AN EPIGENETIC APPROACH TO UNDERSTANDING SEX DIFFERENCES IN AGING

by
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Although aging impacts everyone, individuals vary in pace and severity of age-related decline. Many long-lived primates, including humans, exhibit marked variation in aging patterns between males and females. We know that environment can influence the aging process, but it remains unknown how the environment shapes aging at the molecular level. The epigenome, responsive to biological and environmental changes, presents a unique opportunity to explore mechanisms that may influence aging. To better understand sex differences in the aging epigenome in the hippocampus and liver, two tissues responsive to age-related change, we characterized differential DNA methylation due to age in unmatched banked hippocampus (N=88; females=57) and liver (N=94; females=58) samples from rhesus macaques across the lifespan. We found the majority of age-associated sites are indeed sex-specific; only 3% of age-associated sites are shared between sexes in the hippocampus and 21% of age-associated sites are shared in the liver. We found that differentially methylated sites (regardless of sex or tissue type) overwhelmingly become hypomethylated with increasing age, which is consistent with the

genomic hypomethylation hypothesis of aging. Ultimately, characterizing sex differences in how the epigenome changes with age across tissues will help identify how environmental factors interact with molecular mechanisms to shape variation in the rate of aging in long-lived primates.

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Introduction

No person is immune to aging, the gradual accumulation of wear and tear on our bodies that inevitably culminates in disease, degeneration, and death. It follows as no surprise that human civilizations have fantasized about slowing aging and evading death across centuries and cultures. In the Epic of Gilgamesh, a poem regarded as the oldest surviving piece of literature, the protagonist yearns for physical immortality. From the foundation of youth to anti-wrinkle treatments, the desire to evade aging continues to entice us. This is for good reason; aging is a risk factor that underlies many chronic conditions, including cancer, cardiovascular disease, and neurodegenerative disorders (Niccoli and Partridge, 2012). The push to understand patterns of normal aging and deviations from it will become increasingly relevant as the aging population grows. According to the CDC, the number of Americans that are 65 and older is projected to reach 98 million by 2040. With this influx of aging patients comes an increased burden on the healthcare system and individual families and their elderly loved ones. Identifying molecular targets will be important to implement relevant medical interventions and public health infrastructure.

Although aging impacts everyone, individuals vary in pace and severity of age-related decline. Notably, there is marked variation in how aging presents between men and women. There are widely documented sex differences in incidence, prevalence, age at onset and severity of symptoms in age-related disease (Menger et al. 2010), however the mechanisms that underlie these differences remains unclear. One lens which has the potential to offer insight into the sex gap in aging is epigenetic changes. A better understanding of epigenetic differences in aging between sexes presents a promising avenue to explore the mechanisms that may contribute to variation in aging and longevity.

Sex Gap in Aging

Women have been recorded to live longer than men across countries, generations, and cultures (Sampathkumar et al., 2020). Paradoxically, although women live longer, they exhibit a higher likelihood of age-related degenerative diseases than men (Fischer and Riddle, 2017). There are also widely documented sex differences in incidence, prevalence, age at onset and severity of symptoms in autoimmune, cardiovascular, and cancerous diseases (Menger et al. 2010). Even after adjusting for survival, women are reported to suffer from higher rates of chronic lower respiratory diseases, Alzheimer's, influenza, and pneumonia (Heron 2016). However, men suffer disproportionally from cardiovascular conditions, liver disease, and Parkinson's (Ostan et al. 2016). The magnitude of differences is variable and is shared by complex interactions between biological and environmental factors, including social and cultural forces in humans. The survival gap likely reflects differences in gender roles and expectations; men tend to engage in riskier behaviors and more dangerous occupations, and are less likely to seek medical help, leading to delayed diagnoses and treatment (Oksuzyan et al. 2008; Luy and Wegner-Siegmundt 2015). The general pattern that females are longer-lived than males has been observed across human history and in most mammals, including primates (Austad and Fischer 2016; Bronikowski et al. 2022; Lemaître et al. 2020), suggesting the importance of underlying biology.

Why is there a sex gap in aging?

There are often five mechanistic theories attributed to sexual dimorphism in aging and longevity: a stronger immune response in females, the protective effect of estrogen, reduced hormone activity, the impact of a second X chromosome, and the influence of oxidative stress (Fischer and Riddle 2017). The two best described biological explanations for sex differences in aging trajectories are sex-chromosomal linked mechanisms and hormonal mechanisms (Hägg and Jylhävä 2021). Although not the focus of this project, sex differences in aging trajectories may be driven by mechanisms involving the sex chromosomes. Because mammalian females usually carry two X chromosomes, dosage compensation mechanisms evolved, leading to random X-chromosome inactivation, which represses an X chromosome early in development. Disease is thought to arise from dosage imbalance from X chromosome inactivation and consequently hormone imbalance downstream (Skuse et al. 2018).

Hormonal differences may also be driven by mechanisms outside of the sex chromosomes. The most common groups of sex steroids are androgens (testosterone), which are mostly present in men, estrogen (estradiol, estrone, and estriol), and progestogens highly abundant in women. Estrogen is thought to be protective against a wide variety of diseases, whereas testosterone seems to enhance the risk of disease progression (Clocchiatti et al. 2016; Ostan et al. 2016). Consistently, the risks of hypertension and developing Alzheimer's disease, two major causes of death in females, are inversely correlated with estrogen production (Ostan et al. 2016; Pike 2017).

Differentiating Between Sex and Gender

This project focuses on sex differences rather than gender differences in aging, but it is important to note that both sex and gender contribute to disparate health outcomes. At the most

basic biological level, sex is defined by gamete size, while gender relates to the conceptions societally determined to belong to a sex category. However, this distinction is nuanced, and the terms are often interwoven. Biological sex is not a binary; even sex hormones and other biological markers associated with sex exist on a spectrum (Ritz and Greaves 2022). Statistically significant male-female comparisons are worthy to note, but do not necessarily mean that males and females function in fundamentally different ways. Understanding, accounting for, and addressing the interplay of sex and gender disparities in health is arguably one of the most interesting and important challenges in contemporary research.

Presently, there is a relatively limited amount of information on how biological aging presents differently between sexes. This may be attributed to a long tradition of clinical trials that excluded women in sampling due to hormonal fluctuations and pregnancy regarded as confounding variables. In many instances, female bodies were assumed to operate in the same ways as male bodies, and findings from research conducted exclusively in men were often generalized to women (Hägg and Jylhävä 2021). Consequently, male-biased samples have informed the diagnosis, treatment, and prevention of chronic disease and lead to disparate quality of care and mortality outcomes between the sexes (Mauvais-Jarvis et al., 2020; Ritz and Greaves, 2022). For example, for many decades, research on cardiovascular disease was largely male-biased, resulting in risk calculations and clinical presentations that do not apply to women (Schenck-Gustafsson, 2009), who often present with different symptoms than men. Thus, a better understanding of the biological mechanisms that may drive differences in aging is crucial to effectively tackle variation age-related decline and diseases through an individualized approach.

Why do we age?

To broach questions of variation in human aging, we must understand why aging occurs in the first place. From an evolutionary lens, aging doesn't seem to make sense. Darwin's theory of evolution by natural selection is premised on the idea that evolution acts to increase the fitness of species over time. Why would our bodies be shaped to give out and become increasingly vulnerable to disease with age? How do biological mechanisms drive human aging trajectories and age-related decline? We can explore why aging occurs from both an ultimate (evolutionary theories for why we age) and proximate (mechanistic theories for how we age) perspective.

Evolutionary Explanations for Aging

Most explanations revolve around the notion that natural selection favors reproductive success, not longevity. Past reproductive age, selection pressure weakens. As a result, alleles that impair health in later life are more likely to escape selection and accumulate in populations over the course of evolution, resulting in aging. (Medawar, 1952). This explanation has been widely acknowledged for many years, but the underlying evolutionary mechanism remains contested.

Proposed by Sir Peter Medawar in 1952, the mutation accumulation theory suggests that aging may result from harmful mutations that do not present until later in life, because natural selection fails to eliminate them. This ties into the notion of the selection shadow, a "shadow" of time in which selective pressure becomes less strong. According to this theory, aging arises from a multiplicity of mutations that accrue over successive generations.

The antagonistic pleiotropy model goes one step further and postulates that late-acting deleterious mutations are actively selected for, and do not just accumulate with chance. It rests on two assumptions: 1) a particular gene may influence several traits and 2) combined effects of genes may have opposite impacts on fitness. Aging is thus the genetic trade-off between early

life fitness and late-life mortality. The mutation accumulation theory and antagonistic pleiotropy model both fit the conventional paradigm is that aging is an inevitable byproduct of the evolutionary process. However, this countered by species that do not experience aging or senescence at all (Klimovich et al. 2018). Increasingly, the factors that drive aging and the variation in the shape and pace of the aging trajectory have been revealed to be dynamic and multifarious.

Mechanistic Explanations for Variation in Aging

At the proximate level, several evolutionarily conserved molecular and physiological mechanisms likely underlie the aging process and aging phenotypes.

A general "wear and tear theory" suggests that DNA damage accumulates, and DNA repair mechanisms become less efficient over time. Aging can be assessed in various ways; the nine "hallmarks of aging" include genomic instability, telomeric alteration, epigenetic alterations, mitochondrial dysfunction, the loss of proteosasis, dysregulated nutrient sensing, stem cell exhaustion and altered cellular communication (López-Otín et al. 2013). These be classified into three categories: primary, antagonistic, or integrative. The primary hallmarks are defined as key factors causing cellular damage including genomic instability, telomere attrition, loss of proteostasis, and epigenetic alterations (López-Otín et al. 2013). During aging, there is a continuous increase of epigenetic changes, which might give rise to multiple age-related pathologies (Salameh et al. 2020).

What is Epigenetics?

In recent years, an epigenetics lens has been adopted to study aging via the interaction between our genes and the environment. Epigenetics, stemming from the word "epi", meaning above, refers to the study of phenomena that cause changes to gene expression that are not

dependent on changes to the underlying DNA sequence. Unlike changes to the sequence of DNA, epigenetic changes can be responsive and reversible. A useful framing to understand this is to consider DNA as a book of life, a popular metaphor utilized by high school Biology teachers. DNA methylation, the most well-known epigenetic mechanism, involves the removal and addition of methyl groups to specific sites within the genome. These methyl groups function as punctuation marks, altering how the DNA is read. Changes in DNA methylation do not alter the sequence of DNA itself, as mutations do, but rather, amplify or quiet down how DNA is read. Through this process, gene activity can be regulated, and expressed in varying degrees.

Epigenetics influences gene expression through chemical modifications of DNA or through the chromatin proteins that bind and interact with DNA. The most recognizable and well-researched forms of epigenetic modification are DNA methylation and histone modification. Usually, DNA methylation refers to the addition of a methyl (-CH3) group to the cytosine nucleotide of DNA (Schubeler, 2015). Methylation occurs at cytosine-guanine (CpG) dinucleotides and locations of methylation are often referred to as CpG sites. Differentially methylated refers to sites that undergo changes in methylation status. In this project, I will be referring to differentially methylated CpG sites that change with age.

