SIGNATURES OF AGING AND ENVIRONMENT IN THE DNA METHYLOME OF RHESUS MACAQUES

By

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DISSERTATION ABSTRACT

Elisabeth A. Goldman

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While the link between aging and metabolic function is well recognized, little is known about how variables like diet are able to drive variation in health and longevity through interaction with molecular mechanisms of aging. Because aging does not occur uniformly throughout the body, tissue-specific analyses are necessary to elucidate patterns of age-related decline in organs with distinct physiological roles.

Existing epigenetic clocks have provided some insight into the variables that impact aging, disease- and mortality risk, but the mechanisms underlying the manifestation of their effects in the larger context of aging remain obscure. While multi-tissue clocks have gained popularity in human clinical and biomedical research, these models provide just one estimate of systemic health and decline, and cannot indicate where early or sub-clinical signs of disease may be starting to subtly manifest. Here, I take a targeted, tissue-specific approach to construct two generalizable epigenetic clock models using genome-wide methylation data generated from blood (n=563) and liver (n=96) samples taken from two independent populations of rhesus macaques. I applied the blood clock model to blood samples from an additional population of rhesus macaques (n=43) at Yerkes National Primate Center and a wild population of baboons (n=271) living at Amboseli National Park in Kenya. The model predicted age with high accuracy in both populations.

Next, I tested whether the liver-specific model was able to detect a delayed biological aging effect as a result of long-term dietary restriction, a known pro-longevity intervention, in 63 rhesus macaque from a long-term dietary restriction study carried out over 33-years at the National Institute on Aging (NIA). Monkeys who entered the study at one of three distinct developmental time points (pre-adult, middle-aged, and older adult). I found that males who began a restricted diet later in life (between age 13 and 23 years) appeared to the reap the greatest longevity benefit, but did not show significantly lower rates of epigenetic aging, counter to the expectation that this trend would correlate with longevity. Interestingly, males who entered the study at older ages as control individuals had unusually long lifespans, perhaps owing to higher quality nutrition and supplementation they received to match their diets with that of the experimental group (whose diet was highly supplemented by 70% of the calories of the control diet). Individuals who began the restricted diet as juveniles had slower rates of epigenetic aging but saw no improvement in life expectancy, suggesting the clock may track trade-offs in energy allocation between processes like growth, maintenance and reproduction, and may at some point become decoupled from an individual's risk of mortality. These models are valuable tools for predicting age in non-human primate species and illustrate how connecting behavioral data with the epigenetic clock can uncover the social and environmental determinants of biological age.

This dissertation includes previously unpublished co-authored material.

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DEDICATION

To my grandmothers, Bernice and Elaine.

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CHAPTER I

INTRODUCTION: DEFINING THE SHAPE OF THE AGING TRAJECTORY

Background and Motivation

The demographics of the global population are changing in unprecedented ways. Over the next three decades, the number of adults aged 60 and older will increase two-fold, to 2.1 billion, while the number of adults aged 80 and older will triple to 426 million (World Health Organization 2021). Since the mid-20th century, life expectancy has increased by over twenty years, largely due to advancements in treatment of infectious disease, improved sanitation, and more food security in many parts of the world (Vaupel, Villavicencio, and Bergeron-Boucher 2021; K. Christensen et al. 2009). Disparities in life expectancy between different countries have also sharply declined (Rosen et al., 2019). As the over-65 demographic continues to expand in the United States, so does an already urgent need for a comprehensive social and public health infrastructure capable of supporting the country's aging population.

Assuming a birth year of 1900, the grandparents of Baby Boomers had a life expectancy at birth of 33 years or 47 years, depending whether they were Black or White, respectively (National Center for Health Statistics, United States 2021b). Today, Baby Boomers can reasonably expect that their grandchildren will surpass age 100 (National Center for Health Statistics, United States 2021a). Unsurprisingly, there is a shift underway in the national attitude towards the experience of aging: with a growing segment of the US population having firmly reached retirement age, popular interest in "healthy aging"—maintaining feelings of physical and cognitive health, autonomy and a sense of control, and continued engagement with one's community throughout old age—has grown substantially in recent years (van Leeuwen et al. 2019). However, that gains in life expectancy have not been accompanied by comparable reductions in rates of chronic, non-communicable disease is an immediate and evermore urgent health concern. The lack of public infrastructure to support aging adults and dearth of options for aging-in-

place (Teater and Chonody 2021) make it difficult for aging people to maintain quality of life when in need of long-term assistance or intensive medical care.

Further compounding the issue of a burgeoning population of older adults is the growing incidence of chronic, non-communicable diseases around the world (Arokiasamy et al. 2017). Rates of type 2 diabetes, non-alcoholic fatty liver disease (NAFLD), and other complications related to the dysregulation of energy balance have jumped dramatically while the average age-at-onset for these diseases has also declined, thereby increasing the proportion of the population requiring long-term medical treatment as well as the likelihood of early mortality (Younossi et al. 2016). It is critical that we develop better methods of prevention, early detection, and effective therapeutic interventions to delay or reverse the pathogenesis of age-related and metabolic disease to prevent complete and total exhaustion of an already strained healthcare system. Realization of such ambitious goals requires identifying the proximate mechanisms that functionally enable age-related deterioration as well as ultimate explanations for *why* such mechanisms persist.

Scientists have been considering the nature of cellular aging from various perspectives for over a century (Weissman 1892), and paradigm-shifting work has been carried out in model systems like *Saccharomyces cerevisiae* (yeast), *Caenorhabditis elegans* (nematodes), and *Drosophila melanogaster* (fruit flies) (Mortimer and Johnston 1959; Y. Pan 2011; Pickering et al. 2017; Greer et al. 2007; Klass 1983). However, intrinsic species differences continue to limit the translatability of the aforementioned work, or more recent groundbreaking research carried out in mice (e.g., Browder et al. 2022), to an exceptionally long-lived species like humans. While research conducted in short-lived model organisms has without question advanced the field of aging research, the timing and duration of processes like growth, maintenance, and cellular senescence are different in non-trivial ways among long-lived species (Gluckman, Hanson, and Low 2019). Here, I developed a methodological framework to examine tissue-specific variation in the DNA methylome and test hypotheses surrounding how environmental parameters and life history characteristics influence aging in the epigenome of a long-lived, non-human primate (NHP), the rhesus macaque (*Macaca mulatta*).

Evolution of Aging

One may ask why aging should occur at all. Numerous theories have been proposed to explain *why* organisms age form an evolutionary perspective, intending to decipher an ultimate explanation for the aging process. A few classic theories of aging have endured over the years, and two are briefly summarized here. These theories are not mutually exclusive, but neither one provides a complete or sufficient explanation for the origins of biological aging. Nonetheless, they have provided a framework for much of the aging research conducted over the last half century and thus warrant mention.

The mutation accumulation hypothesis is built upon the concept of a *selection shadow*, and suggests that deleterious mutations in the germline do not become evident until after an organism has already reproduced; therefore, seemingly neutral mutations continue to be passed down across generations and can drift to fixation because they only become apparent later in life, in the *shadow* of natural selection, i.e., when natural selection has little ability to act on them, and manifest as aging phenotypes (Haldane 1941; Medawar 1952). There is some evidence to suggest that in mammals, genes upregulated later in life may be subject to stronger drift (Turan et al. 2019). However, it is relevant that this same study found a negative relationship between later-life gene expression and evolutionary conservation, suggesting genes that are more active in old age may be more likely to be species-specific (Turan et al. 2019).

Another arguably more popular framework for understanding the evolution of aging brings together two complementary paradigms, that of antagonistic pleiotropy (Williams 1957) and the disposable soma theory of aging (DST) (Kirkwood 1977). Expanding on the idea of aging as an inadvertent byproduct of the random accumulation of mutations with detrimental effects, antagonistic pleiotropy proposes that genetic variants which are beneficial early in life may have a cost in that they become deleterious to survival in an organism's post-reproductive years (Williams 1957). Under this view, aging is a compromise for the selection of traits that favor early-life survival and fecundity at the expense of earlier mortality. Findings from several studies support this hypothesis: in laboratory studies of fruit flies, delayed reproduction resulted in delayed

senescence (Luckinbill et al. 1984). Studies in natural populations of birds and mammals have found a positive association between earlier age-at-first reproduction and mortality (Hammers et al. 2013; Hayward et al. 2014; Blomquist 2009). The disposable soma theory further extends antagonistic pleiotropy by arguing that transmission of the germ line the priority of an organism, that essentially serves as only as a vessel, and thus, after one's genes have been transmitted to successive generations, there is no incentive to invest in the expensive processes of somatic maintenance or repair. This framework is thus organized around the notion that aging is an evolutionary trade-off that results from the inherently finite resources an organism can allocate to any one energetically-demanding process, such as growth, somatic maintenance, or reproduction (Bartke, Sun, and Longo 2013; Bogin, Silva, and Rios 2007). While several studies show evidence in support of DST, it is also possible that the observed effects did not result from the allocation of limited energetic resources, but instead from antagonistic pleiotropy of yet-discovered genetic variants (Carter and Nguyen 2011). The lifespan-extending effects of dietary restriction and the detrimental effects of overnutrition on life expectancy stand in apparent contradiction to the pillars of this theory, as one would expect unlimited energetic resources to loosen intrinsic restrictions on lifespan (although arguments have been made to try square these effects with the paradigm of DST, using research in wild mice Shanley and Kirkwood 2000).

