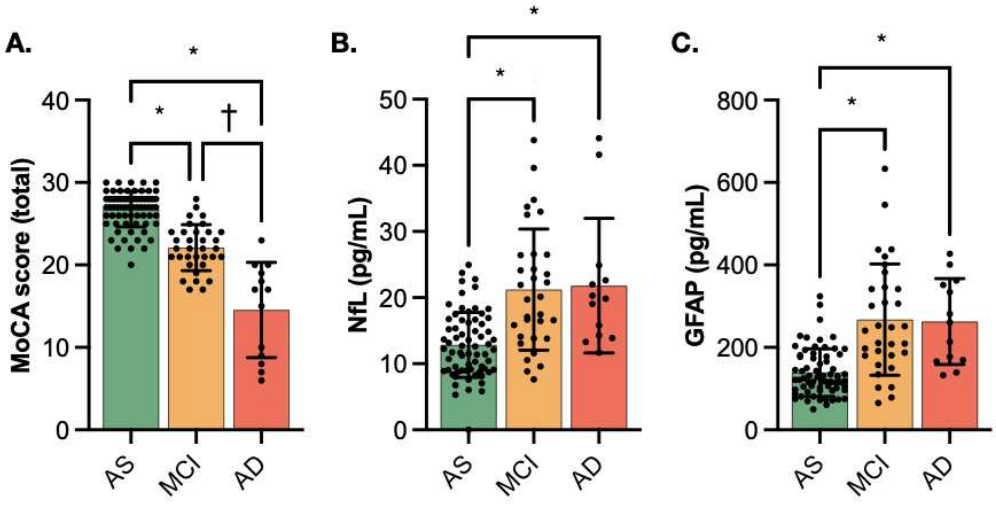

Figure 4.1. Reduced cognitive function is associated with elevated neurodegeneration biomarkers. A) Total Montreal Cognitive Assessment (MoCA) scores and **B-C)** serum concentrations of neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) in 122 older adults. AS: asymptomatic, n=75; MCI: mild cognitive impairment, n=34; AD: Alzheimer's disease, n=13; * p<0.05 vs. AS, † p<0.05 vs. MCI.

3.2 Associations among innate immune sensor gene expression, circulating inflammatory markers, reduced cognitive function, and TE transcripts

To identify systemic transcriptome signatures associated with clinical characteristics in the overall group, we generated total RNA-seq data on whole blood samples from all 122 AS, MCI, and AD subjects. Then, we performed a network analysis (WGCNA) to identify highly correlated transcriptome modules (i.e., genes and TEs) that tracked with clinical measures of health (e.g., blood pressure, body mass index), cognitive function, and blood markers of inflammation and neurodegeneration (Fig. 4.2, next page). We identified a gene/transcript cluster, module J, that was inversely related to both spatial and verbal episodic memory, and positively related to multiple blood markers of inflammation and innate immune activity including C-reactive protein (CRP), intercellular adhesion molecule 1 (ICAM1) and interferon gamma-inducible protein 10 (IP10). Interestingly, module J was also positively related to serum amyloid A (SAA), which is associated with AD pathology[54]. The top genes that drove the clustering of this module included TRIM22, IFIH1 (MDA5), EIF2AK2 (PKR), and DDX58 (RIG1), all of which are innate immune dsRNA and antiviral sensors associated with aging and AD. GO analyses also showed that the transcripts clustered within module J were related to biological processes that are important for innate immunity, viral defense, and external biotic stimulus responses (Fig. S4.1A). We also identified a second cluster of genes/transcripts, module A, that was similarly related to cognitive function. Module A was highly enriched in genes related to histone modulation and chromatin maintenance (e.g., HDAC5, HIST2H2AC, PRR14), and GO analyses showed that gene module members were related to biological processes involving cellular stress responses, autophagy and proteostasis, cell death, and altered intercellular communication (Fig. S4.1B). Notably, module A also contained more than half of the detectable TE transcripts. These data are consistent with the idea that changes to the epigenetic landscape and cellular stress

response may be associated with age/AD-related inflammation and cognitive impairments, and that TE transcript dysregulation coincides with these events.

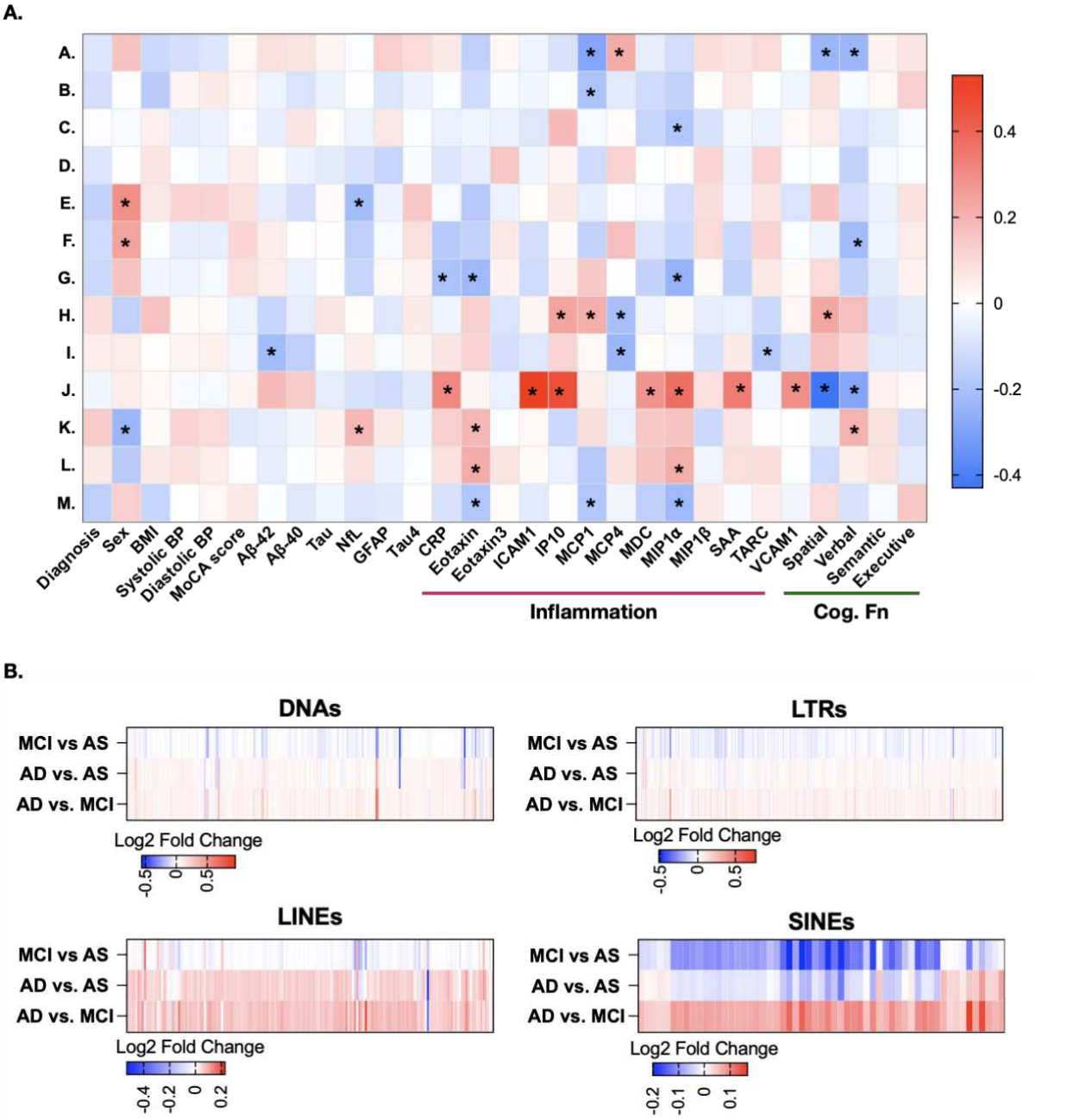

Figure 4.2. Transcriptome signatures that are positively related to inflammation are negatively related to cognitive function, may be related to TE dysregulation. A) Weighted gene correlation networks analysis (WGCNA) of all normalized RNA-seq gene/transcript counts and clinical traits in all subjects. **B)** log2-fold differences of the 4 major TE types among asymptomatic (AS), mild cognitive impairment (MCI) and Alzheimer's disease (AD) subjects. BMI: body mass index; MoCA: Montreal cognitive assessment; NfL: neurofilament light chain; GFAP: glial fibrillary acidic protein; CRP: C reactive protein; ICAM1: intercellular adhesion molecule 1; IP10: interferon-gamma induced protein 10;

MCP1: monocyte chemoattractant protein 1; MCP4: monocyte chemoattractant protein 4, MDC: macrophage-derived chemokine; MIP1α: macrophage inflammatory protein 1 alpha; MCP1β: macrophage inflammatory protein 1 beta; SAA: serum amyloid A; TARC: thymus- and activation-regulated chemokine; VCAM1: vascular cell adhesion molecule 1; Spatial: spatial location memory; Verbal: verbal episodic memory. n=119 (outliers removed); * $p<0.05$.

To further explore TE expression patterns among participants, we performed differential expression analyses on all TE transcripts. In agreement with previous studies that have reported a "retrotransposon storm" preceding AD[29], we found undulating expression patterns of the four major TE types, reflecting progressive dysregulation with MCI/AD, which culminated in increased TE transcript levels in AD when compared to AS and MCI (Fig. 4.2B) [24]. In fact, the decrease in SINEs with MCI agrees with the documented suppression of Alu SINE elements in MCI[29]. Moreover, consistent with our observations above and the idea that these MCI/AD-related TE transcript changes may contribute to immune activation, we observed correlations among TE transcripts by type and the expression of various dsRNA/cDNA sensors that were members of module J in our WGCNA analysis (Fig. S4.1C-Q). These were most pronounced for LTR retrotransposon transcripts, which were strongly related to transcription of the dsRNA sensors DDX58 (RIG-I) and EIF2AK2 (PKR), as well as the cDNA sensor MB21D1 (cGAS). Taken together, these data provide further evidence of a relationship between TE dysregulation, inflammation, and reduced cognitive function with MCI/AD, and for epigenetic dysregulation as a possible upstream mechanism[10-12,24].

3.3 Epigenetic analyses of aging, MCI, and AD subjects

Our group and others have shown that TE transcripts increase with both aging and AD[10,12,20,55]. Additionally, recent studies have shown that DNA hypomethylation and chromatin dysregulation with aging may underlie TE dysregulation that can lead to inflammation[27,56-58]. However, the extent to which these epigenetic changes contribute to TE-

related inflammation with aging vs. disease progression (i.e., MCI/AD) in humans is not fully understood. Therefore, we selected a carefully matched subset of the youngest and oldest asymptomatic (ASY and ASO, respectively), MCI, and AD participants to investigate the contribution of aging and disease to TE transcript dysregulation (Fig. 4.3A). Importantly, these subjects had clinically relevant group differences in cognitive function and markers of neurodegeneration similar to those observed in the larger study group (Table 4.2, Fig. 4.3B-D).