As a result of methylation, additional proteins may bind the region and cause the DNA to be so tightly packed that transcription cannot occur. This is known as “silencing” and is most common in promoter regions of the genome. Promoters, the best-characterized region in which DNA methylation occurs, denote where genes start and regulate the degree to which a particular gene is “turned on” or “turned off.” In general, in promoter regions, hypermethylation, a gain of methylation, is associated with gene silencing and hypomethylation, a loss of methylation, is associated with gene activation---although there are exceptions to this trend (Fernandez et al.,

2012). The end point of both DNA methylation processes usually results in long-term silencing or fine-tuning of gene expression in development and aging (Menger et al. 2010). However, methylation can have a variety of effects depending upon the genomic context in which it is found. DNA methylation can occur in both gene bodies and non-coding stretches of the genome, including in promoters, introns, exons, and intergenic regions.

Epigenetic mechanisms can respond dynamically to environmental factors and behaviors. One of the first groundbreaking studies to explore the role of epigenetic mechanisms found that the amount of licking and grooming provided by a mother rat could produce differences in DNA methylation in her offspring that would persist into adulthood (Meaney and Szyf, 2005). Pups reared by low-licking mothers altered their stress responses of the rats for the rest of their lives. DNA methylation represents a unique opportunity to explore how behavior and environment "get under the skin" to influence health outcomes. Beginning to unravel the sources of variation our genomes may one day reveal ways in which they can be reversed. Pointed research may also uncover critical windows in development and aging and the relative influence of environmental factors.

Inferring Biological Age

Although small changes in CpG sites accrue with age, patterns of methylation with age are largely conserved across individuals. As a result, scientists can use DNA methylation data to infer biological age. Biological age, unlike chronological age, can account for interindividual variation in age-related changes and therefore provides a more comprehensive picture of health and aging. DNA methylation changes emerge early in life and variation in methylation accumulates with age, making it the gold standard biomarker of aging. In 2013, Horvath and

Hannum and colleagues independently published separate versions of the epigenetic clock model, which relies on DNA methylation data to quantify biological age.

The epigenetic clock supposes the existence of conserved epigenetic changes that occur during aging and can infer chronological age to a high degree of precision (Horvath 2013; Hannum et al. 2013). Simultaneously, inter-individual variation accrues with age. Since the clock model was developed, epigenetic clocks have proven to be accurate predictors of chronological age in numerous species including humans (Perna et al. 2016; Marioni et al. 2015), mice (Thompson et al. 2018), and non-human primates (Anderson et al. 2021 for baboons, Goldman et al. 2022 for rhesus macaques). These clocks appear to be sensitive to a number of physiological, psychological, and environmental factors and capture variation in age-related decline. Accelerated epigenetic aging is associated with an increased vulnerability to mortality and age-related disease (Chen et al. 2016; Zheng et al. 2016). DNA methylation, reflective of genetic mechanisms and environmental exposures, provides an avenue to explore the combined effect of underlying sex differences and social and cultural disparities.

Sex Differences in Epigenetic Signatures

A challenge of epigenetics research is that we know surprising little about sex-specific differences in aging. In many studies investigating the aging epigenome, male/female datasets are pooled together, and sex differences remain unexplored. Sex is regarded as confounding variable, rather than an area of interest. Although there are several methylation patterns that seem to be consistent with aging, there is no clear consensus in patterns regarding aging and the epigenome. The data from human DNA methylation studies suggest that alterations to the epigenome occur at a slower pace in females than in males; this gain/loss may occur at a greater rate in males than in females in some tissues, suggesting that this difference might contribute to

the different aging pattern seen in men and women (Austad and Fischer 2018). Previous studies in human cohorts using Hannum and Horvath's epigenetic clocks report sex differences in biological aging that appear in adolescence (Simpkin et al. 2017) and increase with age (Kankaanpää et al. 2022). Human and non-human primate studies also have found that epigenetic aging may be accelerated in males (Horvath et al. 2016, Klein et al. 2019, Anderson et al. 2021). Genome-wide hypomethylation (loss of DNA methylation) in the autosomes as a hallmark of aging has been observed across multiple studies in humans (Marttila et al. 2015; Fernández et al. 2015; Li et al. 2017), but how this pattern tracks across sexes and tissues remains unclear. Many studies note that the role of the epigenome in shaping variation in aging between the sexes remains understudied and underscore its importance in future studies (Fischer et al., 2018; Deegan et al., 2019; Sampathkumar et al., 2020).

Relevance of Macaque Model

Developing relevant biomedical models of human aging has been a central focus of gerontology research for decades. Conventional organisms like yeast, worms, flies, mice have provided valuable insights but are limited by their distant evolutionary relatedness from humans (Chiou et al. 2020). Non-human primates are an intuitive alternative for their comparable anatomical, physiological, and life-history traits. Humans, like other primates, are characterized by life histories of reduced reproductive effort. This entails relatively late onset of reproduction, long lifespans, and low fertility (Jones 2011). Chimpanzees, our genetically closest primate relative, are subject to strict ethical regulation and been phased out from biomedical research in last decade (Colman 2018). Ongoing studies in wild chimpanzee populations offer valuable insight into how aging and disease are shaped by social and ecological factors (Emery Thompson et al. 2020). In a biomedical setting, high costs of care, extremely long lifespans, and the

endangered status of virtually all other great ape species inhibit their use as alternatives to chimpanzees (Chiou et al. 2020).

Given these challenges, rhesus macaques have become the most widely used primate in biomedical research and have been used to construct major resources including high-quality genome assemblies. Rhesus macaques share 93% sequence similarity with humans (Gibbs et al. 2007), and this similarity extends to numerous aspects of anatomy, physiology, neurology, endocrinology, immunology, and behavior. The rhesus macaque lifespan is approximately 3 times shorter than that of humans, yet aging macaques recapitulate the human aging process and development of age-related disease (Roth et al. 2004). Externally, rhesus macaques share many hallmarks of aging with those exhibited in humans. Thinned, wrinkled skin on the face, hunched posture, and loss of muscle mass and overall frailty are characteristic in elderly rhesus macaques, mirroring the aging phenotypes that we see in humans (Simmons, 2016). Rhesus macaques live approximately 25 years in captivity, although they may live up to 40 years (Chiou et al. 2020). One macaque year of life is roughly equivalent to three human years; a 10-year-old rhesus macaque is about 30-years in human equivalence (Colman 2018).

Rhesus macaques also recapitulate sex differences in development and aging that make them useful proxies to disentangle sex variation in lifespan and healthspan. They display similar patterns of puberty, reproductive senescence, and menopause (Chiou et al. 2020). As in human females, wild female macaques demonstrate a slight survival advantage over their male counterparts (Kessler et al. 2015). As a result of shared aging trajectories, comparable sex patterns with age between humans and macaques may also present in the epigenome. An examination of aging signatures in blood revealed age-related DNA methylation changes are

shared between rhesus macaques and humans demonstrated a strong conservation of the aging trajectory between humans and macaques (Chiou et al. 2020).

Rhesus macaque models also offer the potential to study aging in tissue types apart from blood, an opportunity restricted in humans due to ethical and logistical considerations. Many age-related methylation sites are conserved across different regions of the body, but there is distinct variation in the rate of aging between tissues, organs, and even sub-regions within organs (Seale et al. 2022). Epigenetic mechanisms have been implicated in producing differences that are responsible for the aging process of different tissues (Pagiatakis et al. 2021), but heterogeneity in aging across tissues remains underexplored. Rhesus macaques strike the balance between utility and applicability and serve as an ideal model for studying aging in the epigenome.

Utility of a Two-Tissue Model

Whole-blood samples are an accessible, established source for methylation-based predictors of biological age (Goldstein et al. 2021), but a more comprehensive picture of aging could be provided by looking at other tissue types. A growing body of studies suggests that aging is tissue-specific and occurs at distinct rates across organs and tissues (Levine et al. 2015). While blood is a sound choice for systemic assessments of health, certain tissues are better for targeted explorations. Epigenetic mechanisms have been implicated in producing differences that are responsible for the aging process of different tissues (Pagiatakis et al. 2021), but a model of epigenetic sex differences from a multi-tissue perspective remains underdeveloped.

Hippocampus and liver tissue samples rhesus macaques offer a unique opportunity to explore how the aging epigenome may differ between sexes. The liver is an established indicator of metabolic function and whole-body health (Hahn et al. 2018) and may exhibit clinical signs of

dysfunction earlier than other tissues (Goldman et al. 2022). A recent study identified sex differences in molecular mechanisms of aging in the liver, specifically in cell cycle and cell senescence pathways that contribute to the development of aging-induced liver diseases (Lomas-Soria et al. 2021). The hippocampus is useful to explore aging in the context of neurodegenerative disorders and aberrant brain-related pathology that accrues with age (O'Shea et al. 2016), a pattern that is also present in long-lived primates (Freire-Cobo et al. 2021). Hippocampus samples display sexually divergent methylation patterns in mice (Masser et al. 2017) and humans (Choleris et al. 2017), but methylation patterns with age have yet to be characterized in a long-lived primate. Elucidating mechanisms that contribute to sex differences in a tissue-specific manner can reveal subtle, yet critical changes underlying age-related pathologies.

Project Description

This project seeks to better understand sex differences in aging, using DNA methylation data collected from male and female rhesus macaques across their lifespans and across two tissue types. This will make a novel contribution to the epigenetics and gerontology fields, which lack a comprehensive picture of the landscape of differential methylation between the sexes with age. As aging does not occur uniformly through the body, looking at different tissues is necessary to characterize sex-associated methylation differences with aging. Assessing data from rhesus macaques will be a useful proxy for humans to understand normal aging trajectories between the sexes.

Research Objectives and Hypotheses

Broadly, this research was guided by an interest in the molecular mechanisms that shape variation in aging and longevity and why there are differences in aging trajectories between sexes. Sex differences in the epigenome remain under investigated, leaving a gap in our understanding of the mechanisms that shape variation in aging. Within this specific project, I sought to explore whether different patterns of aging between males and females were evident in the epigenome. Using previously generated liver and hippocampus datasets from rhesus macaques, I used bioinformatics techniques to characterize patterns of DNA methylation with age, highlighting differences observed between male and female samples. These larger questions will be targeted through the following specific questions and objectives:

Are there detectable differences in the methylated sites that change with age between male and female datasets? **Objective One: Test** for differential methylation with age as a function of sex in two tissue types, the hippocampus and liver, that are implicated in aging and age-related disease and identify differentially methylated cytosines (DMCs). I hypothesize there

will be a difference in age-dependent methylation patterns between the sexes, with females experiencing a greater proportion of differential methylation with age.

Where are sites that change with age for each sex dataset located in terms of genomic features? **Objective Two: Annotate** sex-specific differentially methylated sites in terms of genomic features, e.g., whether a given site is in a promoter, exon, intron, 5' UTR, or 3' UTR. I hypothesize that a large proportion of differentially methylated sites will fall within promoter regions of the genome but that there will not be discernable difference between male and female datasets. That is, that genomic feature representation will not be variable between sexes.

What biological processes and diseases are associated with the sites that change with age? **Objective Three: Characterize** age-associated sites shared and specific to male and female datasets in both tissue types. Following established theories of sex differences in aging, I hypothesize that differentially enriched pathways are associated with immune response and hormone regulation. Additionally, given increasing evidence that the epigenome is tissue specific, I hypothesize that enriched pathways for differentially methylated sites will be different in the hippocampus and liver.