Ultimately, phenotypic expression in humans and many (if not most) other species is the combined product of perinatal conditions of the environment and a much longer species-specific evolutionary history (Gluckman, Hanson, and Low 2019). In a modern context, the flexibility that exists in the range of phenotypic expression, or *developmental plasticity*, enables deleterious alterations to fetal growth trajectories in response to inaccurate cues of environmental scarcity from the external world.

The effects of altered development programming are worse when the true conditions of the external world at birth do not match the version to which the fetus preemptively acclimated (Treviño et al. 2020). Signals of scarcity restrict fetal growth, reduce nephron density in the kidney, and induce other life-long metabolic alterations that increase the likelihood of cardiovascular disease and type 2 diabetes in adulthood (Barker 1995). Such alterations to the developmental trajectory of different organ systems have not been shown to be reversible when

they occur within the narrow perinatal window.

Other, more proximate explanations have been suggested to provide mechanistic insight into the molecular and physiological mechanisms that underlie the aging process, focusing on changes that occur during the lifetime of an individual, such as the loss of genomic integrity, epigenetic alterations, telomere attrition, the loss of proteostasis, dysregulated nutrient sensing, and mitochondrial dysfunction (López-Otín et al. 2013). Several of these mechanisms are inextricably linked, and in this context, epigenetic alterations are notably powerful because they can directly or indirectly affect any of the other processes (although they are not unique in this ability) (López-Otín et al. 2016). Mitochondrial dysfunction has long been a high-priority target of aging research, as it was initially believed that the reactive oxygen species (ROS) produced as a byproduct of cellular respiration were uniformly damaging. However, more recently it come to light that the response to ROS production appears to be dose-dependent (Berry and Kaeberlain 2021). When present at consistently high levels, ROS can cause damage that accumulates over time, resulting in lower rates of respiration and greater proton loss, both of which are associated with increased cellular aging (Lenaz et al., 2000). However, low levels of ROS are able to act as messenger molecules and have been shown to extend the lifespan of cells, possibly through sustained maintenance of the cellular response to damage (Lenaz et al., 2000). This is particularly salient for highly differentiated cell types like hepatocytes, which depend on the maintenance of cellular machinery over the long-term (rather than cell division to regenerate) to continue a functional and non-pathogenic existence.

Thus far, no singular grand theory of aging has prevailed. Nonetheless, a robust framework has emerged that highlights the intricacy of the phenotype of whole-organism aging and acknowledges the dynamic interplay between processes that contribute to variation in age-related decline and disease incidence (López-Otín et al. 2013; Lemoine 2021; López-Otín et al. 2016). The growing recognition of the complexity of organismal aging suggests that no singular mechanism or theory of aging is likely to explain all aspects of the phenomenon. Once thought to be a universal facet of life on this planet, aging has since been redefined as more flexible in nature, with some species seemingly

able to regenerate indefinitely (e.g., hydra, Chera et al. 2009; Klimovich et al. 2018), and others with atypically long lifespans for their body size (e.g., painted turtles, Warner et al. 2016), underscoring the questions that remain unresolved regarding the evolutionary underpinnings of biological aging.

Life History Strategy and Species-Specific Aging

A species' life history is structured by a series of developmental stages and transitions that together represent a strategy aimed at optimal resource use and reproductive success (Del Giucide et al. 2018). Long-lived organisms like humans and many other non-human primates have "long, slow" life histories that have been fundamentally shaped by particular evolutionary pressures and ecological conditions over millions of years (Jones 2011). Environmental stability and relative resource abundance are two key factors that influence a species' mortality risk and consequently, impact their reproductive strategy and other life history characteristics. An organism in a turbulent, unpredictable environment with high extrinsic mortality risk who attempts to maximize reproductive success by having many large litters as rapidly as possible will not reap the same benefit of a mechanism that (e.g.,) prevents the accumulation of tumorigenic mutations in a taxon whose life is expected to span multiple decades. Indeed, short-lived organisms mature more rapidly, reproduce more frequently, and have larger litter sizes as compared to longer-lived species (Lemaître et al. 2015; Chisholm et al. 1993), reflecting notable differences in life history strategy.

As humans grow older, they become less susceptible to permanent epigenetic alterations, although perhaps not immune (Jagust 2016). It could be argued that the biological and molecular networks whose dynamic activities are critical to quotidian health and survival are more efficient systems that come to replace the more mercurial epigenome as humans mature. In conditions of optimal health, the metabolic, immune, and stress response systems expertly pursue homeostatic conditions, responding to environmental experiences or insults of the kind humans encounter on a daily basis. The action of cortisol under the stress response system (SRS) assists with mounting physiological demands with immediate effects (e.g., alertness, metabolism), as well as those that occur over the short- (e.g., tissue repair, energy storage) and long-term (e.g., growth, development, reproduction) (Bogin 1999; Bartke and Quainoo 2018). Interestingly, while

anatomic and physiological aspects of the SRS are highly conserved across taxa, its temporal activation can vary considerably between species and may also be moderated by an individual's history of experience and exposure (Romero and Gormally 2019), suggesting epigenetic involvement in modulating these effects.

There has long been evidence to suggest that social and familial conditions, particularly during childhood, have far-reaching implications for health and well-being in humans. Prior to the discovery of the dynamic and reversible nature of epigenetic mechanisms, such implications were exceptionally difficult if not impossible to quantify. Today, through the lens of the epigenome it is possible to examine the ways in which different environments and social experiences impact health and longevity through interactions with the genome. Rhesus macaques exposed to certain environmental stressors have been shown to exhibit changes in DNA methylation similar to those observed in humans under comparable stressors, but it is unknown whether the variables associated with the acceleration of epigenetic aging in humans also lead to the same phenomenon in macaques. This is an important question as rhesus macaques are foundational to past and ongoing biomedical research.

Inter-individual Variation in Rates of Aging

For many common chronic health conditions, age is the biggest risk factor. Yet, aging and the rate at which it occurs reflect an incompletely understood biological narrative. With regards to non-inherited sources of variation that modulate the aging trajectory, diet, stress, and their downstream deleterious health effects are among the most potent. Obesity hastens the deleterious effects of age-related decline, and is a common cause of premature aging (Garaulet, Ordovás, and Madrid 2010). The global prevalence of obesity nearly tripled between 1975 and 2016 (World Health Organization 2021), and rates of chronic non-communicable disease related to metabolic dysfunction have been similarly on the rise in countries around the world (Ramos-Lopez et al. 2017).

For some individuals, the onset of cardiovascular disease, diabetes, neurodegenerative disorders, cancer or other age-related diseases can occur early in adult life (e.g., late in the fourth decade of life, or early in the fifth), while others experience

slower rates of decline and lower incidence of serious disease, and thus are able to maintain good health well into (and in some cases through) their ninetieth decade (Didier et al. 2016; Benayoun, Pollina, and Brunet 2015). While there is a well-documented relationship between increasing age and reduced cellular regeneration capacity, dysregulation of the immune and inflammatory responses, and a diminished ability to repair DNA damage, the rate and severity of age-related decline varies widely across individuals (Booth and Brunet 2016; Didier et al. 2016). While exposure to environmental factors like pollution, toxic chemicals, and tobacco smoke are known to cause DNA damage, other sources of cellular stress, like the production of reactive oxygen species by the mitochondria, are a normal and inevitable byproduct of cellular metabolism found in all oxygen-consuming organisms (Lombard et al. 2005). Individuals vary in how effectively they are able to repair DNA damage, which is, in part, dependent on the extent of the damage to which they have already been exposed (Lombard et al. 2005). This variation is partly a function of age, but is also determined by lifestyle and environmental factors that have been repeatedly shown to have significant positive or negative effects on general health and longevity (Dato et al. 2017; Beach et al. 2015; Gao et al. 2017; Ramos-Lopez et al. 2017).

Variability in the progression of aging is partly driven by epigenetic differences (Dirks, Stunnenberg, and Marks 2016). Broadly speaking, biological aging is the result of a decline in cellular regenerative capacity and the accumulation of senescent cells in various tissues of the body after an organism has reached its peak reproductive period (Bhatia-Dey et al. 2016; Lowsky et al. 2014). While some genetic variants found at high frequencies in centenarians have been associated with increased longevity, their role and influence on the aging process remains unclear, in part due to each individual's unique history of gene *x* environment interactions (Sebastiani et al. 2012).