Table 4.2. Characteristics of the middle-aged and older asymptomatic subjects, those with mild cognitive impairment, and those with Alzheimer's disease.

	ASM	ASO	MCI	AD
Age (years ± SD)	61 ± 4.8	76 ± 3.6 *	73 ± 6.0 *	70 ± 8.5 *#
Sex	5 M / 6 F	6 M / 8 F	5 M / 5 F	5 M / 5 F
Education (years)	16.6 ± 2.4	16.5 ± 2.7	16.7 ± 2.6	15.8 ± 2.2
Body mass index (kg/m²)	25.4 ± 3.1	27.3 ± 3.7	28.5 ± 5.4	25.4 ± 3.1
MoCA total	26.9 ± 2.5	27.1 ± 1.6	20.3 ± 2.2 *#	15.3 ± 5.8 *#
Systolic BP (mmHg)	119 ± 9.3	127 ± 8.9	129 ± 12.7	133 ± 12.5
Diastolic BP (mmHg)	71 ± 8.2	69 ± 5.1	70 ± 9.1	73 ± 7.6

Data shown as mean ± SD
* $p < 0.05$ compared to ASY, # $p < 0.05$ compared to ASO, † $p < 0.05$ compared to MCI
ASM: middle-aged asymptomatic; ASO: oldest asymptomatic; MCI: mild cognitive impairment; AD: Alzheimer's disease; MoCA: Montreal Cognitive Assessment

In RNA-seq data on these subjects, we also observed a pattern of TE dysregulation by group/diagnosis similar to that in the larger study sample (Fig. 4.3E), including undulating TE transcript expression patterns in the four major types with MCI/AD consistent with the pre-AD "retrotransposon storm" others have reported. As described in the following sections, to determine if these TE expression patterns may be associated with epigenetic dysregulation, we next performed WGBS and ATAC-seq on isolated white blood cells from this subset of subjects.

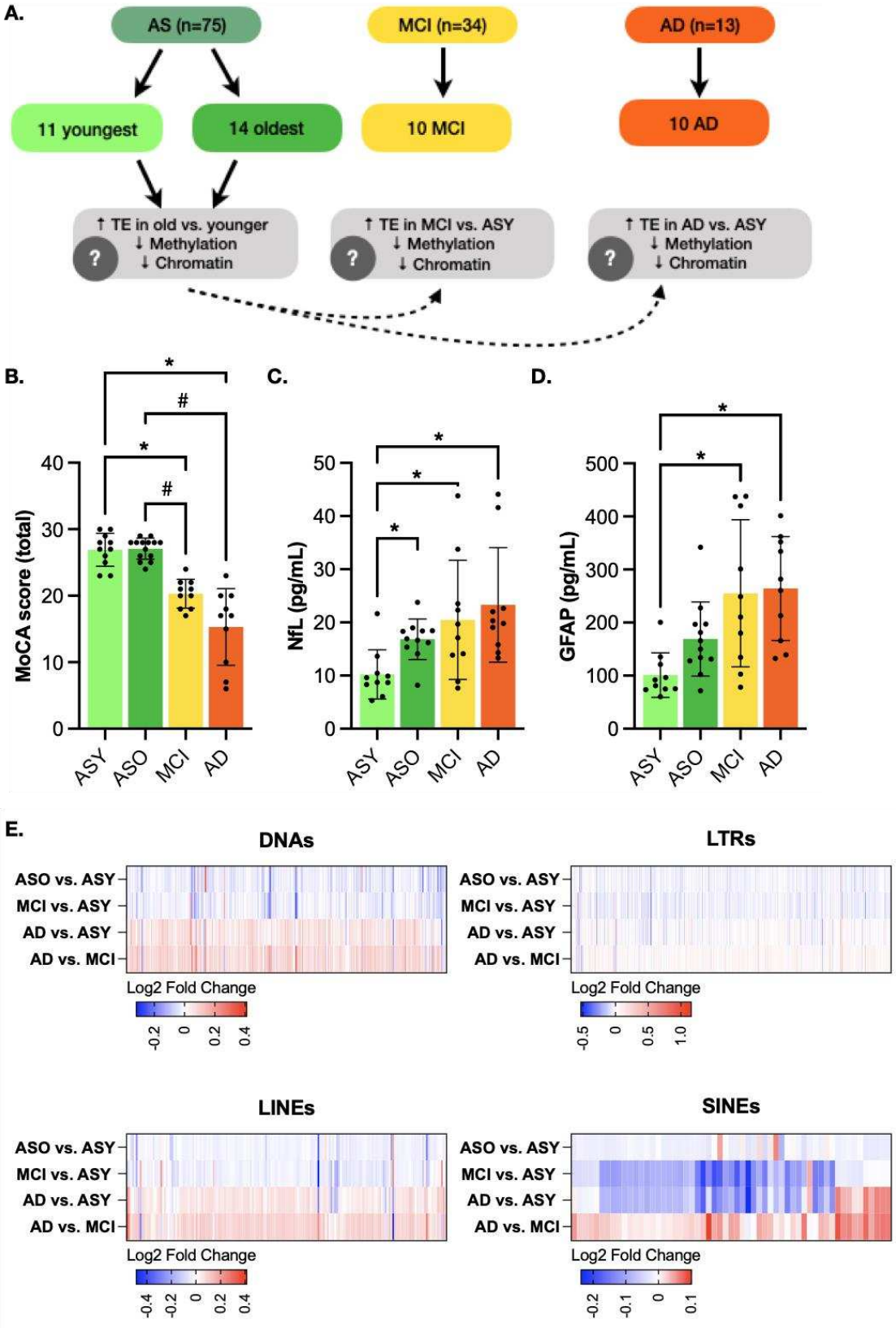

Figure 4.3. Clinical characteristics and TE transcript levels in a subset of subjects for epigenetic analyses. A) Schematic for subject selection and WGBS and ATAC-seq

analyses; **B)** total Montreal Cognitive Assessment (MoCA) scores and **C-D)** serum concentrations of neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) in a subset of subjects by group. ASY: younger asymptomatic; ASO: older asymptomatic; MCI: mild cognitive impairment; AD: Alzheimer's disease. * $p<0.05$ vs. ASY, $^{\#}$ $p<0.05$ vs. ASO; **E)**log2-fold differences in expression of 4 major TE types among groups.

3.4 TE transcripts that increase with aging and disease may originate from hypomethylated DNA

Global DNA hypomethylation is a well-documented consequence of aging and AD, and increasing evidence suggests that TE expression can be enhanced by reduced DNA methylation. To evaluate DNA methylation in our subjects/samples as comprehensively as possible (including in genomic regions that are not captured by promoter-focused methylation arrays), we performed WGBS. With aging alone (i.e., ASO vs. ASY), we found that >50% of TEs that were increased in our RNA-seq data were also located in hypomethylated regions of the genome (Fig. 4.4A). By major type, these TEs included mostly LTRs, and the absolute number of hypomethylated regions coinciding with transcriptionally enriched TEs was highest for LTRs as well (Fig. 4.4B-C). In MCI, the total number of TEs increased in expression and associated with hypomethylated DNA was similar to ASO vs. ASY, and most were also LTRs and DNA transposons (Fig 4.4D-F). However, in AD vs. ASY, the number of transcriptionally enriched TEs that were also associated with hypomethylated DNA was markedly increased, with nearly ~75% within regions of the genome that were hypomethylated (Fig. 4.4G). Of these, the most abundant TEs by type were LTRs, DNA transposons, and LINEs (Fig. 4.4H-I), but LINEs and SINEs were more abundant in the number of different hypomethylated regions to which they could be traced. These broad patterns of TE hypomethylation were similar, although somewhat more exaggerated, in AD vs. MCI (Fig. 4.4J-L). Overall, these data suggest that similar degrees of TE hypomethylation (particularly for LTRs, which are the most common type of

TE) may occur with aging and MCI, but that AD is associated with a marked exaggeration of these events and further dysregulation of other key TE types.