Methods

Sample Information

The datasets used in this project were previously generated as part of a larger study on epigenetics and aging by PI Sterner and colleagues. I inherited these data from the dissertation projects of Dr. Elisabeth Goldman, a recent PhD in the Sterner Lab, and Tanner Anderson, a current PhD student in the Sterner Lab. Banked liver (N=96) and hippocampus (N=96) samples came from a population of rhesus macaques (Macaca mulatta) housed at the Oregon National Primate Research Center (ONPRC). Importantly, individuals differed between the two datasets (i.e., unmatched). Individuals were selected to represent both sexes and timepoints across the lifespan to capture trends in "normal" aging **(Table 1**). No individuals were younger than three years of age, excluding an important developmental period leading up to puberty in rhesus macaques. We removed 2 individuals from the liver dataset (N=94) due to missing datapoints. We removed 6 individuals from the hippocampus dataset due to choroid plexus tissue contamination, identified from the expression of specific choroid plexus marker genes. After performing principal component analysis (PCA) to visualize the overall structure of the data, we removed 2 additional individuals from the hippocampus dataset (N=88) that were extreme outliers. For the hippocampus samples, females ranged in age from 3.42 - 35.05 years (mean = 17.3) whereas males ranged from 4.04 - 28.30 years (mean = 15.1 years). For the liver samples, females ranged in age from 3.3 to 32.01 years (mean = 14.96) and males ranged from 3.2 to 33.09 years (mean = 15.8 years). The ratio of females to males in both the brain and liver dataset was approximately 3:2.

	Brain	Liver
N (Females)	88 (57)	94 (58)

Ages	3.5 – 35 years	3.2 – 33 years
Human Age Equivalents	~9 – 105 years	~9 – 99 years

Table 1: Age and Sex Distribution of Datasets

RRBS Library Preparation and Sequencing

DNA was isolated and quantified from the hippocampus and liver tissue samples at the ONPRC Primate Genetics Core using standard approaches. CpG methylation was measured using reduced representation bisulfite sequencing (RRBS), which targets specific regions that are likely to be methylated to maximize efficiency and avoid sequencing the entire genome (Gu et al. 2011). RRBS requires a specialized library preparation. First, DNA is fragmented into smaller pieces by a restriction enzyme. Fragmented DNA ends are then repaired to generate blunt ends that can be bound by specific adaptor molecules that are necessary for amplification and sequencing. After the adaptor ligation, these fragments undergo bisulfite treatment, in which unmethylated cytosines are converted to uracil. This is a crucial step to differentiate between unmethylated and methylated sites in subsequent differential methylation analysis. Following bisulfite conversion, the DNA fragments are subjected to PCR amplification, in which a target DNA sequence of interest is duplicated many times over in order to selectively amplify a specific region. The resultant collection of gene fragments is referred to as an RRBS library. RRBS libraries were produced by the KCVI Epigenetics Consortium at Oregon Health and Sciences University (Carbone et al. 2019).

Liver libraries underwent quality control by the University of Oregon's Genomics and Cell Characterization Core Facility (GC3F) to assess their integrity and ensure they met required standards and sequenced on an Illumina HiSeq4000 with 75 base-pair read length. Hippocampus libraries were sequenced on a Novaseq S4 by OHSU's Massively Parallel Sequencing Shared Resource (MPSSR).

Data Processing, Alignment, and Filtering

Genomic DNA data was filtered and formatted by Dr. Elisabeth Goldman as part of her dissertation. Briefly, she used TrimGalore! to remove low-quality bases and FastQC and MultiQC as an additional quality check (Goldman et al. 2022). She then aligned trimmed reads to the rhesus macaque reference genome (Mmul10) after performing in silico bisulfite conversion of the reference genome using Bismark v0.19.0. Sites missing in more than 10% of samples were removed as well as all sites on sex chromosomes. Recent research suggests that normalizing methylation data with the sex chromosomes introduces a large technical bias to many autosomal CpGs (Wang et al. 2021). Twenty files, each corresponding to one autosome, were generated that included all the CpG sites located on a given chromosome and present in at least 90% of the samples. Sites were further filtered to remove low coverage regions (i.e., less than 10x median coverage) and exclude sites of constitutive methylation (i.e. where methylation does not vary across the lifespan, and thus the median is either below 10% or above 90%).

As part of this project, I received training in bioinformatics (mainly in the programming language R) and in how to use statistical tools to analyze and visualize genomic data. I performed all subsequent analyses in R version 4.2.3 unless otherwise stated. I received processed data in the form of methylation count and total count matrices corresponding to the same sites for each sample, along with metadata files for each respective tissue that included information about the sex and age of each individual along with other identifiers. We performed principal component analysis (PCA) to visualize the overall structure of the data and detect any outliers or clustering based on batch or group status. The data were loaded into RStudio and processed through a secondary analysis to ensure sites of constitutive methylation and sites missing in more than 10% of the samples were excluded. We also generated a relatedness matrix

for samples within each tissue that represents the degree of relationality between each individual as a decimal between 0 and 1, and controls for relatedness as a variable that may influence similarity of methylation profiles.

Differential Methylation Analyses

To accomplish Objective 1, we used PQLseq (version 1.2.1), an R package that is designed for differential analysis of large-scale RNA sequencing data or Bisulfite sequencing data (Sun et al. 2021). PQLseq fits a Generalized Linear Mixed Model (GLMM) to identify sites that are differentially methylated (DMCs) as a function of age. To investigate sex-associated differences in sites that changed with age, we split males and females into two distinct datasets for each tissue type (i.e., male liver, male hippocampus, female liver, female hippocampus). Input data for PQLseq was formatted into a model matrix that included methylation count matrices (n = 276,840 sites for the hippocampus and n = 154,263 sites for the liver), mean centered ages of each sample, and a relatedness matrix. Mean-centered ages are calculated by subtracting the mean from each individual age value in the dataset as a representation of deviation from the mean. Using mean-centered ages helps mitigate multicollinearity, the presence of high correlations between predictor variables. After these files were prepared, they were transferred to the Talapas supercomputer, UO's high performance cluster that performs operations much more quickly than a desktop computer. I used the PQLseq package on Talapas to run separate analyses for male and female datasets for each tissue type.

PQLseq identified sites that were differentially methylated with age and formatted this output as an. RData file that could be loaded into RStudio for further examination. I found that re-analyses of these input datasets consistently identified the same CpG sites that were differentially methylated with age, ensuring the replicability of the PQLseq output. Additionally,

to explore how both datasets may be influenced by the female-biased ratio, I created smaller female datasets to match the number of males for each analysis. These datasets were representative of the age range of the entire dataset and included all the sites included previously. For the liver, I created a partition of 36 females and for the brain, I created a partition of 30 females. To account for inter-individual variation, I created three different partitions for each tissue type comprised of the same number but different female individuals. I then ran these iterations through the same PQLseq analysis described previously.

The PQLseq output included CpG sites that were differentially methylated with age as well as statistical identifiers including p-value and beta-value. Probabilities (p-values) were corrected for multiple testing using the Benjamini-Hochberg method of False Discovery Rate (FDR), implemented using the package q-value (v 2.26.0) (Storey, 2003). An FDR threshold is used to control the proportion of falsely identified differentially methylated sites among all sites that are identified as significant. For example, an FDR threshold < 0.1 corresponds to a 10% or lower expected proportion of false discoveries among the significant findings. I used an FDR threshold of < 0.1 for the brain and FDR < 0.001 for the liver based on previously used parameters for these tissues.

We then characterized the direction of methylation change to determine whether sites lost methylation with age (i.e., became hypomethylated, corresponding to a negative beta value) or gained methylation with age (i.e. became hypermethylated, corresponding to positive beta value) **(Table 2).**

	Direction of Methylation	**Beta-value**
Loss of Methylation	Hypomethylation	Negative

Gain of Methylation	Hypermethylation	Positive

Table 2: Interpretation of beta-values to detect direction of methylation

To visualize overlap between significant differentially methylated sites in males versus females, I used the RStudio package "ggVennDiagram" to generate Venn diagrams that showcase the number sites that are shared between the sexes and the number of sites that differ. This allowed us a quantitative comparison of age-associated differential methylation in female vs male samples.

Characterization of Age-associated Sites

Genomic Feature Annotation

To gain more insight into the functional role of the significant age-associated sites identified in Objective 1, I identified which features they fell into within the genome (Objective 2). I exported the results of the PQLseq analysis (sites with an FDR < 0.1 for the hippocampus and FDR < 0.001 for the liver) as a .bed file that included the chromosome and position then used the package ChIPSeeker to visualize genomic feature representation. I retrieved annotations for these sites using "org.Mmu.eg.db", the genome wide annotation for rhesus macaques primarily based on mapping using Entrez Gene identifiers (Carlson et al. 2019). Using the "annotatePeak" function in ChIPSeeker with org.Mmu.eg.db" as the annotation package and (-3000, 3000) as the region range, I created bar chart representations of where sites fell in terms of genomic features, including exons, introns, 5'UTR, 3'UTR, promoters, and distal intergenic regions. Sites were grouped by dataset (i.e., sex and tissue; n=4) and further split by direction of methylation.

Gene Ontology Analysis

For Objective 3, I used the gProfiler web interface to conduct Gene Ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes) to test the differentially methylated sites for enrichment against a custom background of all genes associated with the CpG sites included in each tissue dataset (n = 276,840 sites for the hippocampus and n = 154,263 sites for the liver). Ontology analysis enables us to determine if any genetic terms or pathways were significantly overrepresented in the sites that were differentially methylated in each dataset. As input, I used the chromosome and position (e.g., chr1: 12347) of all the sites that were identified in Objective 1. I used all the sites that were differentially methylated in each sex dataset to detect if the same sites in both sexes had similar enrichment terms but opposite directions of methylation. gProfiler ties these sites to associated genes and leverages various databases to identify biological functions (ontologies) and their relationships to one other. GO has three major subontologies: Molecular Functions (MF), Biological Processes (BP), and Cellular Component (CC). It also employs statistical algorithms to assess the overrepresentation of specific functional categories, known as functional enrichment, compared to the background reference set. I used a Benjamin-Hochberg false discovery rate (FDR) < 0.1 to adjust for the multiple hypothesis testing inherent in enrichment analysis. gProfiler yields a list of enriched terms, associated pathways, or biological pathways along with statistical significance. It also generates visualizations such as network diagrams to visualize the enriched functional categories.

Results

To characterize sex differences in the aging epigenome, I first identified sites that were differentially methylated with age. Then, I found which sites were shared vs specific to each sex (Objective 1). From these sex-specific, age-associated sites, I also identified whether they lost or gained methylation with age. I found that **1) a large subset of age-associated sites is sex-specific in both the hippocampus and liver datasets** and **2) the majority of sites become hypomethylated with age.** Then, I identified where these sites were located in terms of genomic features (Objective 2). I found that **3) in both tissue types and sexes, age-associated sites were located within and between genes (**e.g., promoters, exons, introns, and distal intergenic regions). Finally, I determined if these sites were enriched for any GO terms or pathways (Objective 3). I found that **4) Age-associated sites were enriched for similar biological terms between sexes in the liver but different terms in the brain.**

No batch effects were present in either tissue dataset.

Prior to performing differential methylation analysis, I visualized the structure and clustering of methylation data for both tissue types using Principal Component Analysis (PCA). PCAs for both the hippocampus and liver did not display clustering that would indicate batch effects that would need to be corrected for prior to PQLseq analysis. The First Principal Component accounted for 20% of variation in the hippocampus methylation samples (**Figure 1**) and 43% of variation in the liver samples (**Figure 2**), but there was no evident clustering by age or sex in either tissue.