The only known behavioral intervention that has been shown to extend lifespan and healthspan across a number of taxa is dietary restriction (DR, also called caloric restriction [CR]). While the mechanisms by which DR delays aging remains elusive, there are numerous reports indicating that caloric restriction may preserve mitochondrial activity (Zhang et al. 2022; Morgunova, Shilovsky, and Khokhlov 2021). Dietary restriction is typically defined as ~30% reduction in daily caloric intake without malnutrition. One hypothesis posits that it exerts a pro-longevity effect through the regulatory actions of silent information regulator protein

deacetylases (sirtuins), a family of proteins that has been widely shown to extend lifespan in model organisms (Vijg and Campisi 2008; B. Yang, Britton, and Kirchmaier 2008; Kitada et al. 2019).

Dietary restriction has been shown to delay age-related morbidity and extend lifespan repeatedly in short-lived model organisms (Greer et al. 2007; Hahn et al. 2017; Cole et al. 2017; Bitto et al. 2015) as well as in adult rhesus macaques (Colman et al. 2009; Mattison et al. 2012), and even quite possibly in humans: in a six-month human trial, participants who followed a calorically-restricted diet experienced increased expression of *SIRT1*, a regulator of mitochondrial biogenesis, upregulation of the mitochondrial protein TFAM, and showed a greater number of mitochondria when compared to controls (Barja 2004; Berry and Kaeberlein 2021). It is notable that no pro-longevity effect was found among macaques who began DR as juveniles (Mattison et al. 2012; 2017) a finding which I investigate in Chapter III of this dissertation.

Molecular Mechanisms of Aging

Human aging is associated with pervasive molecular change, including altered patterns of gene expression, modifications of the epigenetic landscape, and changes to genomic architecture (Lu et al. 2017; Siebel and Lendahl 2017; Brunet and Berger 2014). Genomic integrity deteriorates as a function of both age and the rate of DNA damage and is associated with substantial changes in the epigenomic landscape (Booth and Brunet 2016). A long-active area of investigation has been the hunt for "longevity genes", of which only one (*APOE*) has been identified as directly influencing risk of developing an age-related condition (Alzheimer's disease) later in life, depending on the variant of the gene an individual has inherited (Walker et al. 2021). There is also considerable interest in determining the relative contribution of the genetics versus the environment to the pace and progression of age-related biological change—this has sometimes been reduced to the "nature versus nurture" debate, reflecting a false dichotomy. While estimates for the heritability of longevity across generations have been proposed (e.g., Herskind et al. 1996), others have argued that such estimates are inflated by assortative mating (Ruby et al. 2018), and the debate is ongoing. Thus, despite substantial progress in the science of

aging over the past decade, few promising molecular targets have been identified for therapeutic intervention. The few that have exhibited staying power thus far include regulatory actions of silent information regulator protein deacetylases (sirtuins), the Forkhead box (*FOXO*) family of transcription factors, and the much broader category of epigenetic mechanisms (Balistreri et al. 2013). While the mechanisms by which dietary restriction is able to delay aging remain elusive, there are reports suggesting it may involve mitochondrial activity (Zhang et al. 2022; Morgunova, Shilovsky, and Khokhlov 2021). As previously noted, low levels of ROS produced by the mitochondria can act as a low-level cellular stressor that promotes the maintenance of cellular mechanisms of self-preservation (Ristow and Schmeisser 2014).

Cells employ a number of compensatory mechanisms to help mitigate the effects of genomic damage and instability caused by epigenetic modifications and telomere attrition (López-Otín et al. 2013). While effective if used intermittently, when chronically activated these compensatory processes become contributing factors to age-related decline (Engelfriet et al. 2013). One such example is cellular senescence, a hallmark of molecular aging (Rakyan et al. 2011; Benayoun, Pollina, and Brunet 2015). While senescence is often seen as a detrimental process, cellular capacity to enter a senescent state appears to be a protective mechanism against tumorigenesis, as malignant cells are more likely to evade detection with increasing age due to a decline in immune system function (López-Otín et al. 2013). However, circulating senescent cells exhibit a pro-inflammatory cytokine profile known as the senescence-associated secretory phenotype (SASP), a cause of tissue damage and dysfunction (Benayoun, Pollina, and Brunet 2015). As senescent cells accumulate with age, their pro-inflammatory properties become increasingly problematic, triggering yet another series of pathological age-related events that contribute to “inflammaging”, a process by which low-grade inflammation accelerates biological aging (Franceschi et al. 2018).

Genome instability is a near-universal hallmark of aging, arising from repeated molecular assaults that induce point mutations, chromosomal abnormalities, translocations, and mitochondrial dysfunction (Lemoine 2021; López-Otín et al. 2013). At peak fitness, cellular repair machinery mitigates these harmful effects by repairing DNA damage and preserving mitochondrial integrity (Rossi et al. 2007; Palikaras, Lionaki, and Tavernarakis 2015). When

cellular repair and maintenance machinery can no longer match the pace at which damage occurs, permanent cellular damage begins to manifest.

Genomic integrity is also compromised by loss of constitutive heterochromatin (usually at telomeres, centromeres, and pericentromeres) and general histone loss that result in transcriptional deregulation (Sen et al. 2016). Abnormal patterns of gene expression are a hallmark of an unstable and thus potentially dysfunctional cell. Epigenetic dysregulation compromises genomic integrity, and in doing so increases the likelihood of replicative senescence or, alternatively, oncogenesis, both of which have negative implications for organismal health (Bhatia-Dey et al. 2016; Sen et al. 2016). Cellular senescence is strongly linked to systemic aging, and the rate at which senescent cells accumulate appears to play a key role in determining how well *and* how rapidly an individual ages at the biological level. However, replicative senescence can be triggered by many different molecular and environmental factors.

Unrepaired DNA damage can reorganize the nuclear architecture, impacting genes and transcriptional pathways of high functional importance (López-Otín et al. 2013). Factors that further precipitate genomic instability with increasing age include telomere attrition and epigenetic alterations (e.g., DNA and histone methylation, histone acetylation, changes to heterochromatin conformation) (Benayoun, Pollina, and Brunet 2015). While human aging is remarkably plastic, increasing age is nevertheless characterized by specific changes in gene expression that weaken global genomic stability, alter genomic architecture and the epigenomic landscape, and increase risk of cancer, neurodegeneration and cardiovascular disease (Sen et al. 2016; Brunet and Berger 2014). Together, these factors manifest in progressive functional deterioration and visible decline of tissue and organ systems, ultimately leading to mortality.

Historical Difficulty of Quantifying Biological Age

Biological age is a measure of the structural and functional state of the body. Unlike chronological age, biological age accounts for interindividual variation in the progression of age- related phenotypic change, and therefore provides a more accurate picture of systemic health (Benayoun, Pollina, and Brunet 2015; Chen et al. 2016;

Sebastiani et al. 2012).

Chronological age is a strong predictor of morbidity and mortality but has long acted as a relatively crude stand-in for biological age (Jylhävä, Pedersen, and Hägg 2017; Bell et al. 2019). Because it has been well established that individuals age differently and at different rates, the utility of chronological age as a predictor of health outcomes and mortality is limited. Quantifying biological age has proved challenging, and further complicating the matter is the lack of an universally agreed-upon definition or standard of measurement among the wider aging research community. Regardless, the concept of biological age offers a more informative and valuable measure of variation in health and disease risk than chronological age can alone.

Ideally, a molecular biomarker of aging should track the progression of age-related physiological change such that it is able to determine the rate at which an individual is aging (akin to the annual rate of increase in the likelihood of mortality) and predict future physical and cognitive capability, disease risk, and mortality (Jylhävä, Pedersen, and Hägg 2017). Nine classic hallmarks of the molecular aging process were proposed by López-Otín and colleagues (2013) and have grown to become a part of the foundational framework of molecular aging research (Lemoine 2021). Several have already been tested as biomarkers or have the potential to be used as such. Those that have already been involved in aging biomarker development include telomere attrition, epigenetic alterations, and to a lesser extent, dysregulated nutrient sensing, but no one measure has been able to claim status as an universal and fully comprehensive biomarker of aging. Each one presents its own advantages and drawbacks: for example, age-associated decline in leukocyte telomere length has been widely investigated as a putative biomarker of aging (von Zglinicki and Martin-Ruiz 2005) but the fragility of telomeric DNA continues to pose a considerable challenge in obtaining reliable telomere length measurements (Goldman et al. 2018).

Molecular measures of biological age should reflect the activity of the processes responsible for age-related change and be capable of differentiating between normal and pathological age-related phenomena (Mamoshina et al. 2018). In addition to biophysiological properties desired in a molecular marker of aging, key methodological factors to be considered include assay cost, replicability, and robustness to variations in sample quality; all must be taken into account when designing molecular biomarkers (Dirks, Stunnenberg, and Marks 2016; Bock

2012; Kurdyukov and Bullock 2016).

Epigenetic Mechanisms of Aging

While aging involves the interdependent activity of numerous external and internal systems, epigenomics has already provided new avenues of exploration to decipher how individual variation and interactions with the external world become biologically embedded to contribute to differences in health and life expectancy. The epigenome refers to a set of biochemical mechanisms that can affect gene regulation and expression without altering the underlying DNA sequence (Bird 2002). Unlike the genome, the epigenome is both dynamic and flexible; it is tasked with unifying information and instructions encoded in the genome with cellular, extracellular and environmental signals to produce modifications to a phenotype (Campbell and Wood 2019). Epigenetic mechanisms are the means by which environmental signals induce changes to the epigenetic landscape, influencing gene expression through biochemical modifications to the genome and altering chromatin structure (Rose and Klose 2014). Changes in DNA methylation, posttranslational histone modifications, rearrangement of the nuclear architecture, and RNA-associated silencing all play a role in determining the molecular, functional, and structural features that constitute the epigenomic landscape (Klemm, Shipony, and Greenleaf 2019).