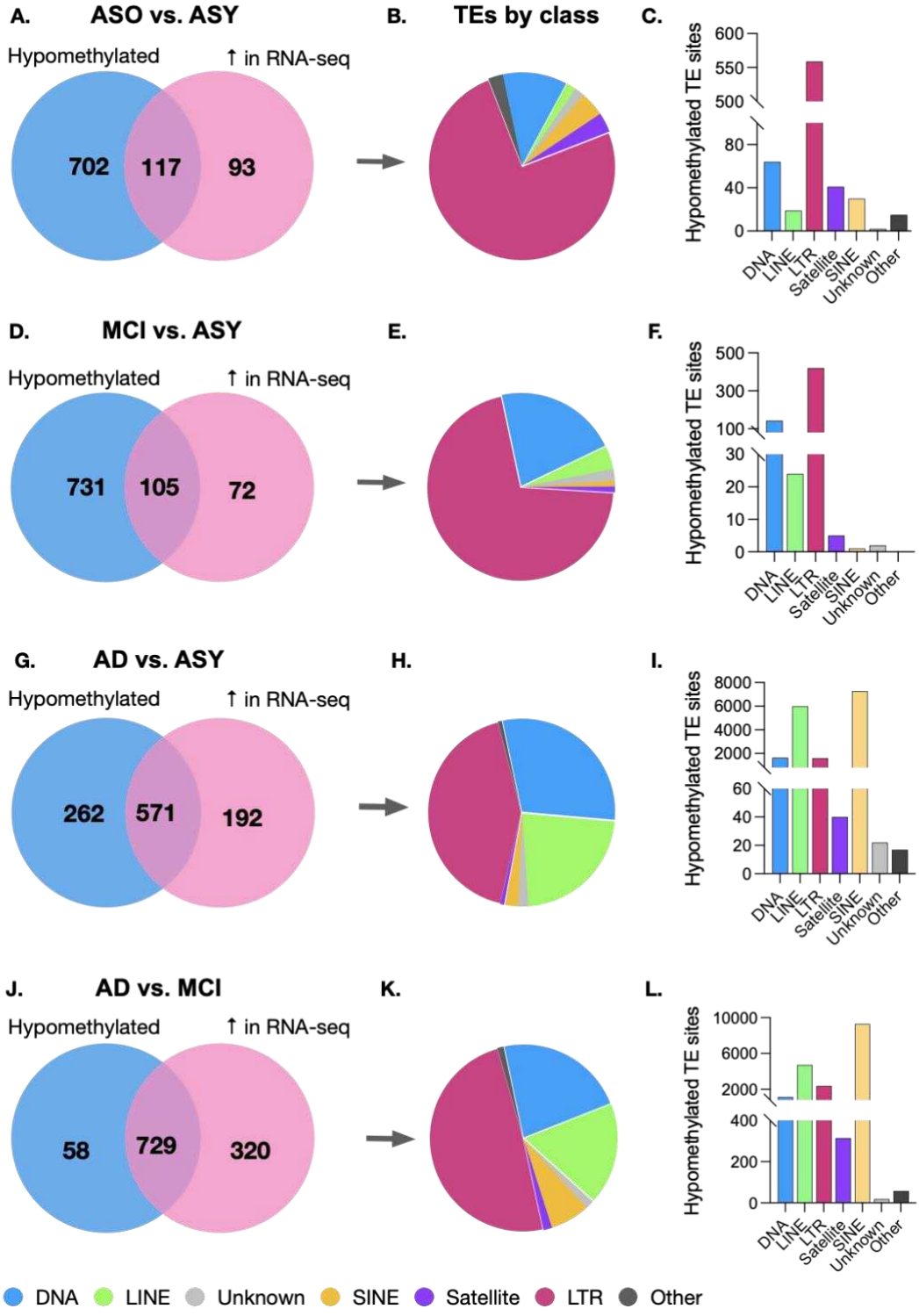

Figure 4.4. Transcriptionally dysregulated TEs are hypomethylated with aging, MCI, and AD. (Left) Venn diagrams showing TE transcripts that are both increased in RNA-seq

data and associated with differentially methylated DNA regions; **(Middle)** pie chart showing the relative percentage composition of enriched TEs by type located in hypomethylated DNA; **(Right)** bar graphs showing abundance of TEs by number of occurrences in hypomethylated DNA regions. **(A-C)** ASO vs. ASY; **(D-F)** MCI vs. ASY; **(G-I)** AD vs. ASY; and **(J-L)** AD vs. MCI.

Next, we examined the contributions of aging, MCI, and AD to hypomethylation-associated TE dysregulation. We found 26 TEs that were dysregulated (increased in terms of expression and association with hypomethylated regions) with advancing age, MCI, and AD (Fig. 4.5). LTRs made up the largest proportion of these TEs (Fig. S4.2A), consistent with current evidence showing a key role for LTR transcripts in aging and disease. These TEs also included L1HS, a LINE that is among the only retrotransposition-competent TE in humans, and LTR5HS, a prominent HML-2 human endogenous retrovirus (HERV) [59,60]. Both L1HS and LTR5HS have been directly linked to inflammation and disease in humans [18,20]. Aside from these central, age- and disease-associated TEs, most methylation-dysregulated TEs were associated with AD, as reflected by 396 dysregulated TEs in AD vs. ASY and AD vs. MCI (Fig. 4.5). Of these TEs, LINEs, LTRs, and DNA transposons made up approximately one-third each, suggesting that these TE types might be involved in AD and cognitive decline but not necessarily brain aging itself (Fig. S4.2B) hypomethylation-related dysregulation of TEs with age/AD is due to the interaction of aging and disease processes (rather than separate contributions from both).

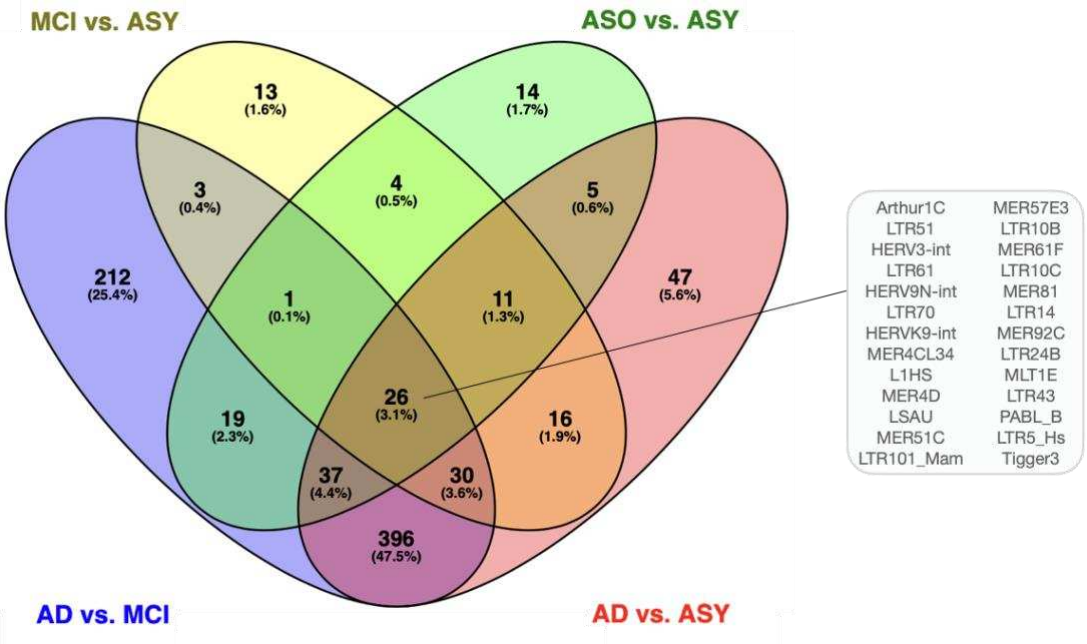

Figure 4.5. Hypomethylation-associated TE dysregulation with aging, MCI and AD. Venn diagram showing TEs that were elevated in RNA-seq data and located in hypomethylated regions of the genome in WGBS data.

3.5 TEs with increased transcription with aging and disease can be traced to open chromatin

Changes in chromatin structure and maintenance are also a well-documented consequence of aging, and both AD patients and preclinical AD models show chromatin dysregulation including increased histone acetylation, which generally increases transcriptional accessibility[25,61]. As TEs are often highly chromatinized to minimize their expression, chromatin changes could be an important epigenetic process upstream of TE transcriptional dysregulation (i.e., in addition to hypomethylation). Therefore, we used ATAC-seq to examine differences in TE chromatin accessibility with aging and disease. In ASO vs. ASY, we found that the majority (~75%) of TEs that were increased in expression in our RNA-seq data could also be traced to chromatin-accessible genome regions (i.e., ATAC peaks, Fig. 4.6A). By major type, most of these enriched, ATAC-accessible TEs were LTRs (Fig. 4.6B), although DNA transposons also frequently occurred in chromatin-accessible genome

regions (Fig. 4.6C). These numbers and patterns were similar in MCI vs. ASY, with ~70% of the TEs with increased expression located in open chromatin regions, and most being LTRs (Fig. 4.6D-F). Similar to our observations regarding DNA methylation, AD was associated with an even greater number of chromatin-dysregulated TEs with increased expression (~90% in AD vs. ASY and AD vs. MCI, Fig. 4.6G-J). Among these, the most common TE by type was LTRs, but LINEs and SINEs occurred the most frequently in chromatin-accessible regions. Collectively, these results suggest that, similar to DNA hypomethylation, increased chromatin accessibility occurs across most TEs with aging and MCI (as reflected by numerous chromatin-accessible LTRs), and that AD is associated a substantial increase in accessibility of additional TE classes (e.g., LINEs and SINEs), consistent with current evidence of a role for LINEs and Alu/SINE elements in neurodegeneration[62].

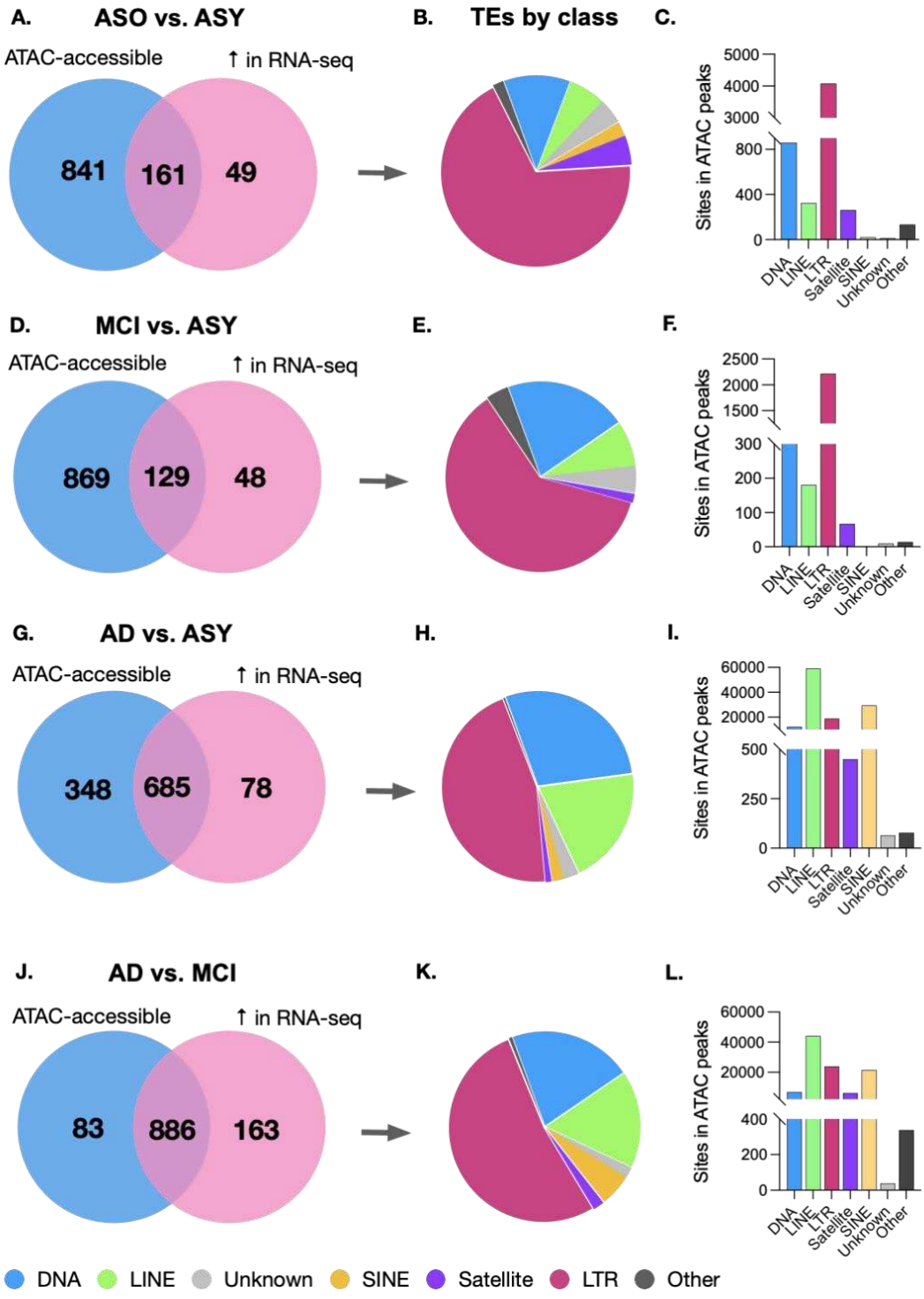

Figure 4.6. Most TE transcripts that increase with age, MCI, and AD can be traced to chromatin-accessible DNA regions. (Left) Venn diagrams showing TEs that both increased in RNA-seq data and associated with differentially chromatin-accessible DNA regions; **(Middle)** pie charts showing the relative percentage composition by type of enriched TE transcripts that may be located within open chromatin regions; **(Right)** bar graphs showing abundance of TEs by number of occurrences in chromatin-accessible DNA regions. **(A-C)** ASO vs. ASY; **(D-F)** MCI vs. ASY; **(G-I)** AD vs. ASY; and **(J-L)** AD vs. MCI.