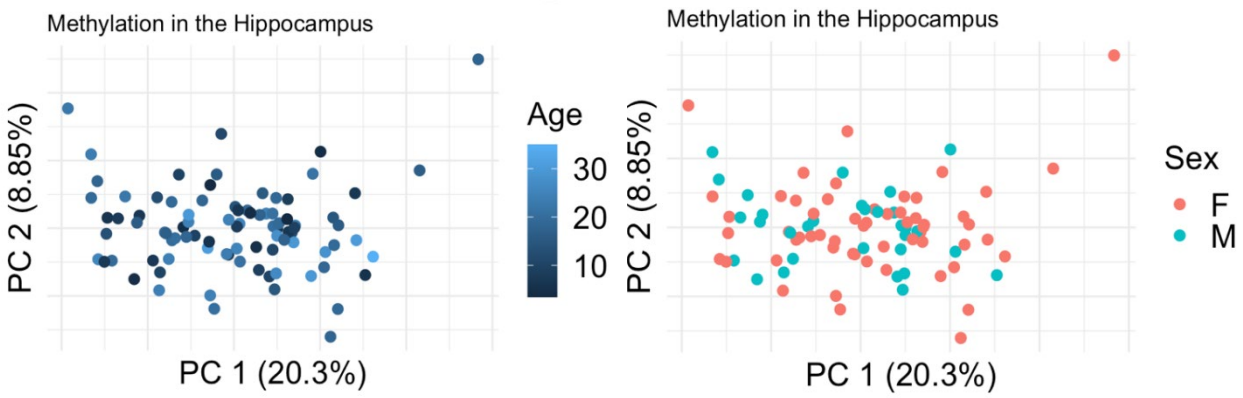


Figure 1: PCA of methylation counts in the hippocampus. Colored by age (left) and sex (right).

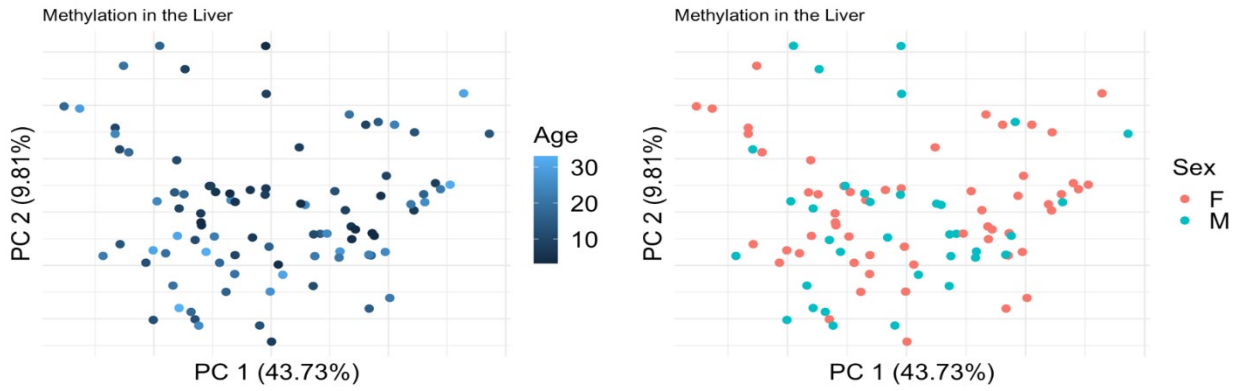


Figure 2: PCA of methylation counts in the liver. Colored by age (left) and sex (right).

A large subset of age-associated sites is sex-specific in both the hippocampus and liver datasets.

In the hippocampus, I identified 1,336 sites in the female dataset (n = 58) and 473 sites in the male dataset (n = 30) that became differentially methylated with age (FDR < 0.1). A greater proportion of sites become differentially methylated with age in females. This remains true for different iterations of equalized sample sizes (n = 30 females; see **Figure S1).** Only 3% of age-associated sites were shared between male and female datasets (**Figure 3).**

In the liver, I identified 4,483 sites in the female dataset (n = 58) and 2,959 sites in the male dataset (n = 36) that changed with age. Like in the hippocampus, a greater number of sites became differentially methylated with age in the female dataset (FDR < 0.001). However, this does not remain true for all partitions of equal sample sizes (n = 34 females; see **Figure S2)**. Only 21% of age-associated sites were shared between the male and female datasets (**Figure 3)**.

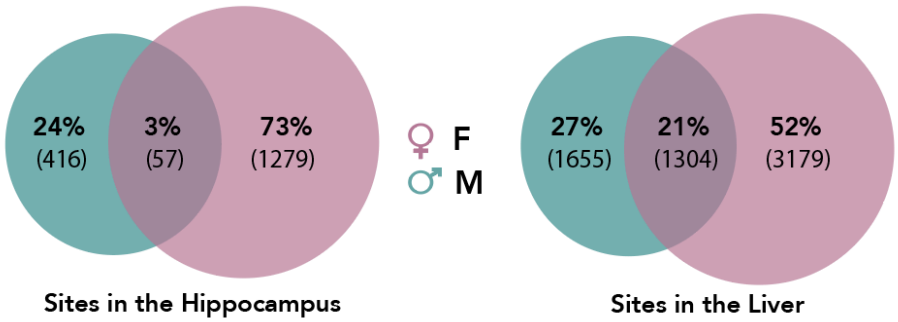
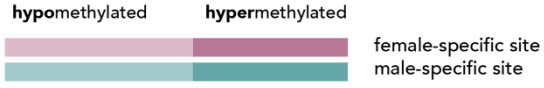


Figure 3: Significant age-associated sites (Hippocampus FDR < 0.1; Liver FDR < 0.001) categorized by sites that are male-specific (turquoise), female-specific (pink), and shared (purple).

The majority of age-associated sites become hypomethylated with age, regardless of sex and tissue type.

A general loss in methylation with age was observed in both male and female datasets in the hippocampus and liver, following a pattern of global hypomethylation with age coupled with targeted hypermethylation in specific regions.



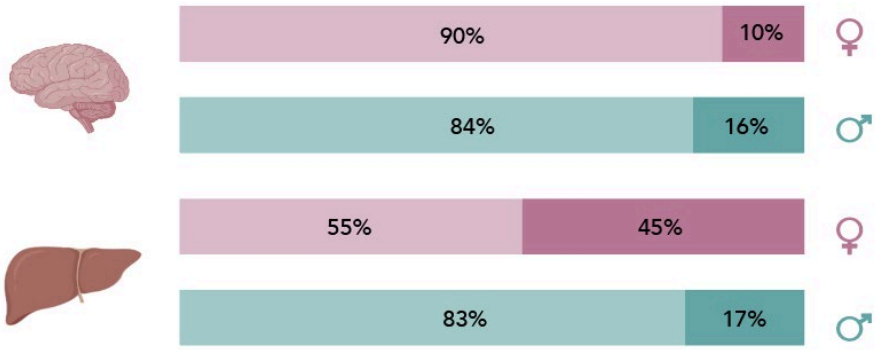


Figure 4: Trends of hypo and hypermethylation. Represents sex-specific, age-associated sites in the hippocampus (FDR > 0.1) and sex-specific, age-associated sites in the liver (FDR < 0.001) determined through beta-values. Pink represents female-specific sites, and turquoise represents male-specific sites. A lighter shade of the color represents a loss of methylation (hypomethylation) with age, while a darker shade represents a gain of methylation (hypermethylation) with age.

Age-associated sites were located within and between genes in both tissue types and sexes. In both the male and female datasets in the hippocampus and liver, more than 25% of age-associated sites fell within distal intergenic regions (i.e., stretches of DNA located between genes). A large proportion of sites in both tissues and sexes was also located in introns (non-protein coding regions of the genome). Interestingly, in the hippocampus, only 8.6% of background sites fell within promoters, while 23.8% of hypomethylated age-associated sites in the female dataset annotated to promoters (**Figure 3**), indicating these sites were overrepresented in promoter regions.

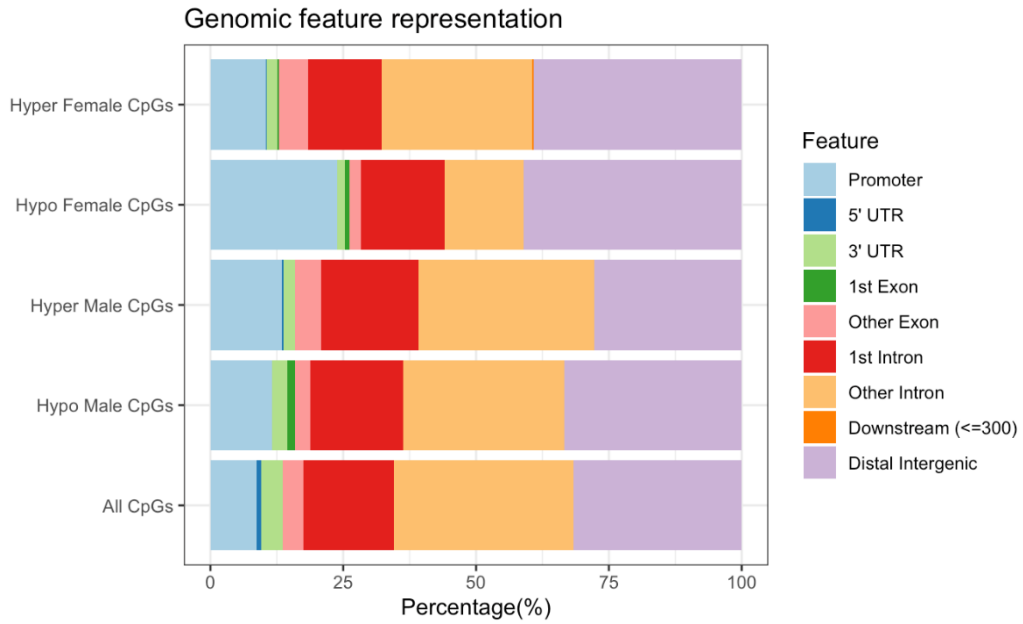


Figure 5:

Functional characterization of sex-specific sites in the hippocampus. Each site is grouped into differentially methylated sites that are unique to each sex, and further divided by direction of methylation. “All CpGs” corresponds to the background of all input CpG sites for the hippocampus.

In the liver, only 7.3% of all background sites were located within promoter regions, while 21.4% of sites in the hypomethylated male sites and 16.1% of sites in hypomethylated female sites were found in promoter regions **(Figure 4).**

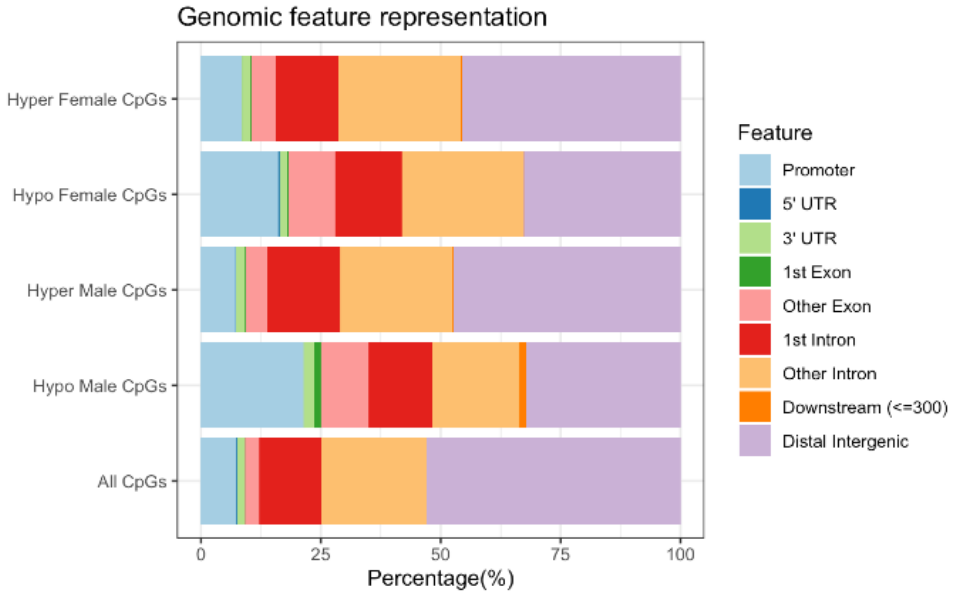


Figure 6: Functional characterization of sex-specific sites in the liver. Each site is grouped into differentially methylated sites that are unique to each sex, and further divided by direction of methylation. “All CpGs” corresponds to the background of all input CpG sites for the liver.