The epigenome mediates gene-environment interactions, and environmental variables can modify gene activity and phenotypic expression via epigenetic mechanisms. However, the extent to which different variables leave a mark on the "aging ledger" kept by the epigenome is not clear. While results from gene expression studies are generally straightforward to interpret in that they provide direct evidence of the up- or downregulation of specific genes, DNA methylation wears different hats depending on the genomic context in which it is found (i.e., its effect on gene regulation or other aspects of molecular function can vary markedly by genomic element): it is associated with the suppression of gene expression in promoter regions (promoters being the best characterized of all regions in which DNA methylation occurs), but its effect in other genomic regions is much more varied and can depend on latent, context-specific

parameters. Thus, while patterns of DNA methylation are known to change with age, the nature of the changes induced by DNA methylation are not well understood. Nonetheless, evidence strongly suggests that DNA methylation plays a crucial role in age-related biological change and variability in the aging trajectory; epigenetic modifications are well poised to act as proxy measures of biological aging due to their sensitivity to behavioral and environmental factors known to affect parameters of individual health.

Epigenomics has already provided new avenues of exploration to decipher how individual variation and interactions with the external world become biologically embedded to contribute to differences in health and life expectancy. Nearly all aspects of cellular function in a multicellular organism require precise regulation of differential gene expression (Booth and Brunet 2016). During prenatal development, DNA methylation acts as part of a broader network of epigenetic mechanisms to establish differences in the structure of posttranslational chromatin states. Modified chromatin states promote functional polarization of chromatin domains, thereby allowing stable commitment to transcriptional activity or inactivity, and the capacity to commit genetically homogenous cells to a wide, heterogeneous variety of cellular fates (Bird 2002).

DNA is densely packaged and wrapped around an octamer of core histone proteins to form the basic unit of a chromatin polymer, the nucleosome (Hochberg et al. 2011). In cells that are not actively dividing, units of chromatin adopt either a euchromatic or heterochromatic configuration (Booth and Brunet 2016). Euchromatin is the transcriptionally active state, in which chromatin is loosely packaged in an open conformation, allowing transcription factors and other regulatory proteins access to DNA. In the heterochromatic state, DNA is tightly condensed, and this closed conformation prevents transcriptional activity (Allis and Jenuwein 2016).

Epimutations (disruptions to existing patterns of epigenetic regulation), accumulate at varying rates with age, increasing rates of aberrant epigenetic change and modifying gene expression (Issa 2014). A global trend of DNA hypomethylation is also observed with increasing age, although there are specific loci often enriched for genes related to development as well as potential oncogenesis which become hypermethylated with age (Kananen et al. 2016; Salminen et al. 2012; Salminen 2021). Age-related changes in histone methylation are known to contribute to the loss of heterochromatin, which disrupts chromatin architecture and increases the likelihood of DNA damage. Chromatin reorganization can modify patterns of gene expression, reduce

transcriptional fidelity, and increase the likelihood of mutations, thereby intensifying genomic and mitochondrial deterioration (Engelfriet et al. 2013). Abnormal restructuring of heterochromatin can also create a more permissive environment for the activation and relocation of mutagenic transposable elements, which disrupt coding and regulatory processes and further contribute to the loss of genomic integrity (Sturm, Ivics, and Vellai 2015). These myriad epigenetic changes drive a growing number of cells towards apoptosis or replicative senescence (Engelfreit et al. 2013).

Transcriptional activation of most protein-coding genes occurs at promoters that contain an unusually high density of CG dinucleotides, known as CpG islands. These are stretches of the genome typically between 500 bp to 2 kb long and with at least 50% guanine-cytosine (CG) content (Lee and Pausova 2013; Day et al. 2013). The accumulation of methyl marks at multiple CpG sites in the same genomic region is known as hypermethylation, while their loss is referred to as hypomethylation (Day et al. 2013). When hypermethylation occurs in promoter regions, expression of the associated gene is typically suppressed (Lee and Pausova 2013). Interestingly, when methylation occurs within gene bodies, it is associated with enhanced rather than repressed gene expression, although in general, its effect outside of promoters is only partially understood (Campbell and Wood 2019).

DNA methylation suppresses gene expression by preventing or facilitating binding of transcriptional activators or repressors, respectively, and through recruitment of repressive histone-modifying enzymes (Booth and Brunet 2016). In most individuals, CpG-rich regions show modest but consistent levels of methylation, but the specific identity of the methylated site varies by individual. In contrast, CpG-poor regions show greater variability in the degree of methylation, but more uniformity in terms of which sites are methylated across individuals (Johnson and Tricker 2010). Studies of identical twins have shown that inter-individual differences in genome methylation patterns increase as a function of age, leading to differences in gene expression and lifespan (Moskalev et al. 2014; Thompson et al. 2010). Twin pairs who exhibited greater lifestyle differences or spent less time together over the course of their lives show greater inter-individual variability, highlighting an environmental influence on the epigenomic

landscape (Lee and Pausova 2013). Variation in epigenetic markers between cells from the same tissue also increases with age (Maegawa et al. 2017; West, Widschwendter, and Teschendorff 2013; Mendenhall et al. 2021). This trend of increasing variability among certain epigenetic markers over time is known as epigenetic drift, and is believed to be driven by both intrinsic and environmental factors (Lee and Pausova 2013; Issa 2014). Epigenetic changes that occur in response to environmental stimuli could underlie certain aspects of inter-individual vulnerability to age-related disease and decline (Perna et al. 2016), but the mechanisms by which this occurs remain obscure.

Predictive Models of Aging Using the DNA Methylome

An epigenetic model with the capacity to quantify biological age in population-based research was first realized in 2013, when Horvath and Hannum and colleagues independently published separate versions of human-specific epigenetic clocks. The epigenetic clock is a biological age predictor model that measure changes in DNA methylation at select CpG sites to predict chronological age with unprecedented accuracy (Horvath 2013; Hannum et al. 2013). In healthy individuals, chronological and biological age are typically well correlated. However, individuals who experience prolonged socioeconomic or psychosocial stress, are pre- disposed to particular age-related diseases, to or who actively manage chronic non-communicable conditions (such as type 2 diabetes or cardiovascular disease), often exhibit signs of pathological or accelerated aging, which is reflected in a predicted biological age that exceeds actual chronological age, indicating increased risk of mortality (Gassen et al. 2017; Horvath et al. 2014). While the two human clock models are based on a similar framework and methodology, each has specific strengths depending on context: as a multi-tissue age predictor, Horvath's epigenetic clock theoretically has a wider range of potential applications, and may be less likely to detect tissue-specific changes (Quach et al. 2017). The epigenetic clock model developed by Hannum and colleagues was correlated with age-related changes in gene expression, and could possibly be used in conjunction with transcriptome dynamics to characterize molecular aging in greater detail (Hannum et al. 2013).

At the site-specific level, it is apparent that methylation levels change only modestly over the course of the lifespan. Despite the small contribution to the aging phenotype of each CpG site

in isolation, when assessed in aggregate, changes in methylation at these sites closely tracks the progression of aging. The cumulative age effects of these small changes suggest that aging may be regulated by a large number of small changes distributed throughout the genome.

It has been proposed that these age predictor models measure the "work" performed by an epigenetic maintenance system whose main function is the preservation of genomic stability (Horvath 2013). The intensity of the "work" performed by this maintenance system corresponds to the pace at which the clock "ticks". Under this premise, the clock is expected to tick most rapidly during growth and development; when growth is largely complete, the ticking rate slows down and assumes a more constant pace during normal aging. However, threats to genomic stability are expected to upregulate activity of the epigenetic maintenance system, theoretically accelerating DNA methylation age. Disruptions to the genomic architecture, mitogenic activity, and carcinogenesis are all expected to activate the epigenetic maintenance system and thus accelerate rates of DNA methylation aging (Nwanaji-Enwerem, Weisskopf, and Baccarelli 2018; Horvath 2013).

An important feature of epigenetic clock models is that they are both accurate predictors of age but can also be used to identify outliers who do not follow the typical pace of normal aging (Hannum et al. 2013). This allows the epigenetic clock to be used both as a measure of chronological age and a putative indicator of disease and mortality risk (Perna et al. 2016; Marioni et al. 2015a; 2015b). Epigenetic age predictor models appear to be sensitive to a number of physiological, psychological, and environmental factors, contributing to their robustness as a method aimed at capturing biological age. While other studies have identified genomic patterns of age-related change in DNA methylation (Bocklandt et al. 2011; B. C. Christensen et al. 2009), they never gained widespread popularity for use as biomarkers, possibly owing to issues related to replicability of performance.