The relative contributions of aging, MCI, and AD to chromatin-associated TE dysregulation were also similar to those we observed with respect to hypomethylation (Fig. 4.7). We found 44 elevated and potentially ATAC-accessible TE transcripts that were common with advancing age and disease. Of these transcripts, more than two-thirds were LTRs (Fig. S4.3A). Interestingly, as in our WGBS data, L1HS and LTR5HS were among these particularly chromatin-dysregulated TEs. Also consistent with our WGBS findings, most TEs that were increased in expression and epigenetically dysregulated were age- and AD-related, but not associated with aging or MCI alone. In fact, 472 TEs were primarily associated with AD/MCI, and these comprised ~25% LINEs, ~38% LTRs and ~31% DNA transposons (Fig. S4.3B). Similar to our WGBS findings, there were few individual TEs associated with age and MCI, but not with AD. The majority of these TEs were LTRs and, interestingly, they included the same three LTRs that were associated with hypomethylated DNA with aging and MCI (Supplementary table 4.3). As such, these data suggest that chromatin-related dysregulation of TEs with age/AD may occur in parallel with TE hypomethylation, and that both of these events are exacerbated in AD.

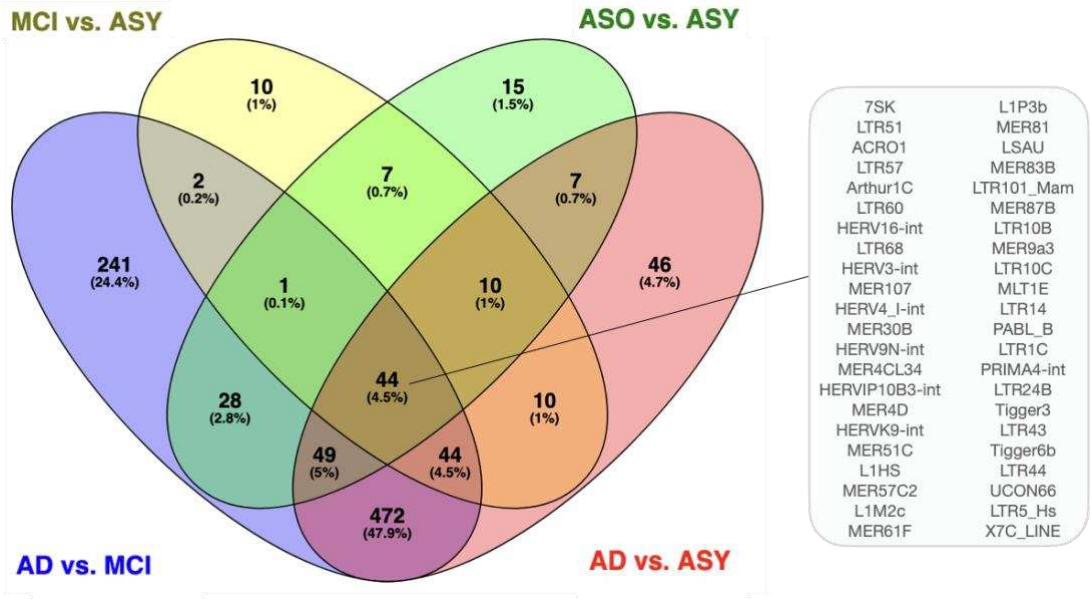

Figure 4.7. Chromatin-associated TE dysregulation with aging, MCI and AD. Venn diagram showing TEs that were increased in RNA-seq data and located in ATAC-accessible genome regions among all group comparisons.

3.6 Epigenetically dysregulated TE transcripts are related to age, cognitive function, and clinical markers of neurodegeneration

To determine if epigenetically dysregulated TE transcripts might be clinically relevant, we identified the most increased/accessible TEs in both our WGBS and ATAC-seq analyses on the subset of subjects above, and we tested the idea that they might predict clinical phenotypes in the larger study group. We found 22 TE transcripts that were enriched with aging/AD in RNA-seq data and traceable to both hypomethylated and open chromatin regions of the genome (Fig. 4.8A, Supplementary table 4.5, Fig. S4.4A-B). Many of these TEs in this group have been implicated in gene regulation, cancer, and other inflammatory conditions (e.g., LTR10B, LTR10C, LTR51, MER4D, MER81) [63-67], including L1HS and LTR5Hs (which have recently been linked with aging and inflammation). TEs that were associated with age and disease but could only be traced to regions of the genome with open chromatin were mostly LTRs, LINEs, and DNA transposons, whereas only LTRs were enriched/hypomethylated but *not* chromatin-accessible (Supplementary tables 4.6-7). These data suggest that TE transcripts that increase in expression with advancing age and MCI may be more closely tied to hypomethylated regions of the genome, or that open chromatin regions may be less "specific" to TE expression. In any case, using the 22 TE transcripts that were increased with age and disease and could be traced to both hypomethylated and ATAC-accessible genome regions, we performed a forward stepwise regression analyses on age, cognitive function, and serum markers of neurodegeneration and inflammation. Briefly, normalized counts for the 22 dysregulated TE transcripts were used to predict clinical data such as blood GFAP levels and determine the correlation with actual collected data. In this subset of subjects, we found that these highly dysregulated TEs contributed to regression models that significantly reflected age (Fig. 4.8B), serum NfL and GFAP concentrations (Fig. 4.8C-D), as well as three subdomains of cognitive function (verbal and spatial memory, and executive function, Fig. 4.8E-G). Additionally, these TE transcripts contributed to regression

models that significantly reflected circulating markers of inflammation associated with cognitive decline such as vascular cell adhesion molecule 1 (VCAM1, Fig. 8H) [68], IP10 (Fig. 4.8I) [69], and macrophage inflammatory protein 1-alpha (MIP1α, Fig. 4.8J) [70], as well as systemic markers of inflammation like CRP and ICAM1 (Fig. S4.5). Finally, in the larger study group, we found that the same dysregulated TE transcripts were also significantly related to the inflammatory markers CRP, ICAM1, IP10, and verbal/spatial memory and executive function measures (Supplementary table 4.8). Collectively, these multi-omics data indicate that declining epigenetic control may contribute to age and disease-relevant TE transcript dysregulation, and that expression of these TE transcripts may play a role in age- and disease-related inflammation and cognitive dysfunction.

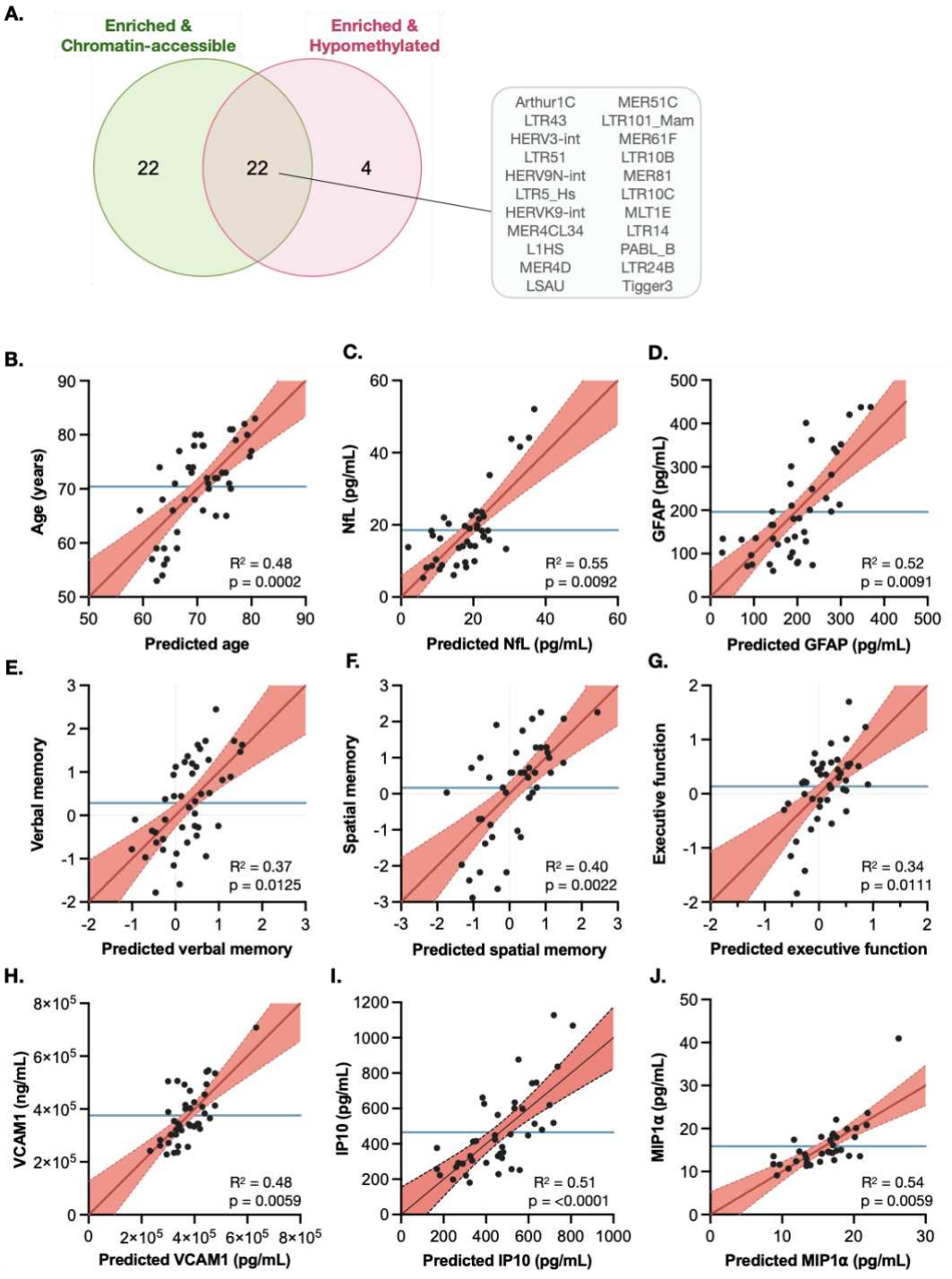

Figure 4.8. Expression of TE transcripts associated with age- and AD-related hypomethylation and chromatin dysregulation are related to markers of neurodegeneration and cognitive function. A) Venn diagram showing overlap and 22 dysregulated TEs in both ATAC-seq and WGBS data. **B-J)** Regression models of actual vs. predicted clinical data, based on the expression of the 22 epigenetically dysregulated TEs for predicting **B)** age, **C)** serum neurofilament light chain (NfL), **D)** serum glial fibrillary acidic protein (GFAP), **E)** verbal episodic memory, **F)** spatial location memory, **G)** executive

function, **H)** vascular cell adhesion molecule 1 (VCAM1) **I)** interferon gamma-inducible protein 10 (IP10), **J)** macrophage inflammatory protein 1-alpha (MIP1α).