Age-associated sites were enriched for broad GO terms and pathways.

Enrichment in the Hippocampus

Functional profiling of sites in the male dataset in the hippocampus (identified in Objective 1 using an FDR < 0.1) revealed 17 enriched KEGG terms (FDR < 0.1 for gProfiler analysis) including: Hepatitis C, transcriptional misregulation in cancer, thyroid cancer, gastric cancer, and the thyroid hormone signaling pathway. There were also 300 enriched Human Phenotype (HP) terms associated with two age-associated sites that were specific to the male dataset (**Table S1**). Both sites became hypomethylated with age. 187 HP terms corresponded to a site on chromosome 7 and 215 terms corresponded to a site on chromosome 20, with 102 terms corresponding to both.

source	term_name	term_id	adjusted_p_value	intersections
GO:MF	retinoic acid binding	GO:0001972	0.06818181818181750	chr15:3798063:3798064
GO:MF	UDP-xylosyltransferase activity	GO:0035252	0.06818181818181750	chr20:16895575:16895576
GO:MF	retinoic acid-responsive element binding	GO:0044323	0.06818181818181750	chr15:3798063:3798064
GO:MF	xylosyltransferase activity	GO:0042285	0.06818181818181750	chr20:16895575:16895576
GO:MF	ligand-activated transcription factor activity	GO:0098531	0.06818181818181750	chr15:3798063:3798064
GO:MF	protein xylosyltransferase activity	GO:0030158	0.06818181818181750	chr20:16895575:16895576
GO:MF	isoprenoid binding	GO:0019840	0.06818181818181750	chr15:3798063:3798064
GO:MF	DNA binding domain binding	GO:0050692	0.06818181818181750	chr15:3798063:3798064
GO:MF	nuclear receptor activity	GO:0004879	0.06818181818181750	chr15:3798063:3798064
GO:MF	retinoid binding	GO:0005501	0.06818181818181750	chr15:3798063:3798064
GO:MF	vitamin D response element binding	GO:0070644	0.06818181818181750	chr15:3798063:3798064
GO:MF	nuclear steroid receptor activity	GO:0003707	0.06818181818181750	chr15:3798063:3798064
GO:MF	LBD domain binding	GO:0050693	0.06818181818181750	chr15:3798063:3798064
GO:MF	nuclear vitamin D receptor binding	GO:0042809	0.06818181818181750	chr15:3798063:3798064
GO:MF	metal ion binding	GO:0046872	0.09101624169292670	chr15:3798063:3798064,chr20:16895575:16895576,chr7:165252211:165252212
GO:BP	response to retinoic acid	GO:0032526	0.08181818181818090	chr15:3798063:3798064
GO:BP	chondroitin sulfate metabolic process	GO:0030204	0.08181818181818090	chr20:16895575:16895576
GO:BP	dendritic cell differentiation	GO:0097028	0.08181818181818090	chr3:176810123:176810124
GO:BP	chondroitin sulfate biosynthetic process	GO:0030206	0.08181818181818090	chr20:16895575:16895576
GO:BP	negative regulation of dendritic cell differentiation	GO:2001199	0.08181818181818090	chr3:176810123:176810124
GO:BP	actomyosin structure organization	GO:0031032	0.08181818181818090	chr7:165252211:165252212
GO:BP	cellular response to vitamin	GO:0071295	0.08181818181818090	chr15:3798063:3798064
GO:BP	response to vitamin	GO:0033273	0.08181818181818090	chr15:3798063:3798064
GO:BP	regulation of response to extracellular stimulus	GO:0032104	0.08181818181818090	chr15:3798063:3798064
GO:BP	negative regulation of leukocyte differentiation	GO:1902106	0.08181818181818090	chr3:176810123:176810124
GO:BP	cellular response to vitamin D	GO:0071305	0.08181818181818090	chr15:3798063:3798064
GO:BP	negative regulation of hemopoiesis	GO:1903707	0.08181818181818090	chr3:176810123:176810124
GO:BP	regulation of dendritic cell differentiation	GO:2001198	0.08181818181818090	chr3:176810123:176810124
GO:BP	regulation of response to nutrient levels	GO:0032107	0.08181818181818090	chr15:3798063:3798064
GO:BP	regulation of thyroid hormone mediated signaling pathway	GO:0002155	0.08181818181818090	chr15:3798063:3798064
GO:BP	response to vitamin D	GO:0033280	0.08181818181818090	chr15:3798063:3798064
GO:BP	thyroid hormone mediated signaling pathway	GO:0002154	0.08181818181818090	chr15:3798063:3798064
GO:BP	retinoic acid receptor signaling pathway	GO:0048384	0.08181818181818090	chr15:3798063:3798064
GO:BP	positive regulation of thyroid hormone mediated signaling pathway	GO:0002157	0.08181818181818090	chr15:3798063:3798064
GO:BP	chondroitin sulfate proteoglycan biosynthetic process	GO:0050650	0.08181818181818090	chr20:16895575:16895576
GO:BP	mRNA transcription by RNA polymerase II	GO:0042789	0.08181818181818090	chr15:3798063:3798064
GO:BP	proteoglycan biosynthetic process	GO:0030166	0.08181818181818090	chr20:16895575:16895576
GO:BP	proteoglycan metabolic process	GO:0006029	0.08181818181818090	chr20:16895575:16895576
GO:BP	mRNA transcription	GO:0009299	0.08181818181818090	chr15:3798063:3798064
GO:BP	chondroitin sulfate proteoglycan metabolic process	GO:0050654	0.08181818181818090	chr20:16895575:16895576
GO:BP	vitamin D receptor signaling pathway	GO:0070561	0.08181818181818090	chr15:3798063:3798064
GO:BP	regulation of vitamin D receptor signaling pathway	GO:0070562	0.08181818181818090	chr15:3798063:3798064
GO:BP	heparan sulfate proteoglycan biosynthetic process	GO:0015012	0.08181818181818090	chr20:16895575:16895576
GO:BP	positive regulation of vitamin D receptor signaling pathway	GO:0070564	0.08181818181818090	chr15:3798063:3798064
GO:BP	peroxisome proliferator activated receptor signaling pathway	GO:0035357	0.08181818181818090	chr15:3798063:3798064
KEGG	Hepatitis C	KEGG:05160	0.05716783762310680	chr15:3798063:3798064
KEGG	Parathyroid hormone synthesis, secretion and action	KEGG:04928	0.05716783762310680	chr15:3798063:3798064
KEGG	Bile secretion	KEGG:04976	0.05716783762310680	chr15:3798063:3798064
KEGG	Transcriptional misregulation in cancer	KEGG:05202	0.05716783762310680	chr15:3798063:3798064
KEGG	Thyroid cancer	KEGG:05216	0.05716783762310680	chr15:3798063:3798064
KEGG	Small cell lung cancer	KEGG:05222	0.05716783762310680	chr15:3798063:3798064
KEGG	Non-small cell lung cancer	KEGG:05223	0.05716783762310680	chr15:3798063:3798064
KEGG	PPAR signaling pathway	KEGG:03320	0.05716783762310680	chr15:3798063:3798064
KEGG	Adipocytokine signaling pathway	KEGG:04920	0.05716783762310680	chr15:3798063:3798064
KEGG	Glycosaminoglycan biosynthesis - heparan sulfate / heparin	KEGG:00534	0.05716783762310680	chr20:16895575:16895576
KEGG	Glycosaminoglycan biosynthesis - chondroitin sulfate / dermatan sulfate	KEGG:00532	0.05716783762310680	chr20:16895575:16895576
KEGG	Gastric cancer	KEGG:05226	0.05716783762310680	chr15:3798063:3798064
KEGG	Thyroid hormone signaling pathway	KEGG:04919	0.05716783762310680	chr15:3798063:3798064
KEGG	Th17 cell differentiation	KEGG:04659	0.07045677935458750	chr15:3798063:3798064
KEGG	Lipid and atherosclerosis	KEGG:05417	0.07671103879380470	chr15:3798063:3798064
KEGG	Chemical carcinogenesis - receptor activation	KEGG:05207	0.07671103879380470	chr15:3798063:3798064
KEGG	Non-alcoholic fatty liver disease	KEGG:04932	0.08624367098057830	chr15:3798063:3798064

Table 4: GO terms for male sites in the hippocampus. Enriched terms for the male dataset in the hippocampus (FDR < 0.1), denoting the ontology database, corresponding function, term identifier, p-value, and chromosome source. The ontology databases include Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Physiology (HP). See **Table S1** for enriched HP terms.