Over the past nine years, epigenetic clocks has been applied in a variety of contexts to examine the impacts of lifestyle, behavior, diet, hereditary disorders, chronic infection, and non-communicable chronic diseases on the pace of aging. Results from

these analyses have provided consistent if somewhat enigmatic support for the clock's ability to capture true variation in the progression of aging and age-related decline. Contributing to the enigma is the "black box" nature of the type of machine learning used to produce these models—while widespread application and testing of the human clocks provide substantial evidence for their capacity to capture (some of the) variation in biological aging that chronological age cannot, attempts at identifying the molecular mechanisms underlying their functionality have yielded no definitive results. It is also notable that hazard ratios for the relationship between clock-based estimates of age acceleration/deceleration and the incidence of disease or age-related mortality are very modest (e.g., Perna et al. 2016), suggesting these models capture an incomplete picture, despite being one of the most robust biomarkers of aging in use today. The "next-generation" of human clocks have incorporated biochemical and behavioral data to produce multi-level age predictor models that more accurately model human risk of mortality. While this is not typically feasible for studies involving non-human animals, empirical application of non-human clocks to the study of putative pro- or anti-aging interventions or behaviors can elucidate the variables to which such models are more or less sensitive. This, in turn, will shed more light on the physiological systems and molecular networks that play active roles in DNA methylation changes that promote healthy or pathogenic processes of aging.

Dissertation Research

The molecular pathways involved in the process of biological aging are multitudinous, and the environmental and genetic factors that influence the activity of these pathways, thereby driving inter-individual differences in rates in biological aging, are equally numerous and complex. Many of the physiological processes that ultimately result in age-related decline and mortality are inescapable; however, they are not entirely immutable. It is clear that different individuals age at different rates, although it is not clear why or how this variation manifests. This dissertation research was aimed at elucidating sources of variability in the pace and shape of the aging trajectory using the lens of the DNA methylome to systematically investigate the molecular nature of aging in a targeted, tissue-specific approach. As has been recognized previously (e.g., Bell et al. 2019), targeted tissue-specific models are expected to be more

valuable to the advancement of our understanding of aging and age-related disease.

Description of Chapter II: An Epigenetic Clock Captures Variation In Molecular Aging In Two Cercopithecoid Species

The research constituting this chapter had two main objectives. The first was to develop an epigenetic age predictor model for rhesus macaques. To this end, I used DNA methylation data from whole blood from 563 rhesus macaques living on the island of Cayo Santiago to develop an epigenetic clock model using data from a free-ranging, long-lived non-human primate.

The second objective of this research was to apply the clock to evaluate its predictive performance using independent datasets. However, due to the nature of the assay, DNA methylation data generated using reduced representation bisulfite sequencing (RRBS) are never uniform between independent datasets and this heterogeneity in genomic coverage complicates inter-study application of epigenetic clock models. Thus, to increase the generalizability of my model to other studies and datasets, I develop a simple but highly effective method ("sliding window method") to increase the degree of shared coverage between datasets. I tested the efficacy of this "sliding window-based" approach and the functional capacity of my model to estimate epigenetic age with high accuracy using previously collected data from two cercopithecoid species living in highly disparate environments: the first, a captive group of 43 female rhesus macaques housed at Yerkes National Primate Research Center, and the second, a wild population of 271 male and female baboons living in Amboseli National Park, in Kenya. In doing so, I was able to present a new, updated method with greater cross-study applicability, enabling more powerful comparative analyses from existing and future RRBS datasets. I also demonstrated that the RheMacAge model is applicable not just to rhesus macaques, but also predicts age with high accuracy in a second closely-related and biomedically relevant species, baboons (*Papio* sp.). Finally, I test the model's sensitivity to the effects of social status in a pilot analysis of data from macaques on Cayo Santiago, and show that the RheMacAge model recapitulates the effect of male rank on epigenetic age in

baboons that was first reported in the publication of a baboon clock by Anderson et al. (2021), demonstrating its sensitivity to factors that modulate the pace of aging even when utilized in a separate species.

The research in this chapter is yet unpublished but includes contributions from multiple co-authors. Co-author Marina Watowich (MW) ran the statistical test to determine if there was a significant association between social status (rank) and the pace of epigenetic aging, and contributed written text to the Results section under the sub-heading "Effects of Socio-environmental Variables on Residual Epigenetic Age". MW also generated the figure showing the relationship between rank and epigenetic aging (Figure 5). Co-author Kenneth Chiou generated the PhastCon scores that I used to test the clock loci for enrichment for evolutionarily conserved sequences. He contributed text describing the methodology for PhastCon score generation in Appendix B**,** under the sub-heading "Enrichment Analysis for Evolutionarily Conserved Sequences". Apart from the two aforementioned sections, all work in this document is my own. Additional co-authors who had the opportunity to review the work in Chapter II are Jordan A. Anderson, Lauren J.N. Brent, James P. Higham, Julie E. Horvath, Melween I. Martínez, Arianne Mercer, Michael J. Montague, Michael L. Platt, Sierra N. Sams, Noah Snyder-Mackler, Kirstin N. Sterner, and Jenny Tung.

Description of Chapter III: Characterization of the Rhesus Liver Methylome by Sex, Age, and in Response to Dietary Restriction

Diet is one of the most consequential behavioral variables in the human environment, with profound effects on health and longevity. However, the relationship between diet and aging is not well understood, and efforts to characterize this relationship in humans have struggled to disentangle the effects of diet from confounding variables. Because nutrition is arguably the most powerful environmental determinant of health over the long lifespan typical of primate species, characterizing the effects of diet on longevity in a primate model is an important task. The research described in this chapter thus had three primary objectives. The first was to build an epigenetic clock specifically for liver tissue; the second objective was to apply the liver-specific

clock model to a study of long-term dietary restriction to test the hypothesis that DR delays the rate of biological aging. The third objective was to compare the effect of DR on the aging methylome in age- and sex-matched samples to better understand the molecular effects of DR and the functional underpinnings of its pro-longevity effects.

Two major studies of long-term DR have been conducted in long-lived primates. One was performed at the University of Wisconsin, Madison, and investigated how DR affected risk and incidence of age-related disease and life expectancy in a population of adult rhesus macaques using a case/control study design. The second was conducted at the National Institute on Aging (NIA); analysis of this population is the focus of this chapter. Unlike the UW study, results from the NIA study of long-term DR are more ambiguous and involve individuals who started on a calorically-restricted diet at multiple developmental time points and ages. To address this gap in knowledge, I constructed an epigenetic clock using banked liver tissue samples from 96 rhesus macaques from the Oregon National Primate Research Center (ONPRC). I subsequently tested the effect of dietary modification, specifically a calorie-restricted diet, on the pace of epigenetic aging using this model. Importantly, this is the only research to date to examine the effect of caloric restriction on lifespan when started during the early juvenile stage in a long-lived non-human primate.

Finally, I characterized patterns of epigenomic aging across the adult lifespan in the liver of healthy rhesus macaques, as well as in each sex independently, providing a novel window into the epigenetic changes associated with tissue-specific aging in both sexes of this species through differential methylation analyses. I comparatively examined the degree of epigenetic age-related change in the liver between normal aging and DR study populations to shed light on the processes of epigenetic aging in a critically important but underexplored tissue.

Description of Chapter IV: Concluding Summary

In the final chapter, I summarize the intellectual contributions that have been made through the completion of the research I describe here. I propose potential explanations for the most salient findings from these analyses, with a particular focus on

the outcomes from Chapter III. I also briefly describe and exhibit a tissue-specific clock I constructed using 96 hippocampus samples from rhesus macaques but was not able to formally include in this dissertation. Finally, I conclude by suggesting future directions in which this research may go to continue to reveal novel and interesting insight into the relationship between the environment, the epigenome, and aging.

Bridge to Chapter II

Blood is often the preferred biological sample type for periodic evaluation of general health in humans and non-human animals. Several aspects of blood and blood sample collection make it a logical choice for this purpose. Blood circulates throughout the body and interfaces with all tissues and organs; for this reason, it is relied on as a proxy measure of systemic health. Additionally, changes in cell type proportions among immune cells found in blood can be indicative of an underlying risk factor for disease or disease state. Venous blood draws are a quick and efficient means of obtaining a biological sample from a healthy living organism especially compared to most other tissues in the body. Compared to biopsy, this is a minimally-invasive procedure that can be completed by a phlebotomist in minutes. In the following chapter, I investigate age-related changes in the methylome of semi-wild rhesus macaques living on the island of Cayo Santiago using whole blood samples collected after darting and briefly anesthetizing an animal before re-releasing them back into the group. I built and tested an age predictor model on two additional cercopithecoid populations and examined the putative epigenetic response to environmental variables expected to influence the pace of aging, finding support for a relationship between male social status and the rate of epigenetic aging. The shape and strength of this relationship differed between male rhesus macaques and male baboons in ways that are putatively reflective of the different ways that males of each species structure of their respective dominance hierarchies.