4. DISCUSSION

Age is the greatest risk factor for AD, and both aging and AD are characterized by chronic, systemic inflammation that coincides with neuroinflammation in the brain. The exact upstream mechanisms that lead to inflammation/neuroinflammation with age and AD are not fully understood, but growing evidence shows that TEs are systemically dysregulated with both aging and AD-related pathology, and may play a role in cognitive dysfunction. Recent work also suggests that TEs stimulate inflammation via the formation of dsRNA and cDNA in various tissues. As such, TE dysregulation could play a central role in brain aging and AD, and the loss of epigenetic TE control during aging/AD could be an important, upstream mechanism of neurodegeneration. Our primary findings in the present study are that the majority of TE transcripts that are elevated in expression with advancing age, MCI, and AD can be traced to regions of the genome that are hypomethylated and lacking chromatin structure, making the DNA at these loci more transcriptionally accessible. We also identified a subset of these TEs that are especially epigenetically dysregulated and showed that transcripts from these TEs are related to age, cognitive function, and clinical markers of neurodegeneration.

Cognitive decline occurs during normal brain aging, and it has the potential to lead to MCI, which may further develop into AD[71,72]. The factors that cause some people to develop MCI and some MCI cases to convert to AD are poorly understood, but cognitive assessments combined with emerging neurodegeneration biomarkers have provided some biological insight in this context. In the present study, we examined gold standard markers of cognitive function and neurodegeneration in a larger study group (n=122) and a subset of subjects for

downstream analyses. In both groups, we found that MCI participants had lower MoCA and other cognitive function scores than AS participants, as expected, and these differences were even more pronounced in participants with AD. The neurodegeneration and neuroinflammation biomarkers NfL and GFAP were also increased with MCI and AD in our study. These observations are in line with current data, as NfL has been used as a plasma biomarker for diagnosis of neurodegerative diseases including AD, amyotrophic lateral sclerosis, and frontotemporal dementia (FTD) [73-75], and growing evidence shows that NfL can predict the progression from MCI to AD in longitudinal studies[76]. GFAP is a classic marker of astrocytes and is often used specifically as a marker of activated astrocytes and gliosis[77]. Recent work on neurological injuries and diseases suggest plasma GFAP as a potential biomarker of neuronal trauma and degeneration including in AD[78]. GFAP is released into the blood from damaged astrocytes and is increased in AD when compared to healthy, asymptomatic subjects and even other neurodegenerative diseases (e.g., FTD) [78-80]. In fact, blood levels of GFAP correlate with amyloid deposition in the cortex of AD patients with the association growing stronger with more severe stages of disease, suggesting that damage to astrocytes occurs prior to symptomatic AD and GFAP levels are associated with advancing AD pathology[79]. Thus, we observed clinical characteristics in our subjects consistent with what others have reported for cognitive aging, MCI, and AD.

To identify potential contributors to inflammation/neuroinflammation and cognitive decline/AD in the subjects in our study using an unbiased approach, we performed RNA-seq and a WGCNA. Using this approach, we found a strong relationship between transcripts related to innate immune signaling (e.g., dsRNA sensors like MDA5 and PKR) and several blood markers of inflammation. We also observed a pronounced negative correlation between these same immune sensor transcripts and multiple measures of learning and memory. These data are in agreement with previous studies showing that biofluid detection of dsRNA

sensors like MDA5 is associated with inflammatory conditions including autoimmune diseases and cancer[81,82]. Cellular dsRNA responses have also been shown to drive cognitive impairment during aging, infection, and neurodegeneration[17,83,84], and although our data were based on peripheral blood samples, our findings are consistent with growing evidence that events like this occur systemically. However, our blood samples were of mixed cell populations (a limitation of the PaxGene RNA collection tubes), so we do not know which, if any, cell types are responsible for these observations. As such, future studies should characterize changes in specific white blood cell populations such as neutrophils, which are among the most abundant blood cell and known to be key mediators of inflammation that can aggravate AD[85-87]. One important caveat to this study is that these data capture only one specific time point and may not track with disease progression and severity. Moreover, we did not control for apolipoprotein E (APOE, a key lipid transporter) genotype in our analyses and a single copy of the APOE4 allele is associated with a three-fourfold increase in AD risk[88]. Therefore, it is possible that current AS subjects may yet develop AD in the future. As such, additional, ideally longitudinal, studies will be required to determine if our findings are reproducible, and a larger sample of AD patients will be important for evaluating the potential role of epigenetically dysregulated TE transcripts throughout disease progression. We also identified a second WGCNA transcript module that negatively correlated with the same measures of learning and memory, and this module was enriched for transcripts involved in chromatin structure and modulation, as well as many TEs. Together, these data suggest that dsRNA sensors, immune/inflammatory signaling, epigenetic maintenance mechanisms, and TE dysregulation may all be interrelated with reduced cognitive function in older age.

Importantly, age-related cognitive can begin around age 50[71], and when MCI or AD begin to develop, age-related declines and disease processes can overlap[89,90]. Therefore, to examine

the effects of both age and disease on TE dysregulation, from our overall study group we selected: 1) the youngest and oldest healthy subjects to capture any subtle changes in gene and/or TE expression associated with aging prior to detectable cognitive impairment; and 2) matched groups of MCI and AD subjects to evaluate the contribution of disease processes. In these subjects, we first assessed age- and MCI/AD-related differences in DNA methylation. Global DNA hypomethylation is a well-documented consequence of aging and age-related diseases. In fact, age-associated changes in DNA methylation are the foundation of many "biological clocks" that estimate biological age (vs. chronological age in years), and groups using these clocks have found accelerated methylation-based aging in AD patients[91-95]. DNA methylation during aging has also been shown to especially decline in TE-rich regions[27,96-98]. Although previous studies have shown differential methylation in AD vs. control brains[92], most of these findings are based on DNA methylation microarrays that only capture genes/coding regions, gene enhancers, and CpG islands**,** whereas TEs may be located throughout the genome[99,100]. Therefore, we employed WGBS to profile genome-wide methylation and provide higher resolution data on TEs and hypomethylated genome regions[101]. We found that the >50% of the TEs with increased expression with normal aging could be traced to hypomethylated DNA regions, and that these TEs consisted of largely LTRs and DNA transposons. A similar proportion of TEs enriched in our RNA-seq data with MCI could be traced to hypomethylated DNA, although other TE types (e.g., LINEs) were more enriched in MCI. This is consistent with previous studies showing that LINE transcripts are elevated in MCI prior to conversion to AD, while transcripts of other TE classes such as SINEs may be reduced[29]. With AD, we also found that most TE transcripts with elevated expression could be traced to regions of the genome that were hypomethylated, and this included a particular increase in hypomethylated LINEs and SINEs. Taken together, these data suggest that the effects of DNA hypomethylation on TE transcript dysregulation are similar with normal aging and MCI, but there is a marked shift in dysregulated TE transcript

profiles with AD. These data suggest an important role for DNA hypomethylation in age and AD-related TE dysregulation, and perhaps in pheno-conversion from MCI to dementia.

Interestingly, among the dysregulated TEs identified within hypomethylated DNA, we found 26 TEs (mostly retrotransposons) were common to aging, MCI, and AD. These TEs included an active LINE and an important HML-2 HERV (or HERV-K) transcript. HERV-K TEs are evolutionarily young HERVs that are highly active and may be capable of coding for and producing retroviral-like particles[102]. In fact, transcription of HERV-K has been shown to induce innate immune pro-inflammatory signaling and is directly associated with neuronal injury and neurodegeneration[103]. However, most TE studies, including those on HERVs, have used cultured animal or human cell lines rather than primary samples taken directly from study participants[104], which is especially important as expression and consequences of HERV-K are specific to humans. The present study is the first to examine dysregulated DNA methylation and the potential impact on TE transcript expression with age and AD using WGBS in human subjects, and our data suggest that important TEs (e.g., HERVs) identified in these other models may be epigenetically dysregulated and relevant in humans, too.

Like DNA methylation, chromatin maintenance is also known to decline with age, and evidence shows that chromatin is dysregulated in AD, specifically[25]. ATAC-seq is a relatively new method for assessing chromatin accessibility, and there are very few studies that have used ATAC-seq specifically to profile TE accessibility[105]**.** Furthermore**,** to our knowledge, none have used ATAC-seq to characterize TE accessibility within the context of cognitive aging and AD. In our ATAC-seq analyses, we found that many TE transcripts with increased expression during normal aging, as well as AD development and progression, could be traced to open chromatin regions. Consistent with our WGBS findings, we found a smaller total number of transcriptionally enriched TEs that could be traced to chromatin-accessible

regions in MCI, whereas AD was associated with a broad increase in most chromatin-accessible TEs. This finding is in line with the concept that certain TE classes are suppressed during MCI prior to pheno-conversion to AD[29]. Interestingly, while the proportion of epigenetically and transcriptionally dysregulated TEs were similar between our WGBS and ATAC-seq data, more copies of dysregulated TEs tended to be located within open chromatin regions than hypomethylated DNA. These findings could be due to different sizes of hypomethylated vs. chromatin-accessible regions, as identified by current WGBS and ATAC-seq analysis pipelines, and/or they could suggest that chromatin modulation may be a key factor (perhaps more so than DNA hypomethylation) in TE accessibility and expression with advancing age and AD.