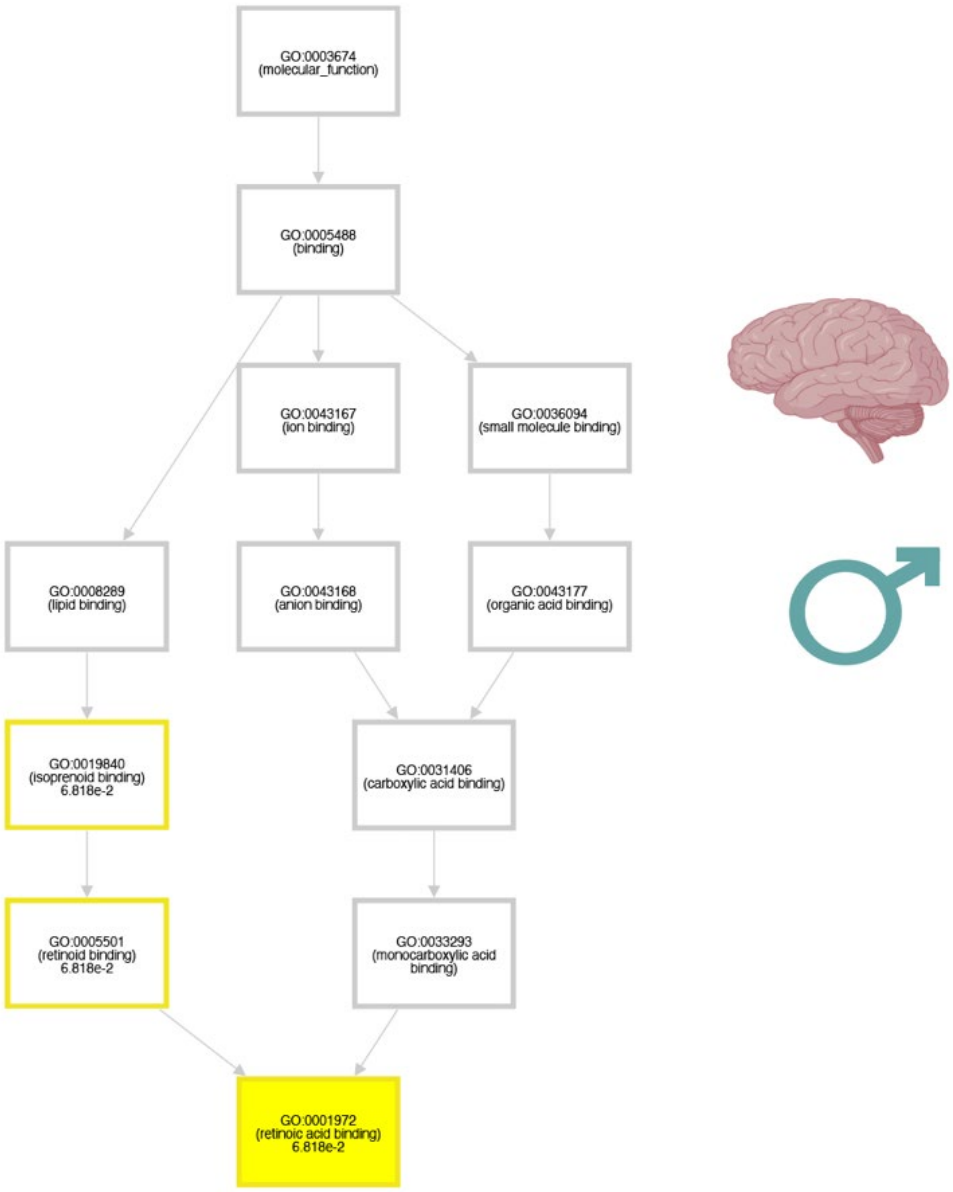


Figure 7: GO:MF diagram for male sites in the hippocampus. Molecular function context diagram for male sites in the hippocampus (FDR < 0.1), created by gProfiler using the approach described in Figure 5. Terms that share GO defined relation are grouped together, using manually curated GO resources to summarize results in the same connected component. Driver terms have a yellow background, significantly enriched terms are displayed with a colored border, and terms with a grey border provide a broader context and connect enriched terms to the root of the domain.

In the hippocampus, sites in the female dataset were enriched for 1 Biological Process (BP) term: vesicle-mediated transport in synapse and 4 Cellular Component (CC) terms: synapse, glutamatergic synapse, cell junction, and presynapse **(Figure 6)**. There were no enriched KEGG terms or HP terms (FDR < 0.1) for the female brain dataset.

Table 3: GO terms for sites in the female hippocampus. Enriched GO terms for female age-associated sites in the hippocampus.

source	term_name	term_id	adjusted_p_value	intersections
GO:BP	vesicle-mediated transport in synapse	GO:0099003	0.057706058191141800	chr19:47544387:47544388,chr20:26184351:26184352,chr5:990703:990704
GO:CC	synapse	GO:0045202	0.008721293368135220	chr16:7957951:7957952,chr19:47544387:47544388,chr20:26184351:26184352,chr3:15078842:15078843,chr5:990703:990704
GO:CC	glutamatergic synapse	GO:0098978	0.008721293368135220	chr19:47544387:47544388,chr20:26184351:26184352,chr5:990703:990704
GO:CC	cell junction	GO:0030054	0.04051095893161310	chr16:7957951:7957952,chr19:47544387:47544388,chr20:26184351:26184352,chr3:15078842:15078843,chr5:990703:990704
GO:CC	presynapse	GO:0098793	0.04051095893161310	chr16:7957951:7957952,chr19:47544387:47544388,chr5:990703:990704

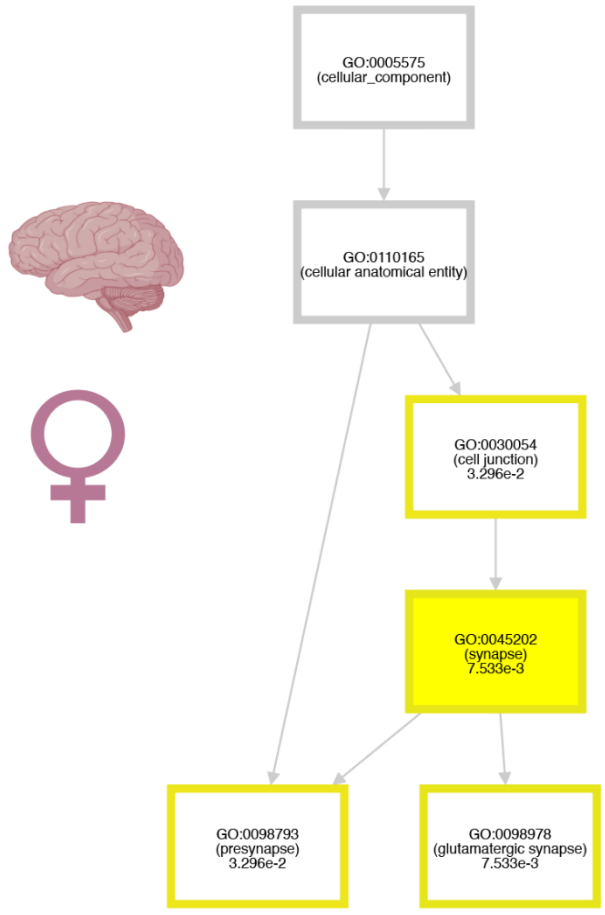


Figure 8: GO:CC Context Diagram for female sites in hippocampus. Represents enriched Cellular Component terms for female sites in the hippocampus (FDR < 0.1) created by gProfiler.

Enrichment in the Liver

In the liver, sites in both sexes were enriched for KEGG term thyroid hormone synthesis. The sites in the male liver dataset were also enriched for the KEGG term glycosphingolipid biosynthesis. There 44 HP terms that were enriched, including male sexual dysfunction, urinary incontinence, amyloidosis, and hypotension. All enriched terms for the male dataset in the liver were associated with a site on chromosome 18 that became hypomethylated with age.

This site in the female liver dataset also lost methylation with age yet was not enriched for any HP terms. The female liver dataset was enriched for only 1 KEGG term: thyroid hormone synthesis. The site associated with this term was also linked to *DUOX1*, a gene related to liver pathophysiology and previously found to be subject to epigenetic regulation.

source	term_name	term_id	adjusted_p_value	intersections
KEGG	Glycosphingolipid biosynthesis - lacto and neolacto series	KEGG:00601	0.08694718763417760	chr4:159398129:159398130
KEGG	Thyroid hormone synthesis	KEGG:04918	0.08694718763417760	chr18:50943506:50943507
HP	Reduced left ventricular ejection fraction	HP:0012664	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal male reproductive system physiology	HP:0012874	0.09306569343065680	chr18:50943506:50943507
HP	Abnormality of enteric nervous system morphology	HP:0025028	0.09306569343065680	chr18:50943506:50943507
HP	Left ventricular systolic dysfunction	HP:0025169	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal CSF protein concentration	HP:0025456	0.09306569343065680	chr18:50943506:50943507
HP	Cardiac amyloidosis	HP:0030843	0.09306569343065680	chr18:50943506:50943507
HP	Impairment of activities of daily living	HP:0031058	0.09306569343065680	chr18:50943506:50943507
HP	Impaired continence	HP:0031064	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal B-type natriuretic peptide level	HP:0031138	0.09306569343065680	chr18:50943506:50943507
HP	Increased circulating NT-proBNP concentration	HP:0031185	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal aortic valve physiology	HP:0031652	0.09306569343065680	chr18:50943506:50943507
HP	Leptomeningeal enhancement	HP:0032070	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal circulating vitamin B6 level	HP:0032476	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal left ventricular ejection fraction	HP:0034314	0.09306569343065680	chr18:50943506:50943507
HP	Male sexual dysfunction	HP:0040307	0.09306569343065680	chr18:50943506:50943507
HP	Tendon rupture	HP:0100550	0.09306569343065680	chr18:50943506:50943507
HP	Erectile dysfunction	HP:0100639	0.09306569343065680	chr18:50943506:50943507
HP	Vitreous floaters	HP:0100832	0.09306569343065680	chr18:50943506:50943507
HP	Increased circulating troponin T concentration	HP:0410174	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal autonomic nervous system physiology	HP:0012332	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal autonomic nervous system morphology	HP:0012331	0.09306569343065680	chr18:50943506:50943507
HP	Digital flexor tenosynovitis	HP:0012276	0.09306569343065680	chr18:50943506:50943507
HP	Constrictive median neuropathy	HP:0012185	0.09306569343065680	chr18:50943506:50943507
HP	Urinary incontinence	HP:0000020	0.09306569343065680	chr18:50943506:50943507
HP	Impotence	HP:0000802	0.09306569343065680	chr18:50943506:50943507
HP	Orthostatic hypotension	HP:0001278	0.09306569343065680	chr18:50943506:50943507
HP	Congestive heart failure	HP:0001635	0.09306569343065680	chr18:50943506:50943507
HP	Aortic valve stenosis	HP:0001650	0.09306569343065680	chr18:50943506:50943507
HP	Restrictive cardiomyopathy	HP:0001723	0.09306569343065680	chr18:50943506:50943507
HP	Thromboembolism	HP:0001907	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal thrombosis	HP:0001977	0.09306569343065680	chr18:50943506:50943507
HP	Abnormality of the autonomic nervous system	HP:0002270	0.09306569343065680	chr18:50943506:50943507
HP	Stroke-like episode	HP:0002401	0.09306569343065680	chr18:50943506:50943507
HP	Hypotension	HP:0002615	0.09306569343065680	chr18:50943506:50943507
HP	Increased CSF protein concentration	HP:0002922	0.09306569343065680	chr18:50943506:50943507
HP	Abnormality of vitamin B metabolism	HP:0004340	0.09306569343065680	chr18:50943506:50943507
HP	Orthostatic hypotension due to autonomic dysfunction	HP:0004926	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal left ventricular function	HP:0005162	0.09306569343065680	chr18:50943506:50943507
HP	Amyloid deposition in the vitreous humor	HP:0007841	0.09306569343065680	chr18:50943506:50943507
HP	Reduced circulating vitamin B6 level	HP:0008326	0.09306569343065680	chr18:50943506:50943507
HP	Amyloidosis	HP:0011034	0.09306569343065680	chr18:50943506:50943507
HP	Entrapment neuropathy	HP:0012181	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal cardiac test	HP:0500015	0.09306569343065680	chr18:50943506:50943507
HP	Abnormal cardiac biomarker test	HP:0500020	0.09306569343065680	chr18:50943506:50943507

Table 3: GO terms for male sites in liver. Corresponds to male sites in the liver (FDR < 0.1), denoting the ontology database, corresponding function, term identifier, p-value, and chromosome source. The ontology databases include Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Physiology (HP), which uses gene orthology information from Ensembl to pair gene databases of other organisms to HP annotations of human genes.

Discussion

In this project, I sought to characterize age-associated DNA methylation changes in male and female datasets of a long-lived primate to see if these patterns reflected sex divergence in aging and longevity. I found that there were evident sex differences in the epigenome with age, and that most sites that changed with age were specific to each sex. When I explored these sites further, most sites lost methylation with age, across sex and tissue type. In both tissues, there were more age-associated sites in the male dataset that were linked to genes and consequently ontology terms. In the hippocampus, male and female sites were enriched for different ontology terms, suggesting that sex-specific sites of methylation may be associated with sex-dimorphic gene expression in the brain. In the liver, both sex datasets were enriched for thyroid hormone regulation. Overall, my findings suggest that differential methylation between sexes could contribute to variation in aging trajectories and disease vulnerability and re-affirms the need for further research into these differences.