CHAPTER II

AN EPIGENETIC CLOCK CAPTURES VARIATION IN MOLECULAR AGING IN TWO CERCOPITHECOID SPECIES

Introduction

Research in this chapter includes unpublished co-authored material. Marina Watowich (MW) and Kenneth Chiou (KC) made substantial contributions to the work in this chapter. MW performed statistical analysis of the relationship between social status and epigenetic aging and generated Figure 5. KC generated the data used for the analysis of evolutionarily conserved sequences. Each wrote a paragraph describing the work performed. All other writing and analyses are my own.

Chronological age is the strongest risk factor for the development of most chronic, non-communicable diseases (Wagner et al. 2016). However, chronological age cannot capture individual variation in health and disease risk beyond that associated with the passage of time. Measures of biological age aim to capture this variation to improve predictions of individual morbidity and mortality risk. The epigenetic clocks constructed by Horvath (2013) and Hannum and colleagues (2013) were the first models of biological age to be successfully used outside their study of origin and have gained widespread use as biomarkers of health (see Nwanaji-Enwerem, Weisskopf, and Baccarelli 2018; Horvath and Raj 2018 for review). Recent work in humans has shown that a more rapid rate of age-related physiological decline compared to the average for one's birth-year cohort (i.e., accelerated aging) is associated with a greater risk of death (Chen et al. 2016; Levine et al. 2018; Marioni et al. 2015) and greater predisposition to several major diseases of aging (Ambatipudi et al. 2017; Z. Yang et al. 2016; Zheng et al. 2016). Epigenetic clocks may also capture socio-environmental effects on the pace of aging, such as exposure to traumatic events during military combat (Boks et al. 2015), adverse childhood experiences (Miller et al. 2015) or the long-term health implications of alcohol and tobacco use (Beach et al. 2015). Yet, the way in which different social and ecological

factors “get under the skin” to regulate shifts in disease and mortality risk is not well understood. Development of epigenetic clocks in non-human animal models provides opportunities for comparative analyses across species using data from controlled experimental settings or from multigenerational field studies.

Macaques and baboons are two ideal models for humans because they are close evolutionary relatives with similar life history strategies to humans. This is particularly relevant for the study of aging because environmental effects may not manifest in a similar manner, if at all, in short-lived species (e.g., rodents) with different life history strategies from humans. Indeed, questions that have been notoriously challenging to study in humans can often be brought to fruition in these closely related non-human primate relatives, such as those related to the long-term health effects of diet and nutritional intake or chronic stress (Kanthaswamy et al. 2017; Rawlins and Kessler 1986). Socially-living non-human primate species like macaques and baboons offer an excellent model for examining how differences in social status contribute to disparities in health and well-being (Snyder-Mackler et al. 2020). Among gregarious NHP species, social relationships and interactions are structured by dominance hierarchies. Individuals with high social status (dominant or high-rank individuals) have greater access to environmental (food, water, resting sites safe from predators) and social (mates, grooming partners) resources (Sapolsky 2005). Low social-status individuals (subordinate or low-rank individuals) are more often the targets of aggressive behavior, are less frequently groomed by other group members, and exhibit more fear- and anxiety-related behaviors compared to dominant individuals, such as grimacing, lip smacking, and vigilant scanning of the social environment (Sapolsky 2005; Shively and Day 2015). Importantly, low social status in human and non-human primates is associated with greater risk and incidence of inflammatory diseases and shorter lifespan (Snyder-Mackler et al. 2020). Studies in captive female rhesus macaques show differences in feeding behavior, body fat storage, and rates of obesity between dominant and subordinate individuals: low-status individuals are more likely to eat at night, consume more calories, and are predisposed to store visceral rather than subcutaneous fat, which contributes to higher levels of disease-associated inflammatory cytokines and adipokines (Wilson et al. 2008; Shively and Day 2015). Given that many of these health effects mirror those associated with chronic social stress and health inequity in humans, the relationship between social status, disease- and mortality risk has

translational relevance to the study of biological aging and to the characterization of what determines resilience or vulnerability to the effects of stress on long-term health outcomes.

The most extensively applied DNA methylation clocks to date were built using DNA methylation data from humans generated on Illumina Infinium microarrays (Hannum et al. 2013; Horvath 2013). By contrast, many of the non-human and non-model organism studies of DNA methylation conducted over the last decade have used high-throughput bisulfite sequencing (BS-seq) approaches due to the high cost of developing new species-specific arrays. These studies have thus generated a large amount of BS-seq data from which additional value might be extracted with the appropriate tools. However, sequence-based clock models are often less generalizable than array-based clocks because of variability in precisely which CG-rich regions of the genome are covered. This characteristic can limit comparative analyses or external application of a clock model because CpG sites cannot be used for prediction if they are absent from the dataset to which the model is being applied.

In developing a non-human primate epigenetic clock, we aim to facilitate comparisons among species whose aging trajectories are more like humans both in duration and timing of key developmental events compared to shorter-lived model organisms like rodents. To this end, our study had two primary objectives: (1) to develop and validate a generalizable epigenetic clock model for use with BS-seq data, and (2) to use this model to explore social environmental sources of variation in biological aging in a pilot analysis. First, we built a sequence data-based epigenetic clock model using blood samples from a population of free-ranging rhesus macaques living on Cayo Santiago. Because CpG sites within close physical distance to one another tend to have similar methylation levels, we partitioned the rhesus macaque genome into 2.85 million non-overlapping windows (each 1 kb in length) and subsequently grouped CpG sites in our dataset into these 1 kb-long windows. Using this sliding window-style approach (as opposed to individual sites) substantially improved our model's generalizability across datasets. Finally, we applied our model to two independently generated datasets, demonstrating not only the cross-*study* but cross-*species* applicability of our approach in two non-human primates (NHP) with exceptional research importance, rhesus macaques

(*Macaca mulatta*) and baboons (a heavily admixed population yellow (*Papio cenocephalus*) and anubis (*Papio anubis*) baboons living at Amboseli National Park, hereafter referred to using the more colloquial "Amboseli baboons", due to the relatively high prevalence of admixed individuals [see Vilgalys et al. 2021]).

Methods

Study Populations and Sample Collection

Our primary dataset consisted of 563 whole blood samples (n unique individuals=493) drawn from sedated rhesus macaques by veterinary staff as part of routine capture-and-release efforts on Cayo Santiago, an island located 1 kilometer off the eastern coast of Puerto Rico. This colony of free-ranging rhesus macaques was first established on Cayo Santiago in 1938 with a founder population of approximately 400 individuals of Indian origin (Rawlins and Kessler 1986). Demographic, socio-behavioral, and biological data have been routinely collected on the island since 1956. As of 2022, the population has grown to more than 1,600 individuals, organized into 11 extant social groups. The data used in this study came from 273 female and 220 male rhesus macaques aged 1.32 months to 28.82 years, and were collected from 2010 to 2018. Age at sexual maturity for rhesus macaques on Cayo Santiago is approximately four years, and median lifespan is approximately 18 years (this is lower than the average lifespan for captive rhesus macaques, whose longevity is benefited by the routine medical care and extensive health monitoring that typically occurs at research institutes). A 20-year old female rhesus macaque in captivity has a remaining life expectancy approximately comparable to a 60-year old woman in the United States. Sixty-six individuals were sampled more than once during this study: sixty-two individuals were sampled twice and four individuals were sampled three times. Blood was collected into K3 EDTA vacutainer tubes (BD Biosciences) which were stored at -80°C within eight hours of collection. Ethical approval was granted by the University of Puerto Rico, Medical Sciences Campus (protocol number A400117) for all samples collected.

RRBS Data Generation

Genomic DNA was isolated from whole blood using the Qiagen Blood and Tissue DNA kit (QIAGEN, Hilden, Germany). To measure CpG methylation, we used reduced representation

bisulfite sequencing (RRBS) (Gu et al. 2011). To prepare RRBS libraries, we followed the library preparation protocol detailed on the Snyder-Mackler Lab website (wp-content/uploads/2020/03/SMack_Lab_RRBS-with-Zymo-EZDNA-MagBead.pdf). Briefly, we digested extracted DNA using the MspI restriction enzyme, which cuts at CCGG sites, ligated NEBNext methylated adapters (Illumina Inc., San Diego, CA), bisulfite converted the DNA using the Zymo EZDNA Methylation- Lightning™ Kit (Zymo Research, Irvine, CA), and amplified the final fragments using PCR with NEBNext Multiplex Oligos to barcode each library. Libraries were sequenced in two batches. Batch 1 contained 104 samples (2x50bp reads) sequenced on an Illumina NovaSeq S2 flowcell. Batch 2 was made up of 527 samples (2x100bp reads) and sequenced on a NovaSeq S4 flowcell.

Secondary RRBS Datasets

We tested the generalizability and performance of our model using two independently generated RRBS datasets. To test generalizability across populations and study systems of the same species, we used samples from 43 female rhesus macaques, aged 3.1 to 20.1 years, housed at Yerkes National Primate Research Center (YNPRC). RRBS libraries were generated from purified classical monocytes (CD3-/CD14+) collected as part of an unrelated study examining dominance rank effects on gene regulation and immune function (Snyder-Mackler et al. 2016a).