Similar to our analyses of TE hypomethylation, we found 44 TE transcripts that were enriched in our RNA-seq data and could be traced to ATAC-accessible sites in both normal cognitive aging and MCI/AD. Once again, these chromatin-dysregulated TEs included L1HS and LTR5HS. Previous studies have employed chromatin immunoprecipitation sequencing (ChIP-seq) to characterize changes in chromatin structure as a mediator of age/AD-related TE transcript dysregulation[28,106], and both AD patients and preclinical AD models show chromatin dysregulation including increased histone acetylation (i.e., activation)[25,61]. However, these are the first data to use ATAC-seq to study TE accessibility in human subjects with aging and AD, providing important insight into TE dysregulation that may contribute to systemic and neuroinflammation.

Finally, among the hypomethylated and chromatin-accessible TEs with aging and MCI/AD that we identified, we found 22 common, “particularly epigenetically dysregulated TEs” (i.e., that were associated with DNA hypomethylation and open chromatin regions). Again, these TEs included L1HS and LTR5HS, and their transcript levels were significantly related to the

age of participants, the primary risk factor for non-familial AD cases. These same TE transcripts were also related to not only NfL and GFAP, but also cognitive function scores in three different subdomains (i.e., verbal episodic and spatial location memory, executive function), as well as circulating markers of inflammation. VCAM1 is a cell-adhesion mediator of leukocyte-endothelial cell signaling and is upregulated by increased release of pro-inflammatory cytokines including tumor necrosis factor-alpha (TNFα) and interleukin-1. Importantly, VCAM1 can be upregulated by nuclear factor-kappa B (NFκB), which can be activated by dsRNA sensors like MDA5 and RIG1[17]. Others have shown that VCAM1 is a systemic marker of aging blood, and that it is associated with both impaired CNS and cognitive function, including that observed in MCI and AD patients[107-110]. IP10, also known as CXCL10, is an important cytokine that is released from monocytes and endothelial cells in response to interferon gamma, a key cytokine that is produced in response to TE-derived dsRNA. Previous studies show that peripheral IP10 is associated with cognitive decline during normal brain aging and AD[109,111]. However, the present study is the first to show that epigenetically dysregulated TE transcripts are significantly associated with circulating markers of aging and inflammation, and may be directly linked with antiviral/dsRNA immune responses. Given that these analyses were done in peripheral blood, future studies should determine if these findings are conserved within the brain, and if the transcriptome signature of epigenetically dysregulated TEs identified here can directly lead to neuroinflammation by determining if their transcripts form cDNA and/or dsRNA. Nevertheless, these data suggest that age-associated declines in epigenetic control contribute to TE transcript dysregulation and may underlie systemic inflammation observed in cognitive aging and AD.

5. Conclusions

To our knowledge, these data are the first to demonstrate TE dysregulation as a mechanism of age- and AD-associated inflammation using both ATAC-seq and WGBS. Most TE

transcripts that are elevated with aging and AD may originate from hypomethylated or chromatin-accessible regions of the genome. Moreover, TEs that may be subject to DNA hypomethylation *and* chromatin dysregulation are related to age, cognitive function, and several circulating markers of aging, [neuro]inflammation, and neurodegeneration.

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CHAPTER 5: MULTI-OMICS APPROACHES TO TARGET BRAIN AGING—A HYPOTHESIS BASED ON MY FINDINGS: CAN WE VACCINATE AGAINST DEMENTIA?

1. INTRODUCTION

This dissertation investigated the hallmarks of brain aging from multiple vantage points through transcriptomics. The results of these studies provide mechanistic insight into accelerated brain aging, potential strategies for "anti-aging" protection in the brain, and novel sources of age-related inflammation that may impact cognitive function. Central hallmarks of brain aging implicated in my findings were declining neuronal health/function, genomic damage accumulation, cellular senescence, and especially immune activation. Immune activity and inflammation are essential during infection; however, these processes can become harmful if they do not resolve, or are inadvertently activated. The importance of inflammation in brain aging is underscored as a central mechanism in each of the three studies in this dissertation. Doxorubicin chemotherapy is associated with decreased cognitive function and increases biological processes related to DNA damage and wounding responses connected to inflammation[1,2]. Apigenin improves cognitive function in old mice and reverses age-related transcriptome changes linked with inflammation and immune activity. Lastly, in human subjects, inflammatory markers are increased with cognitive impairment and gene expression of antiviral immune sensors correlates with circulating markers of inflammation, and is negatively related to cognitive function. Furthermore, epigenetic dysregulation of immunogenic transposable element transcripts with age and Alzheimer's disease (AD) is significantly related to markers of systemic inflammation, neuroinflammation, and neurodegeneration, as well as cognitive function. Because inflammation plays an integral role in age-associated disease, identifying the underlying

mechanisms of inflammation with advancing age is critically important. Then, we may be able to exploit these mechanisms to modulate age-associated immune dysfunction.

One potential method for targeting age- and AD-related immune dysfunction in the future is vaccination. Vaccines are a safe and effective way to prevent infectious diseases, and the earliest example dates back to 1796, when Edward Jenner discovered a way to prevent smallpox. In the nearly 230 years since, we have developed an arsenal of safe and effective vaccines to protect against severe illness, disability, and death. Considering the essential role that immune (dys)function plays in nearly all diseases of brain aging, it begs the question: can we manipulate the immune system such that we can vaccinate against dementias like AD?

2. RATIONALE

2.1. Vaccines can prevent age-related diseases

This concept may sound like science fiction, but there is evidence suggesting that it may warrant further investigation. For example, cancer is the second leading cause of death in the United States and age is a key non-modifiable risk factor for most/all cancers. There are now two vaccines that protect against cancer by preventing viral infection.

(1) Hepatitis B Virus (HB) is very common, with an estimated 296 million cases worldwide and there is no cure. HB is associated with liver cancer (accounting for 15% of liver cancer incidence in the United States). However, the HB vaccine provides >90% protection from infection and was the first vaccine capable of preventing cancer[3,4].

(2) Human Papilloma Virus (HPV) is the most common sexually transmitted infection and 80-90% of sexually active individuals will be infected in their lifetime[5]. Chronic

HPV infection can lead to various genital cancers including cervical cancer, for which it is implicated in >99% of cases[6,7]. Vaccinating against HPV prevents cervical cancer and helps prevent the development of other cancers as well[8].

Given that there is causal evidence for infection in age-related diseases like cancer, the concept of an AD vaccine is not entirely novel. Increasing evidence supports a potential role for viral (and some bacterial, spirochetal) infection in AD (e.g., herpes simplex 1, HPV, influenza) and vaccines can lower AD risk by preventing infection[9-13]. A recent preprint from May 2023 found that vaccinating for shingles (caused by reactivation of the varicella chickenpox virus) has a protective effective on AD risk in women[14]. These are the first data showing a causal link between preventable viral infections and dementia, and they may present a new frontier in the field of dementia prevention.

2.2. Intravenous antibody therapies can reduce AD-related proteinopathy

Much of the protection from vaccines comes from immunological memory in the form of pathogen-specific antibodies. Upon exposure to a previously encountered antigen (immunogenic microbial fragment), antibodies bind to the source and flag it for degradation. While vaccine-preventable infections continue to be of interest in the field of AD, recent work has shifted focus towards using synthetic antibodies to target AD pathology specifically. Donanemab and lecanemab are intravenous antibody therapies that target amyloid beta (Aβ) aggregates in the brains of early AD patients. When administered biweekly, Lecanemab reduced Aβ burden, and modestly improved select clinical dementia ratings[15]. Like Lecanemab, Donanemab was administered once every 4 weeks, and it reduced brain Aβ and cognitive decline[16]. However, these therapies come with risk of potentially fatal brain swelling and hemorrhage. Moreover, Aβ alone is not *necessarily* a marker of AD—Aβ burden increases with aging in cognitively healthy older adults while tau (a key microtubule

protein that aggregates within neurons during AD), is more closely associated with AD pathogenesis and progression. Advanced tau pathology is associated with more severe AD stages, so drugs that can target or prevent the formation of tau neurofibrillary tangles may be more successful in halting AD progression[17]. The primary limitation of Aβ- and tau-targeting therapies is that these therapies do not show efficacy until underlying processes of AD have begun[18]. Thus, although these therapies leverage vaccine-related immune responses (i.e., antibody production), they are still focused on the treatment of an existing pathology rather than prevention. These treatments may *slow* AD progression, but if prevention is the goal, research may be better served by targeting the age-related processes that underlie AD prior to Aβ and tau pathology.

3. HYPOTHESIS

While immune dysfunction with aging is often synonymous with aberrant inflammation, it is also characterized by increased susceptibility to infection, including those associated with dementia. Because this increased infection risk, there is interest in developing vaccines against opportunistic pathogens associated with aging. One such pathogen is *Chlamydia pneumonia* (*C. pneumoniae*), an obligate intracellular, gram-negative bacterium and leading cause of pathogenic pneumonia. *C. pneumoniae* is extremely common, with >75% of people being exposed by age 70, and the risk of developing severe illness and complications is significantly higher in older adults[19]. Moreover, *C. pneumoniae* promotes to the pathogenesis of various age-associated inflammatory diseases such as atherosclerosis and AD[20]. Although *C. pneumoniae* is typically a respiratory pathogen, it can infect the cranial nerves to access the central nervous system where it plays a role in AD risk[21]. Interestingly, one of the most elevated genes with age, mild cognitive impairment, and AD in our human subjects was peptidase inhibitor 3 (PI3), an antimicrobial peptide against gram-positive and gram-negative bacteria like *C. pneumoniae*. The pronounced increase in PI3 expression with age

and cognitive impairment may implicate its important in disease etiology. In fact, a recent study found that plasma levels of PI3 were significantly associated with an increased risk of cognitive impairment or dementia[22]. Together these data suggest that innate immune profiles associated with gram-negative bacterial infections (like *C. pneumonia*) may be a targetable pathway for dementia-prevention therapeutics. Therefore, I suggest prophylactic vaccination against *C. pneumonia* as a potentially viable vaccine target to prevent (or minimize risk of) dementia.