General Hypomethylation with Age Across Sexes

Overall, my results support a trend of global hypomethylation with age, regardless of sex or tissue type. This conforms to the global hypomethylation hypothesis, which argues that a decrease in global DNA methylation is characteristic of aging (Pogribny and Vanyushin 2010). Patterns of loss of methylation appear to occur in most if not all tissues with age. However, current literature suggests that rather than being a direct product of mechanistic aging, an increase in errors with time results in more relaxed and consequently aberrant regulation of gene expression (Unnikrishnan et al. 2021).

More Differential Methylation with Age in Females

In both tissues, there were more sites in the female datasets that became differentially methylated with age. In the hippocampus, I found that more sites change with age in the female dataset. In the liver, this finding is nuanced by the female-biased composition of these data (see **Figure S2**). However, studies in human cohorts have reported that females display higher levels of methylation overall (Liu et al. 2010, Hall et al. 2014, Yousefi et al. 2015) and that females often outpace males in the number CpG sites that lose and gain methylation with age (Grant et al. 2022). However, other studies on sex differences in age-associated methylation in autosomes have found conflicting results. In a study of DNA methylation in human liver tissue, males displayed higher average genome-wide methylation in the autosomes, while females had a higher DNA methylation than males in X-chromosome sites (García-Calzón et al. 2018). Still others have found no significant difference in DNA methylation with age on autosomes between males and females (Zhang et al. 2018). My results suggest that a large fraction of CpG sites that change with age are sex-specific and may contribute to differential aging and disease phenotypes between sexes.

Hypomethylation in Promoter Regions

In both the hippocampus and liver dataset, a greater proportion of hypomethylated sites mapped to promoter regions than in the background datasets. Decrease in methylation with age at promoters is consistent with previously established patterns in the aging epigenome (Gensous et al. 2017). In promoters, hypomethylation is associated with gene activation, while hypermethylation is associated with gene silencing (Bird 2002). A study on frailty and changes in DNA methylation found that a worsening in frailty status was associated with a significant decrease in genome-wide DNA methylation (Bellizzi et al. 2012). Interestingly, a similar study

found that subjects who presented lower levels of DNA methylation at promoter specific CpG sites had decreased odds of frailty (Collerton et al. 2014). Loss of methylation at promoters may contribute to phenotypic age-related decline, and this phenomenon could contribute to physiological decline with age in rhesus macaques. However, the causative nature of this relationship remains unclear. Frailty is associated with aging, but it is unknown whether DNA methylation functions to increase susceptibility to frailty with age. There were no differences between sexes in proportion of promoters or other genomic regions to suggest that patterns of genomic distribution in methylation may be sex-specific in either tissue.

Differential Methylation in Intergenic Regions

I found that a large proportion of DMCs in both tissue and sex datasets were located in distal intergenic regions of the genome, stretches of DNA that lie between genes and do not directly encode proteins. The effect of DNA methylation in regions outside of promoters remains poorly characterized, specifically in intergenic regions (Yan et al. 2016). However, increasingly evidence suggests that methylation at intergenic regions can regulate gene expression (Thomas et al. 2012, Lu et al. 2012, Schlesinger et al. 2013). Intergenic regions may contain many regulatory elements, such as enhancers, silencers, and noncoding RNAs, that may be impacted by DNA methylation (Reschke et al. 2014). Recent research suggests epigenetic control of enhancers, which facilitate regulation of gene expression by bringing distant regulatory elements in proximity to their target genes, alters neuronal functions and may be involved in age-related diseases like Alzheimer's (Li et al. 2019). The role of epigenetic modifications and how they act as regulatory elements in intergenic regions at intergenic regions remains unclear and demands further investigation.

Tissue- and Sex-Specific Enrichment

Based on preliminary findings, DMCs that are associated with enrichment terms may be specific to each sex and each tissue. In the brain, two sites that are not differentially methylated with age in the female dataset are associated with many HP terms in the male dataset. These sites fell on chromosome 20 and chromosome 7, and were associated with pathologies that disproportionally affect men, including gastric cancer and (National Cancer Institute) and Hepatitis C (Baden et al. 2014). In the liver, the specific site associated with all enriched HP terms was not differentially methylated with age in the female dataset. This site was located on chromosome 18 and was associated with several age- and sex-associated phenotypes, including male sexual dysfunction, urinary incontinence, stroke, and hypotension.

Retinoic Acid Binding and Synaptic Plasticity

Male sites in the hippocampus that were associated with retinoic acid binding **(Table 5)** became hypomethylated with age. Retinoic acid contributes to a wide-ranging host of biological processes, but in the hippocampus is crucial for synaptic plasticity and adult neurogenesis, the process of generating functional neurons (Jacobs et al. 2006). GO terms involving synaptic activity were also enriched in the female liver dataset. Accumulation of errors in the epigenome during aging progression increases the risk for onset of age-related pathologies, such of those involving brain deterioration and neurodegeneration. The most common brain disorders affecting elderly individuals are those causing dementia through loss of synaptic plasticity, leading to memory impairment and defective learning capabilities (Salameh et al. 2020). A recent study in a human cohort suggests that hypomethylation of retinoic acid receptor related genes induce persisting neuropsychological consequences (Glad et al. 2017). Epigenetic regulation of retinoic acid binding and synaptic activity with age fits previously established findings, but further

research is needed to explore the differences between female and male datasets to see if they may account for differences in aging phenotypes.

Thyroid Hormone Regulation

A recurring ontology term across tissues and sexes was thyroid hormone regulation. It is well-known that the prevalence of thyroid disorders increases with age (Gesing 2015), and the enrichment of age-associated sites for thyroid hormone regulation suggests the epigenome could be a regulatory mechanism that contributes to susceptibility of thyroid diseases with age. According to the American Thyroid Association, women are five to eight times more likely to develop thyroid disease, suggesting that epigenetic regulation of thyroid hormones could be valuable to further explore in a sex-specific context.

Previous studies examining sex differences in methylation have revealed enrichment among differentially expressed genes involved in hormone regulation in the brain and testis (McCartney et al. 2021), supporting the idea that epigenetic differences can regulate hormone differences that may account for sex-based differences in aging. One site that changed with age in the female liver dataset was associated with the *DUOX1* gene, associated with liver pathophysiology across mammals (Ashtiwi et al. 2021). *DUOX1* mediates the production of reactive oxygen species (ROS), which has been correlated with many diseases that tend to occur later in life (Ashtiwi et al. 2021). The epigenetic silencing of *DUOX1* by hypermethylation of a promoter has been directly implicated in human liver cancer (Ling et al. 2014). In this macaque dataset, I found the site associated with *DUOX1* becomes hypomethylated with age, so the impact of this change remains unclear.

The nature of ontological analysis for differentially methylated sites is inherently difficult to explore and interpret. Unlike gene expression data, DNA methylation does not correlate to

direct genes or pathways and its effects on gene regulation is much less straightforward. The role of DNA methylation on gene expression can vary depending on the genomic context and specific regulatory elements involved, which, as aforementioned, remains complex and in need of further characterization. As previous studies have noted, a lack of a standardized approach for determining sex-associated sites results in limited reproducibility between studies (Gatev et al. 2021 and Grant et al. 2021). Particularly with ontology analysis, a uniform set of statistical parameters has not been defined. Here, I used a Benjamini Hochberg FDR cutoff of < 0.1 for both tissues in gProfiler analyses.

Limitations

Previous studies have predominantly focused on blood-based DNA methylation data from human cohorts, while this project focuses on hippocampus- and liver- based data and focuses on autosomes in rhesus macaques. Interestingly, some studies report the largest sex differences in age-associated DNA methylation in sex chromosomes compared to autosomes (McCartney et al. 2021). There is also a recent emergence of a trend towards hypermethylation for female specific CpG sites that change with age (Kananen et al. 2021, Li et al. 2021), but these studies included sex chromosomes and are thus not directly comparable to my project.

Although the existence of sex differences in the epigenome is well-established, a robust and consistent characterization of these differences is still lacking. Comparisons between my results and results from the literature are complicated by the differing dataset composition and the lack of an established catalogue for exploring differentially methylation across species. Additionally, cross-comparisons are limited by a lack of comparable datasets, such as matched tissue type, model organism, and study demographics across studies.

Future Directions

Epigenetic Drift

As a next step, characterizing the variation in DNA patterns over lifespan and whether these patterns differ between sexes may illuminate other ways in which sex differences in aging are reflected in the epigenome. Future analyses could measure epigenetic drift, in which the epigenome accrues more inter-individual variation with age and becomes more prone to errors (Hernando-Herraez et al. 2019). Epigenetic drift is also referred to as entropy, the measure of disorder in a system. Epimutations, a measure of drift, are rare methylation changes that are specific for one or few individuals within a specific population. Epimutations have been found to increase with age and have been reported in cancer (Teschendorff et al. 2016) Variability, epimutations, and entropy of autosomal DNA methylation with age between sexes were assessed in Yusipov et al. 2020, but no detectable difference in epimutations were found. However, this discrepancy could be attributed to analytical approaches or cohort-specific effects, according to the authors. Further studies are necessary to determine if there may be sex-specific trends present in patterns of epigenetic drift.

Region-Based Analysis

Characterizing and annotating differentially methylated sites in terms of differentially methylated regions (DMRs) may reveal other differences between sex datasets and elucidate how differential methylation may contribute to dimorphism in aging phenotypes between males and females. DMR analysis takes into account neighboring CpG sites and identifies regions where the overall methylation levels are consistently different between groups. By considering multiple CpG sites within a region, DMR analysis provides a more robust and comprehensive assessment of differential methylation. It can capture coordinated changes in methylation patterns and

provide insights into functional genomic regions that are differentially regulated (Yousefi et al 2015).

Sex Chromosomes

Future studies should also include DNA methylation data from sex chromosomes. Exploring DMCs within sex chromosomes in a tissue-specific context would be a novel contribution to the field of epigenetics, which often excludes the sex chromosomes in non-sex focused analyses.

Matched Tissue Datasets

Matched datasets would enable the comparison between tissues, which we avoided in this project due to the two tissue datasets coming from different individuals. However, cross-tissue comparisons would elucidate whether differences in DNA methylation between sexes are uniform or whether these differences present differently or to different degrees across tissues and organs.

Conclusions + Significance

This project sought to focus on sex differences in aging through the lens of DNA methylation, recognized to capture the nuanced interplay of environment and biology and an important component of the aging process that may be a good target for quantifying biological age. Although sex differences have long been acknowledged in the field of epigenetics, only a small body of studies have examined sex-specific patterns of autosomal DNA methylation to elucidate the mechanisms that may reflect sex differences in aging and longevity. Previous studies have predominantly focused on blood-based DNA methylation data from human cohorts, so these findings contribute to an understanding of sex differences in the epigenome of a long-lived primate from a unique, two-tissue approach.

Additionally, this project capitalizes on a pre-existing data from rhesus macaques to perform novel analyses. Historically, few methylation datasets have been available for rhesus macaques. The representation of sexes and the breadth of the age range of these samples make this a valuable dataset for exploring patterns of epigenetic aging in a long-lived primate. It is important to integrate functional genomics and evolutionary theory to nuance our understanding of the aging process. Investigations into the epigenome of non-human primates like rhesus macaques can help elucidate theories of aging for long-lived primates, in contrast to short-lived organisms like mice.