To test the generalizability of our clock to another species that is highly important in biomedical research, we applied our model to a second RRBS dataset generated from 271 whole blood samples collected from wild baboons (from Anderson et al. 2021, SRA project accession PRJNA648767). This dataset contained samples collected from 138 females and 133 males, aged 1.93 to 26.34 years.

Alignment and Preprocessing

We trimmed sequenced reads with Trim Galore! (v0.4.5) (Martin 2011), and used MultiQC (Ewels et al., 2016) to evaluate the quality of the trimmed reads and to check for additional adaptor contamination or low-quality sequences. Mean sequence quality was assessed using Phred scores, which exceeded the minimum acceptable threshold (>20) for all libraries. For both our newly generated data and the two external datasets, we used Bismark (v0.20.0) (Krueger and Andrews 2011) for alignment and methylation calling to the rhesus reference genome (Mmul10). We aligned trimmed reads to the converted rhesus reference genome using default settings for all but two parameters (--score-min and -R; see Appendix B under "Supplemental Methods"). We generated methylation coverage files by summing methylated and unmethylated read counts for each site. We parallelized downstream filtering and processing of the coverage data with GNU parallel (Tange 2018) and used BedTools (v2.24.0) (Quinlan and Hall 2010) to remove features missing in more than 10% of samples and those with < 5X median coverage. For the Yerkes and Amboseli datasets, the data processing workflow was nearly identical apart from modifications for processing the single-end reads in both these datasets. Unless otherwise stated, all subsequent analyses were carried out in RStudio (v1.4.1106) (RStudio Team 2015).

Site-Based Modeling Approach

To limit the inclusion of invariant or uninformative sites, we removed CpG sites that were missing in more than 10% of the training samples and excluded samples that were missing data at >25% of CpG sites in the dataset. We removed constitutively hypo- or hypermethylated sites (those with median percent methylation less than 10% or greater than 90% across samples), and sites with < 5X median coverage, leaving 196,345 CpG sites in the site-based dataset. A detailed description of sample filtering criteria can be found in Appendix B under "Supplemental Methods".

We then imputed missing and low coverage (< 5X) sites in our dataset using BoostMe (Zou et al. 2018). On average, 12.5% of sites were imputed per sample. After removing sites mapping to sex chromosomes and those containing one or more inadmissible values (non-real numbers that result from when BoostMe attempts to divide by zero), the dataset contained

185,153 sites.

Sliding Window-Based Modeling Approach

To improve our model generalizability, we compared performance of the traditional site-based method to a sliding window-style approach with the intent of capturing more shared loci across samples and datasets. For each window containing at least one read in our dataset, we summed methylated reads and divided them by the total number of (methylated + unmethylated) reads to obtain methylation ratios corresponding to each region. We implemented the same filtering strategy described above by excluding windows that were missing in more than 10% of samples (leaving 279,052 windows), samples that were missing >25% of features in the dataset, windows that were constitutively hypo- or hypermethylated, and those with < 5X median coverage, leaving a final set of 161,289 windows.

Missing values and those with < 5X coverage at a given feature were imputed using BoostMe. The average proportion of imputed windows was 1.98% per sample. Notably, the average proportion of features imputed for the window-based approach was six-fold lower than that of the site-based dataset. Following imputation, the removal of windows containing non-real numbers and those mapping to sex chromosomes, 159,472 windows remained. The three datasets used to train, validate and test the model were processed in an identical manner.

Model Training and Optimization via Cross Validation

Each step described in this section was performed independently on both the site-based and window-based datasets.

We used elastic net regression implemented using the R package glmnet (v4.1-1) (Friedman et al. 2010) and leave-one-out cross validation (LOOCV) to evaluate the predictive performance of our DNA methylation data. To perform LOOCV, one sample is held out at a time, and 'proto-models' are generated on the remaining N-1 samples using 10-fold cross validation. The "best" proto-model (the proto-model with the lowest

mean absolute error) is then used to predict the age of the held-out sample. This process is repeated for each sample in the dataset. Once all 563 age predictions were generated, we regressed all predicted age values onto observed age values (chronological age) to evaluate the predictive performance of the model generated from our primary dataset.

After confirming the capacity of our primary dataset to predict age, we proceeded to generate the final model. We quantile normalized methylation values across features and across samples independently for the Yerkes macaque and baboon datasets. Next, we determined optimal hyperparameter settings using the caret package (v6.0-86) (Kuhn, 2008; https://topepo.github.io/caret/) by performing a grid search across two hundred combinations of alpha and lambda using repeated (3x) 10-fold cross validation on all 563 samples. Once the optimal hyperparameter values were determined, we performed an elastic net regression on the entire dataset using the optimized values and applied this model to the two external datasets.

Gene Annotation and Enrichment Analyses for the Window-based Dataset

We tested whether our clock windows were overrepresented in CpG islands and shores using annotation files downloaded using UCSC's TableBrowser tool (Kent et al. 2002). We used BedTools to determine the number of windows that overlapped CpG islands (CG-rich parts of the genome that often co-localize with promoter regions) and CpG island shores (2 kb flanking regions on either side of the island) and used two-sided Fisher's exact tests to test for enrichment. To test whether clock windows were overrepresented in enhancer regions, we downloaded chromatin state annotations for human peripheral blood mononuclear cells (PBMCs) from the Roadmap Epigenomics Project (https://egg2.wustl.edu/roadmap/web_portal/index.html) and used UCSC's LiftOver tool (Kent et al. 2002) to convert these coordinates to the rhesus macaque genome. We retained annotations for genic enhancers (chromatin state 6, frequently occurring in gene bodies and sometimes in exons), bivalent enhancers (chromatin state 12, characterized by both activating and repressive histone marks) and other enhancers (chromatin state 7, occurring less frequently in gene bodies [compared to state 6] and very rarely in exons). We used BedTools to determine the number of windows that overlapped enhancers and two-sided Fisher's exact tests to test for enrichment of hypo- and hypermethylated clock windows in enhancer regions. To determine whether any Gene Ontology (GO) terms or KEGG (Kyoto Encyclopedia of Genes and

Genomes) pathways were significantly overrepresented in the clock windows, we used the gProfiler (v0.2.0) (Raudvere et al. 2019) R package. To test for enrichment of evolutionarily conserved regions in clock windows, we calculated conservation scores for 148,339 of the 155,347 windows in our dataset using phastCons (Siepel 2005) (see Appendix B).

Model Applications

Quantifying the Pace of Epigenetic Aging

We defined a measure of age acceleration, termed "residual age" (similar to Horvath's [2013] "delta age") by taking the residuals from a loess (locally estimated scatterplot smoothing) regression of predicted onto chronological age to enable identification of individuals who may be aging more (or less) rapidly than expected based on their calendar age. By taking the residuals from a loess regression, we can detect putatively meaningful deviation from the expected rate of aging while accounting for systemic effects of the model (e.g., the influence of chronological age) and the non-linear pace of the aging process (see **Figure S1**).

Examining the Effects of Socio-environmental Variables on Residual Epigenetic Age

We tested if low social status, a proxy for social adversity, accelerated the pace of epigenetic aging in the Cayo Santiago rhesus macaques. Rhesus macaques live in social groups composed of adult females from several matrilines, their offspring, and mostly unrelated adult males. Females inherit their mothers' dominance rank, and thus offspring take the position just below their mothers, with younger sisters superseding older sisters. Males typically emigrate from their natal social groups when they reach sexual maturity (approximately age four) to join new social groups, where any status conferred by their matriline is lost and they become low-ranking members in the new group. Contrary to some other social NHPs (e.g., baboons), male rhesus macaques do not attain high rank through direct competition, but "queue" for dominance such that their rank increases in accordance with their length of tenure in a social group (Maestripieri and Hoffman 2012). While female rank tends to be stable over long periods and males do not generally fight

for high rank, dominance hierarchies for both sexes are determined and reinforced via antagonistic interactions between individuals (Maestripieri and Hoffman 2012). For both sexes, high rank confers greater access to resources. Among females, low-rank animals are harassed more frequently and chased away from food. By contrast, high-rank females show higher annual survival, although it is unclear whether rank consistently affects lifespan in female rhesus macaques (Maestripieri and Georgiev 2016; Brent, Ruiz-Lambides, and Platt 2017; Ellis et al. 2019). For males, high rank confers greater access to resources and mates. Prior to the breeding season, high-rank males gain body fat and weight, which they then lose at a rapid pace once breeding commences (Bercovitch 1997). Thus, low-ranking individuals may experience different forms of social adversity dependent on sex.

For this study, we acquired behavioral data and quantified dominance rank for 81 males and 116 female rhesus macaques. Rank was calculated separately for females and males within a social group using dyadic win-loss interactions between individuals, with the highest rank scored as 100 (i.e., dominating 100% of other individuals) and the lowest as 0. Behavioral data used to determine social rank were collected in the year before blood was drawn (i.e., we calculated rank for animals whose blood was drawn between October and December using behavioral data collected from that same calendar year, while the rank of animals with blood drawn between January and March was calculated using behavioral data collected the preceding year).