While *C. pneumoniae* vaccine research is ongoing, there are currently no approved vaccines to prevent human infection. The major outer membrane protein (MOMP) is a primary external marker of the *Chlamydiae* family and is immunogenic during *in vivo* infection with *C. pneumoniae*[23]. However, successful clearance of *Chlamydiae* infection requires both antibody- and cell-mediated (i.e., T cell, macrophage, cytokine responses) adaptive immune function[24], which makes vaccine development much more difficult as a "single epitope [antigen]" like MOMP does not provide sufficient protection. To address this, vaccine research has turned towards structural and computational biology to identify multiple antigens that can stimulate a robust, long-lasting immune response. Instead of using the bacterium itself to identify additional epitopes, research has embraced reverse vaccinology that uses pathogen genome information and pathogen-associated membrane proteins (like MOMP) to identify potential target epitopes that are recognized by B cells, and cytotoxic and helper T lymphocytes[25-27]. Admittedly, these designs are often generated computationally and will require extensive *in vitro* and *in vivo* testing to determine safety and functional efficacy, but these new methods represent hope on the horizon for *C. pneumoniae* vaccination and, perhaps, even a reduction in pathogen-related dementias.

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1. SUPPLEMENTARY DATA—PROTECTIVE EFFECTS OF APIGENIN

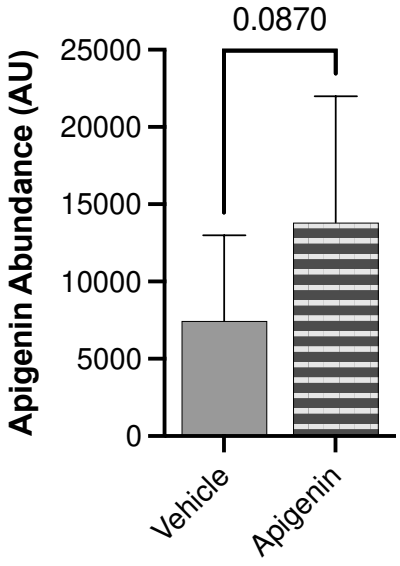

Figure S3.1. Apigenin accumulates in the brain of treated animals. Relative abundance of apigenin in cortical tissue from vehicle-treated controls (n = 5) and apigenin-treated mice (n = 6).

Supplementary table 3.1. Complete GO pathway names and significance for Old vs. young controls.						
source	**term name**	**term id**	**adj. p value**	**term size**	**query size**	**intersection**
KEGG	Complement and coagulation cascades	KEGG:04610	3.4178E-05	92	57	8
KEGG	Antigen processing and presentation	KEGG:04612	0.00027389	82	158	10
KEGG	Phagosome	KEGG:04145	0.00126563	173	158	13
KEGG	Pertussis	KEGG:05133	0.00711449	77	85	6
KEGG	Staphylococcus aureus infection	KEGG:05150	0.00713058	120	57	6
KEGG	Epstein-Barr virus infection	KEGG:05169	0.00886345	223	158	13
REAC	Antigen Presentation: Folding, assembly and peptide loading of class I MHC	REAC:R-MMU-983170	0.00082548	44	145	8
REAC	ER-Phagosome pathway	REAC:R-MMU-1236974	0.00131228	36	145	7
REAC	DAP12 signaling	REAC:R-MMU-2424491	0.00449143	55	46	5
REAC	DAP12 interactions	REAC:R-MMU-2172127	0.00593648	67	46	5
REAC	Neutrophil degranulation	REAC:R-MMU-6798695	0.00593648	492	74	15
REAC	Antigen processing-Cross presentation	REAC:R-MMU-1236975	0.00593648	92	110	8
REAC	Innate Immune System	REAC:R-MMU-168249	0.01115391	981	74	21
REAC	Endosomal/Vacuolar pathway	REAC:R-MMU-1236977	0.01115391	28	25	3
REAC	Activation of C3 and C5	REAC:R-MMU-174577	0.01553328	6	24	2
REAC	Immune System	REAC:R-MMU-168256	0.03268262	1604	92	32

Supplementary table 3.2. Complete GO pathway names and significance for Old apigenin-treated vs. old controls.						
source	**term name**	**term id**	**adj. p value**	**term size**	**query size**	**intersection**
KEGG	RIG-I-like receptor signaling pathway	KEGG:04622	6.1904E-05	70	17	3
KEGG	Hepatitis B	KEGG:05161	0.00251352	163	4	2
KEGG	Viral carcinogenesis	KEGG:05203	0.00462169	221	4	2
KEGG	Epstein-Barr virus infection	KEGG:05169	0.01170326	223	6	2
KEGG	Hepatitis C	KEGG:05160	0.01192795	165	8	2
KEGG	NOD-like receptor signaling pathway	KEGG:04621	0.04120144	205	50	3
REAC	Vasopressin-like receptors	REAC:R-MMU-388479	0.0025358	6	52	2
REAC	TRAF6 mediated IRF7 activation in TLR7/8 or 9 signaling	REAC:R-MMU-975110	0.00725176	1	2	1
REAC	DDX58/IFIH1-mediated induction of interferon-alpha/beta	REAC:R-MMU-168928	0.00804031	31	17	2
REAC	TICAM1-dependent activation of IRF3/IRF7	REAC:R-MMU-9013973	0.01450338	2	2	1
REAC	TRAF6 mediated IRF7 activation	REAC:R-MMU-933541	0.02175488	3	2	1
REAC	TRAF3-dependent IRF activation pathway	REAC:R-MMU-918233	0.02175488	3	2	1
REAC	DEx/H-box helicases activate type I IFN and inflammatory cytokines production	REAC:R-MMU-3134963	0.04350862	6	2	1

2. SUPPLEMENTARY DATA—EPIGENETICALLY DYSREGULATED TRANSPOSABLE ELEMENTS

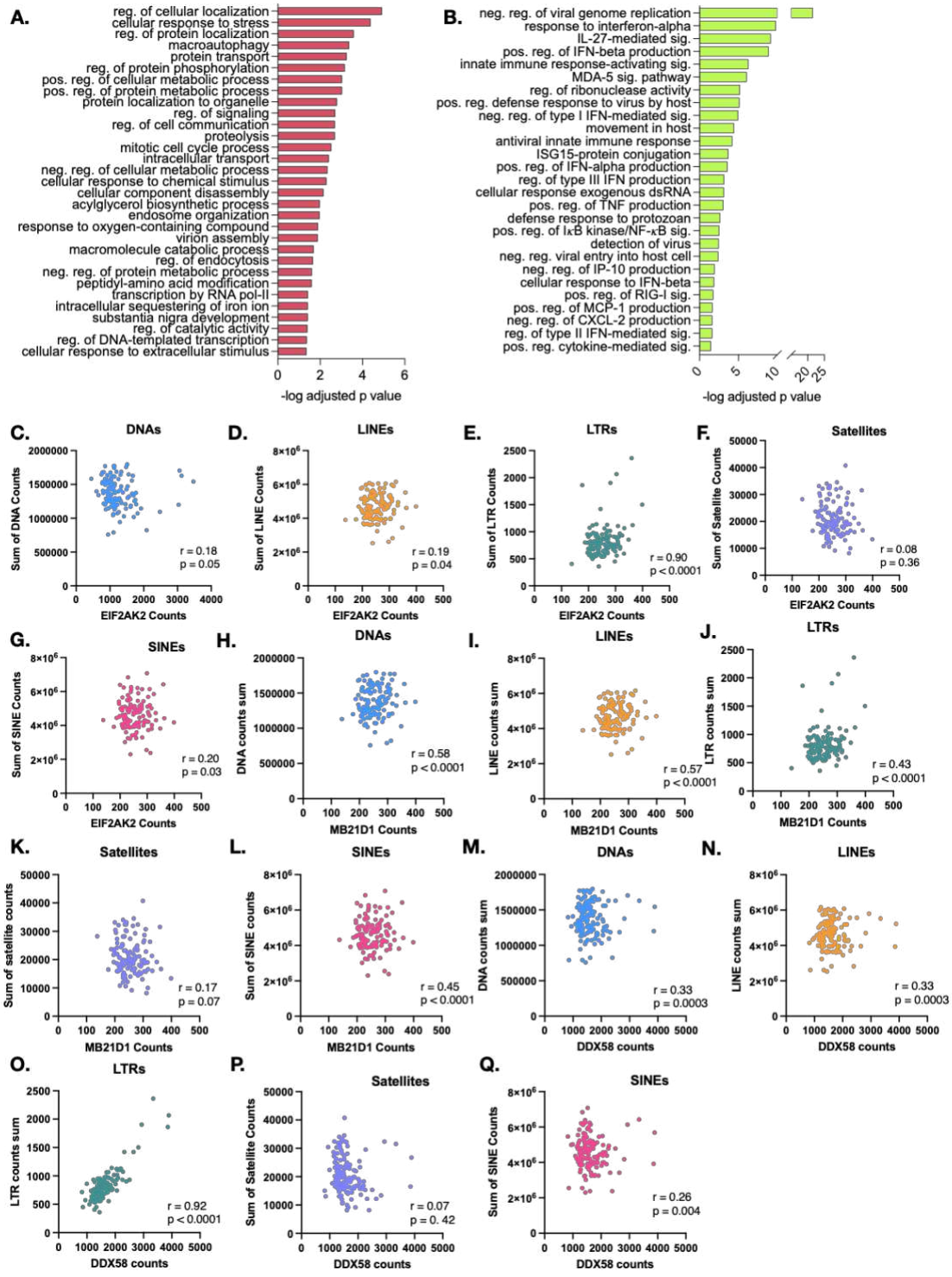

Figure S4.1. TE transcripts are related to multiple antiviral sensors, including those in modules that were associated with inflammation and reduced cognitive function. A) Top biological processes from gene ontology of genes/transcripts in module A; **B)** Top

biological processes for genes/transcripts in module J. **C-G)** correlations of EIF2AK2 (PKR) and summed TE transcript counts by class; **I-L)** correlations between TE transcripts by class and MB21D1 (cGAS); and **M-Q)** correlation of different TE transcript classes and DDX58 (RIG1).