In this project, I learned how to use bioinformatics to analyze DNA methylation data and consider both the statistical and biological validity of different analyses. I found that hippocampus and liver tissue in rhesus macaques have sex-specific methylation profiles that change with age in the autosomes. Overall, these sites lose methylation with age, and are located predominantly in distal intergenic regions, where they may serve to regulate expression of genes

downstream. Differences in methylation regulate sexually divergent pathways that shape variation in aging and age-related outcomes in the sexes. This project may support the overarching hypothesis that sex differences in autosomal DNA methylation data may account for some of the sex differences seen in aging and age-associated disease prevalence, onset, and progression. However, further characterization and elucidation of underlying mechanisms is necessary.

How can we manage aging?

By characterizing the sex-specific DNA methylation changes that occur with age, researchers can better understand underlying mechanisms of aging and the development of interventions to promote healthy aging. Through projects like this one, we can pinpoint specific genomic regions and genes that may serve as molecular targets for anti-aging interventions. The field of biogerontology relies on a holistic, integrative approach to aging. These targets can include genes and gene regulation mechanisms that become dysregulated with age and disease. Uncovering the epigenetic variation that underlies vulnerability to morbidity and mortality is crucial to the development of treatment and management strategies. For example, epigenetic age acceleration was shown to be associated with Alzheimer's Disease (AD) neuropathological markers such as neuritic plaques, diffuse plaques, and amyloid load in the prefrontal cortex (Levine et al. 2015b). Identifying epigenetic signatures of disease may aid in early detection and risk assessment in conditions like AD which lack diagnostic biomarkers. Interestingly, drug pro-longevity interventions in mice have displayed sex-dimorphic responses, such as rapamycin treatment and metformin (Sampathkumar et al. 2020), further validating the need to conduct sex-specific analyses in processes that govern aging, longevity, and morbidity.

Epigenetic insights extend beyond therapeutic interventions---they also support the importance of everyday habits to enhance aging and improve healthspan, which represents the number of quality years an individual enjoys, compared to lifespan. The epigenome demonstrates the ways in which the environment can "get under the skin" to shape variation in disease and mortality risk. Recent studies fuse nutrition with DNA methylation, demonstrating that healthy diets can restore dysregulated DNA methylation markers that accrue with age (Fitzgerald et al. 2021). Blue zones, regions around the world known for having higher proportions of centenarians and lower rates of age-related disease, offer valuable insight into how environment and behaviors can promote longevity and well-being. These zones reveal the importance of diet, strong social bonds, and exercise. Diet plays a vital role in promoting healthy aging, and residents of blue zones set an example in a predominantly plant-based diet that is rich is whole foods, including fruits, vegetables, legumes, and whole grains. Reduction in processed foods and red meat consumption is associated with a lessened risk of heart disease, diabetes, and certain cancers. Additionally, blue zone residents typically consume fewer calories than average Americans. Mindful eating helps maintain health and reduce risk of chronic disease. Blue zones are also characterized by strong social bonds, which have been attributed to the longevity and vitality of their residents. Regular social interaction and support networks contribute to improved mental well-being, reduced stress levels, and a sense of purpose, all of which are crucial for healthy aging.

Appendix

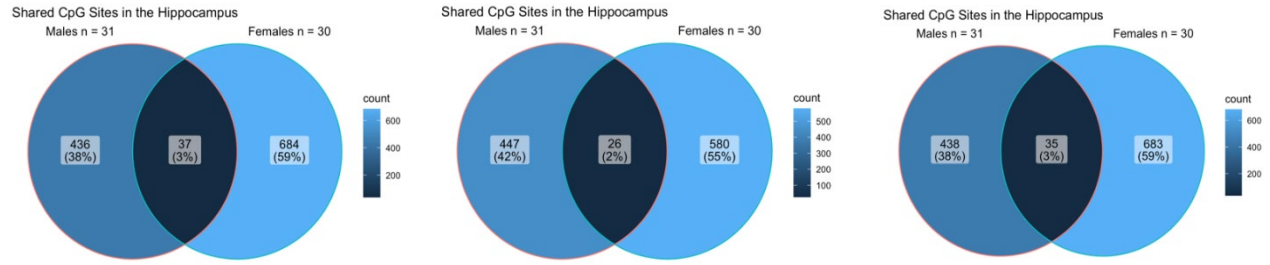
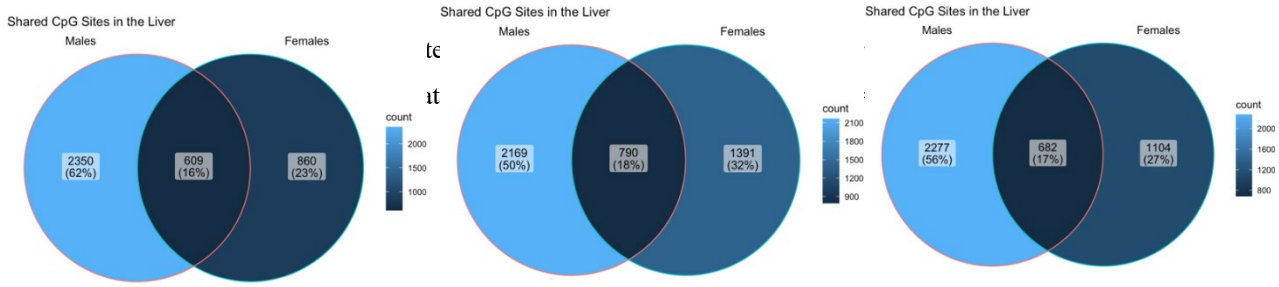


Figure S1: Age-associated sites for equalized sample sizes in the hippocampus. Represents the quantity of sites that change with age that are shared vs sex-specific in males (n = 31) and females (n = 30).



HP	Short clavicles	HP:0000894	0.03988743805263290	chr20:16895575:16895576,chr7:165252211:165252212
HP	Broad foot	HP:0001769	0.03988743805263290	chr20:16895575:16895576,chr7:165252211:165252212
HP	Fetal onset	HP:0011461	0.03988743805263290	chr20:16895575:16895576,chr7:165252211:165252212
HP	Aplasia/Hypoplasia of the clavicles	HP:0006710	0.03988743805263290	chr20:16895575:16895576,chr7:165252211:165252212
HP	Antenatal onset	HP:0030674	0.03988743805263290	chr20:16895575:16895576,chr7:165252211:165252212
HP	Elbow dislocation	HP:0003042	0.06313539802155540	chr20:16895575:16895576
HP	Short long bone	HP:0003026	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal patella morphology	HP:0003045	0.06313539802155540	chr20:16895575:16895576
HP	Patellar dislocation	HP:0002999	0.06313539802155540	chr20:16895575:16895576
HP	Radioulnar synostosis	HP:0002974	0.06313539802155540	chr20:16895575:16895576
HP	Abnormal morphology of the nasal alae	HP:0000429	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Facial hypertrichosis	HP:0002219	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Obstructive sleep apnea	HP:0002870	0.06313539802155540	chr7:165252211:165252212
HP	Low-set ears	HP:0000369	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Short fourth metatarsal	HP:0004689	0.06313539802155540	chr7:165252211:165252212
HP	Short fifth metatarsal	HP:0004704	0.06313539802155540	chr7:165252211:165252212
HP	Crossed fused renal ectopia	HP:0004736	0.06313539802155540	chr7:165252211:165252212
HP	Low anterior hairline	HP:0000294	0.06313539802155540	chr7:165252211:165252212
HP	Abnormality of the philtrum	HP:0000288	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Epicanthus	HP:0000286	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Knee dislocation	HP:0004976	0.06313539802155540	chr20:16895575:16895576
HP	Macrocephaly	HP:0000256	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Aplasia/hypoplasia involving bones of the hand	HP:0005927	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Radial head subluxation	HP:0003048	0.06313539802155540	chr20:16895575:16895576
HP	Anteverted nares	HP:0000463	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Cystic hygroma	HP:0000476	0.06313539802155540	chr7:165252211:165252212
HP	Abnormal epiphysis morphology	HP:0005930	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal clavicle morphology	HP:0000889	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Hypertrichosis	HP:0000998	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Congenital diaphragmatic hernia	HP:0000776	0.06313539802155540	chr7:165252211:165252212
HP	Abnormality of the diaphragm	HP:0000775	0.06313539802155540	chr7:165252211:165252212
HP	Abnormal palmar dermatoglyphics	HP:0001018	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Soft, doughy skin	HP:0001027	0.06313539802155540	chr20:16895575:16895576
HP	Pectus excavatum	HP:0000767	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal sternum morphology	HP:0000766	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Angioid streaks of the fundus	HP:0001102	0.06313539802155540	chr20:16895575:16895576
HP	Tooth malposition	HP:0000692	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Alternating esotropia	HP:0001137	0.06313539802155540	chr7:165252211:165252212
HP	Communicating hydrocephalus	HP:0001334	0.06313539802155540	chr7:165252211:165252212
HP	Joint hypermobility	HP:0001382	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Broad finger	HP:0001500	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Synophrys	HP:0000664	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Intrauterine growth retardation	HP:0001511	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal rib cage morphology	HP:0001547	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Genu recurvatum	HP:0002816	0.06313539802155540	chr20:16895575:16895576
HP	Abnormal eyelash morphology	HP:0000499	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Episodic vomiting	HP:0002572	0.06313539802155540	chr7:165252211:165252212
HP	Limb tremor	HP:0200085	0.06313539802155540	chr7:165252211:165252212
HP	Long eyelashes	HP:0000527	0.06313539802155540	chr7:165252211:165252212
HP	Abnormal eyebrow morphology	HP:0000534	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormality of the neck	HP:0000464	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal bleeding	HP:0001892	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Abnormal delivery	HP:0001787	0.06313539802155540	chr7:165252211:165252212
HP	Pes planus	HP:0001763	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Restrictive cardiomyopathy	HP:0001723	0.06313539802155540	chr20:16895575:16895576
HP	Blue sclerae	HP:0000592	0.06313539802155540	chr20:16895575:16895576
HP	Oligohydramnios	HP:0001562	0.06313539802155540	chr7:165252211:165252212
HP	Asymmetry of the thorax	HP:0001555	0.06313539802155540	chr7:165252211:165252212
HP	Toe clinodactyly	HP:0001863	0.06313539802155540	chr20:16895575:16895576
HP	Bifid uvula	HP:0000193	0.06313539802155540	chr20:16895575:16895576
HP	Abnormal dermatoglyphics	HP:0007477	0.06313539802155540	chr20:16895575:16895576,chr7:165252211:165252212
HP	Cleft soft palate	HP:0000185	0.06313539802155540	chr20:16895575:16895576
HP	Hooded eyelid	HP:0030820	0.06313539802155540	chr7:165252211:165252212
HP	Abnormality on pulmonary function testing	HP:0030878	0.06313539802155540	chr20:16895575:16895576
HP	Joint subluxation	HP:0032153	0.06313539802155540	chr20:16895575:16895576
HP	Abnormal pulse	HP:0032552	0.06313539802155540	chr20:16895575:16895576
HP	Weak pulse	HP:0032553	0.06313539802155540	chr20:16895575:16895576
HP	Aplasia of the right hemidiaphragm	HP:0032592	0.06313539802155540	chr7:165252211:165252212
HP	White oral mucosal macule	HP:0033026	0.06313539802155540	chr20:16895575:16895576

Table S1: HP terms enriched in the male liver. Human Phenotype terms in the age-associated male sites in the liver.

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