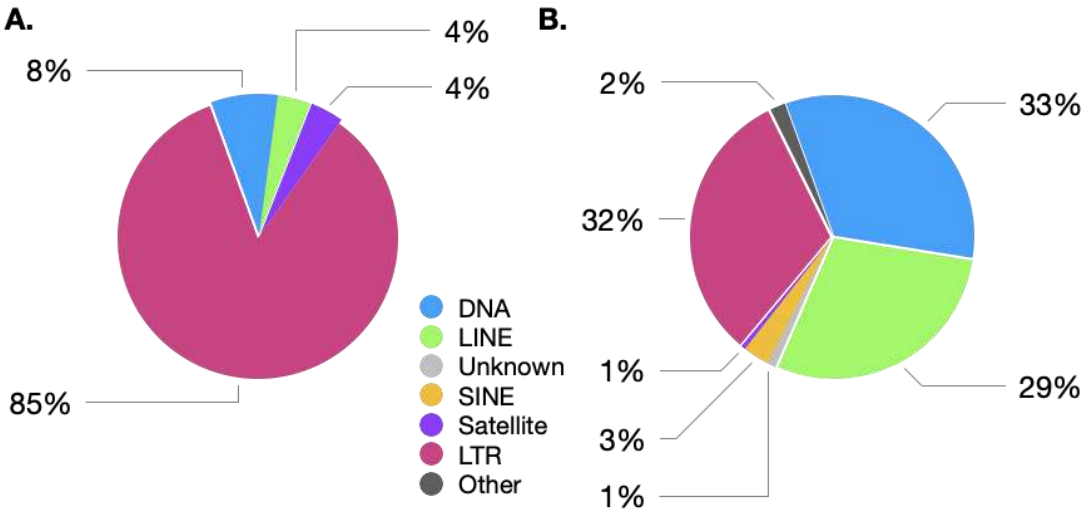

Figure S4.2. Composition of age and cognitive decline-related enriched TE transcripts that can be traced to hypomethylated genome regions. A) TE transcripts common across all 4 comparisons (n=26); **B)** TE transcripts that are associated with AD, but not MCI or normal aging (n=396).

Supplementary table 4.1. Potentially hypomethylated TE transcripts associated with aging and cognitive impairment, but not AD.

Individual TE	TE Class
HERVFH19-int	LTR
LTR06	LTR
LTR25	LTR
UCON69	DNA

Supplementary table 4.2. Potentially hypomethylated TE transcripts associated with age but not cognitive decline.	
Individual TE	**TE Class**
MER125	DNA
Charlie6	DNA
Eulor5B	DNA?
LTR86C	LTR
LTR19B	LTR
LTR68	LTR
LTR77	LTR
PABL_B-int	LTR
MER76-int	LTR
LTR53-int	LTR
SVA_C	Retroposon
ACRO1	Satellite
MSR1	Satellite
UCON25	Unknown

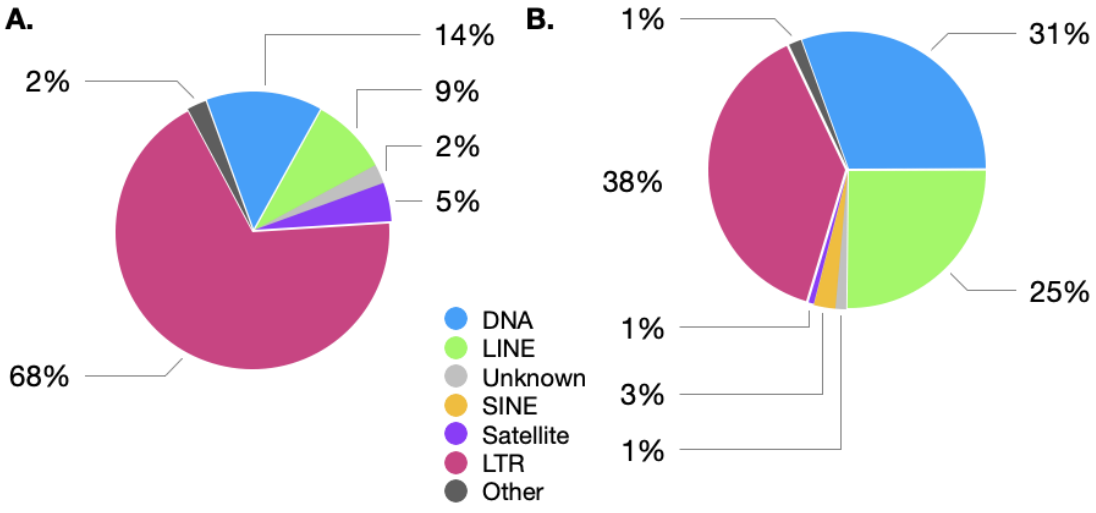

Figure S4.3. Composition of age and cognitive decline-related, enriched TE transcripts that can be traced to open chromatin regions of the genome. A) TE transcripts common across all 4 comparisons (n=44); **B)** TE transcripts that are associated with AD, but not MCI or normal aging (n=472).

Supplementary table 4.3. Potentially chromatin-accessible TE transcripts associated with aging and cognitive impairment, but not AD.

Individual TE	TE Class
CR1-L3A_Croc	LINE
HERVFH19-int	LTR
LTR25	LTR
LTR69	LTR
LTR70	LTR
MER92C	LTR
Ricksha_b	DNA

Supplementary table 4.4. Potentially chromatin-accessible TE transcripts associated with age but not cognitive decline.

Individual TE	TE Class
(CATTC)n	Satellite
HERV-Fc1_LTR3	LTR
HERV-Fc2-int	LTR
HERV1_LTRa	LTR
LTR108d_Mam	LTR
LTR19B	LTR
LTR22A	LTR
LTR77	LTR
MER92A	LTR
MLT-int	LTR
MSR1	Satellite
PABL_A-int	LTR
SAR	Satellite
SVA_C	Retroposon
UCON46	Unknown

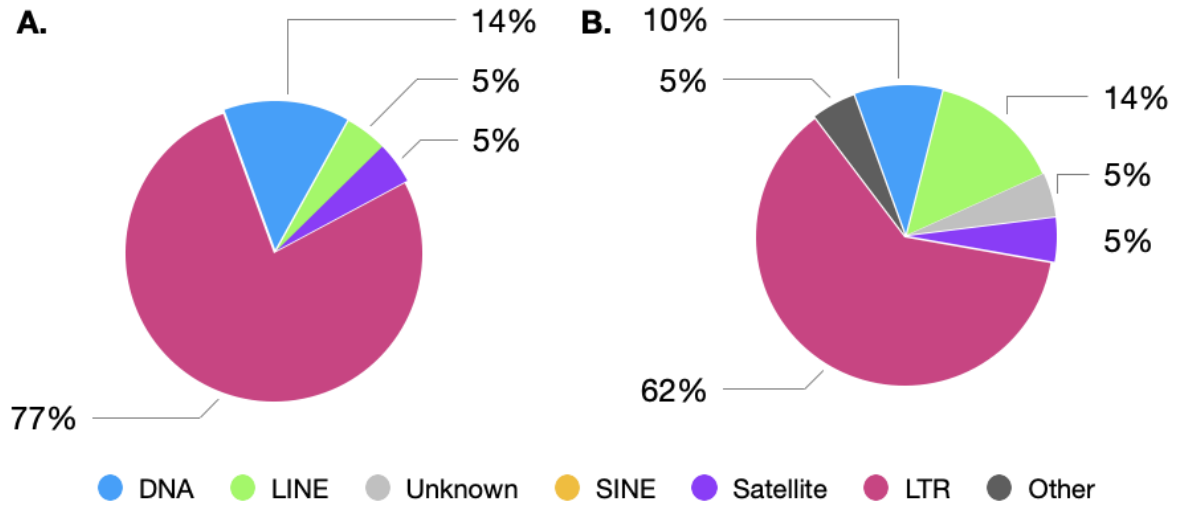

Figure S4.4. Composition of age and disease-related, enriched TE transcripts. A) TE transcripts common across all ages and disease development in both ATAC-seq and WGBS data (n=22); **B)** TE transcripts that are enriched in RNA-seq data and transcriptionally accessible but can only be traced to open chromatin regions (n=22).

Supplementary table 4.5. Epigenetically dysregulated TE transcripts that are associated with age, cognitive decline, and AD development.

Individual TE	TE Class
Arthur1C	DNA
HERV3-int	LTR
HERV9N-int	LTR
HERVK9-int	LTR
L1HS	LINE
LSAU	Satellite
LTR101_Mam	LTR
LTR10B	LTR
LTR10C	LTR
LTR14	LTR
LTR24B	LTR
LTR43	LTR
LTR51	LTR
LTR5_Hs	LTR
MER4CL34	LTR
MER4D	LTR
MER51C	LTR
MER61F	LTR
MER81	DNA
MLT1E	LTR
PABL_B	LTR
Tigger3	DNA

Supplementary table 4.6. Elevated TE transcripts that can be traced only to open chromatin regions and are associated with age, cognitive decline, and AD development.

Individual TE	TE Class
7SK	RNA
ACRO1	Satellite
LTR57	LTR
LTR60	LTR
HERV16-int	LTR
LTR68	LTR
MER107	DNA
HERV4_I-int	LTR
MER30B	DNA
HERVIP10B3-int	LTR
MER57C2	LTR
L1M2c	LINE
L1P3b	LINE
MER83B	LTR
MER87B	LTR
MER9a3	LTR
LTR1C	LTR
PRIMA4-int	LTR
Tigger6b	DNA
LTR44	LTR
UCON66	Unknown
X7C_LINE	LINE

Supplementary table 4.7. Elevated TE transcripts that can be traced only to hypomethylated regions of the genome and are associated with age, cognitive decline, and AD development.

Individual TE	TE Class
LTR61	LTR
LTR70	LTR
MER57E3	LTR
MER92C	LTR

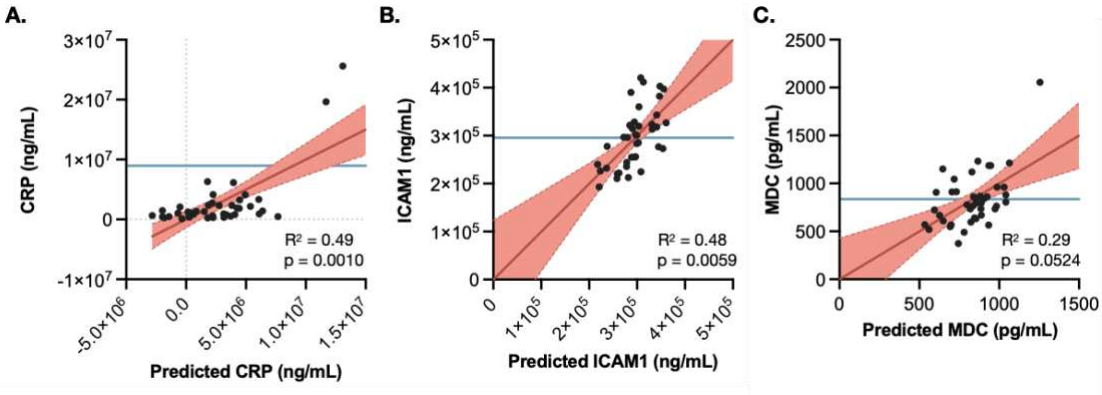

Figure S4.5. Epigenetically dysregulated TE transcripts that increase with age and AD are related to circulating markers associated with disease. A-C) Prediction regressions for serum markers of inflammation including **A)** C reactive protein (CRP), **B)** intercellular adhesion molecule 1 (ICAM1), and **C)** macrophage-derived chemokine (MDC).