We tested whether dominance rank was associated with less rapid epigenetic aging by modeling residual age as a function of rank as a linear variable separately for females (n = 116, aged 6.01 to 27.9 years) and males (n = 81, aged 5.9 to 22.8 years). Because longer tenure is associated with higher dominance rank, we tested whether the pace of epigenetic aging was affected by length of tenure in one's social group among males (n = 230, aged 3.9 to 21.7 years).

Results

The Window-Based Model Outperformed the Traditional Site-Based Model

Despite the fact that both the site- and window-based datasets contained a similar number of loci after filtering (~180K and ~160K, respectively), the overlap in features retained in both the Cayo and Yerkes datasets increased substantially when we used the sliding window-based approach. Of the 185,153 CpG sites in the filtered Cayo Santiago dataset, 38% (70,439) of sites were also found in the Yerkes dataset, compared to 97% (155,347) shared features for the window-based dataset (see **Figure S2** in **Appendix A**). This increase in generalizability across datasets was bolstered by the fact that the window-based model showed superior performance in predicting age in the Cayo population using LOOCV. Our site-based model was able to accurately predict age (Pearson's r = 0.82 MAD = 2.11 years) **(Figure 1A)** but was significantly outperformed (t = 5.52, df = 545, p = 5.36 x 10^{-8}, mean difference = 0.32, paired t-test) by the window-

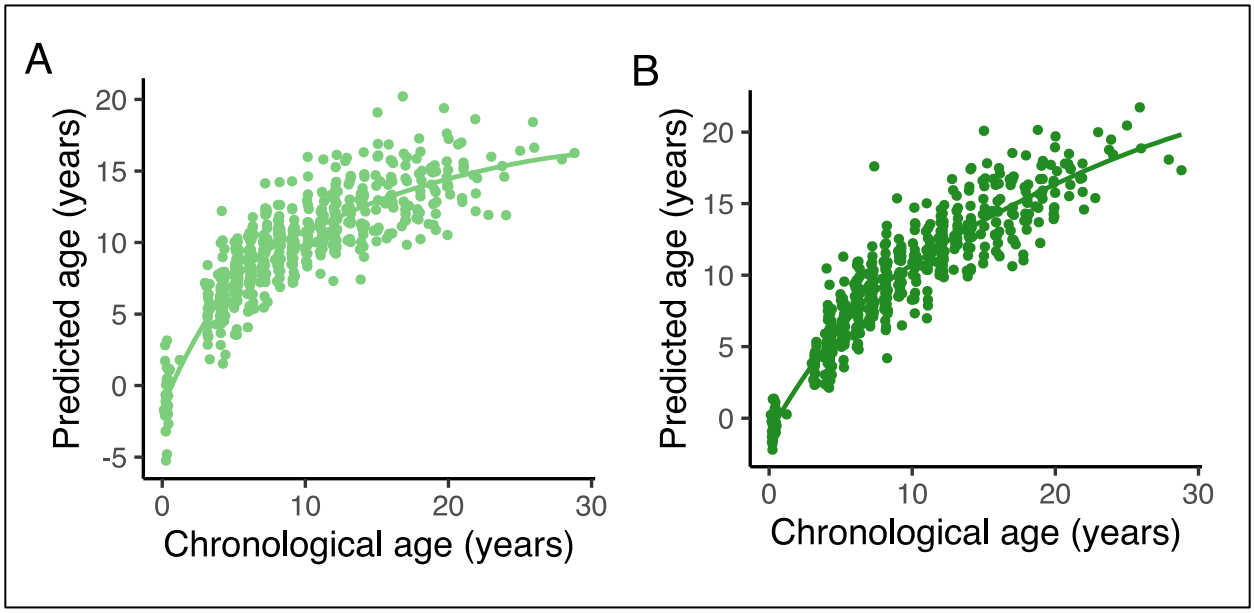


Figure 1. (A) *Site-based model of methylation age successfully predicts known chronological age.* ***Known*** *chronological age is highly correlated with epigenetic age predictions from our site-based epigenetic clock (Pearson's r = 0.82, median absolute deviation between predicted and chronological age [MAD] = 2.11 years). Methylation data used to generate the site- and window-based clocks are from whole blood from a cross-sectional sample of rhesus macaques living on Cayo Santiago (n samples = 549; n unique females = 267). Curved line shows line of best fit from univariate loess regression.* ***(B)*** *Window-based model of methylation age successfully predicts known chronological age and outperforms the site-based model.* ***Known*** *chronological age is even more highly correlated with epigenetic age predictions in the window-based epigenetic clock (Pearson's r = 0.9, MAD = 1.42 years) than the site-based clock. The model was generated using whole blood samples from rhesus macaque living on the island of Cayo Santiago (n samples = 563; n unique females = 273). Curved line shows line of best fit from univariate loess regression.*

based model (Pearson's r = 0.9, MAD = 1.42 years) (**Figure 1B**). It performed equally well in males and females (p = 0.71, two-sample t-test). While both models predicted chronological age well, they did not scale linearly. We found that age predictions began to plateau at older ages (> 20 years), as reported in other species, including humans (e.g., Horvath, 2013; Levine et al., 2020).

We were also able to test if our model could track aging longitudinally using data from 66 individuals sampled more than once. For these 66 individuals (n = 70 paired samples due to the 4 individuals sampled three times), 88.6% (62/70) displayed an increase in biological age in accordance with advancing chronological age (**Figure 2**). Thus, samples collected later in time were more likely to be accurately identified as older than those collected at earlier points in time (p = 9.13 x 10^{-12}, one-sided exact binomial test).

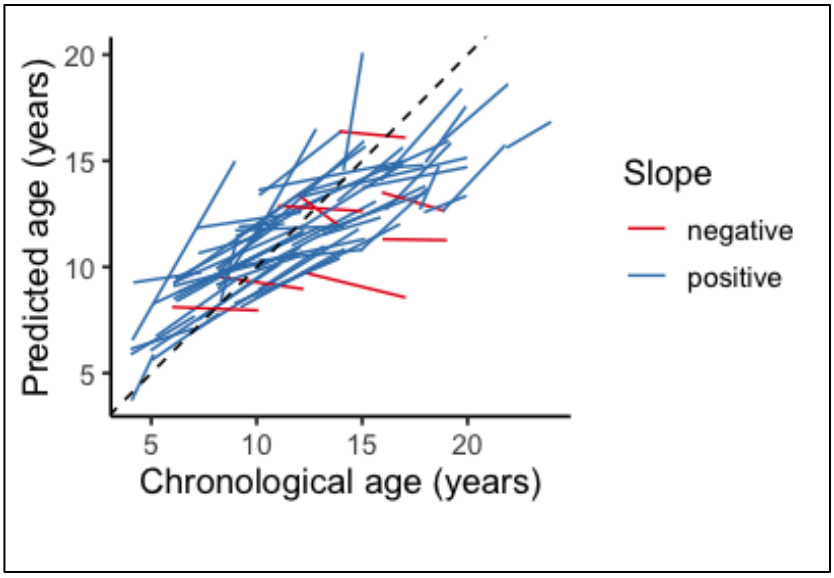


Figure 2. *Predicted aging trajectories for repeatedly sampled individuals overwhelmingly increase over time (n = 66 individuals, 70 total predictions because 4 individuals were sampled 3x). The vast majority of epigenetic age predictions increased as calendar age increased within individuals (blue lines; 62/70 predictions). Very few predictions went against this expected trend and decreased with advancing chronological age (red lines; 8/70; 11.4%). The model was significantly more likely to correctly predict the age from the later sample was greater than the age(s) of the sample(s) collected at earlier points in time (p = 9.13 x 10^{-12}, one-sided exact binomial test). Dashed line shows x=y.*

Characteristics of RheMacAge Clock Loci

The final window-based model (RheMacAge blood clock), generated using all 563 samples, included 359 windows. Of these windows, 164 decreased in methylation with age ("hypomethylated windows") and 195 increased in methylation with age ("hypermethylated windows") . Hypermethylated windows were significantly enriched for CpG islands (Odds Ratio [OR]: 3.58 [95% CI: 2.05, 5.97], $p = 1.09 \times 10^{-10}$) but not CpG island shores (OR: 1.04, $p = 0.8$). Hypomethylated windows were not enriched for CpG islands (OR: 1.09, $p = 0.74$) and were significantly underrepresented in CpG island shores (OR: 0.26, [95% CI: 0.14, 0.46], $p = 4.9 \times 10^{-8}$). Bivalent enhancer regions were significantly overrepresented among hypomethylated clock windows (OR: 4.12 [95% CI: 1.63,8.71], $p = 0.002$), while hypermethylated windows were not significantly enriched for enhancer regions.

To determine whether any biological pathways, processes or molecular functions were significantly overrepresented in our clock, we performed GO and KEGG pathway analyses. We evaluated the 359 clock windows against the background of 155,347 windows that were included in model training. We found no GO terms or KEGG pathways were significantly overrepresented among our clock windows.

CpG sites exhibiting age-dependent changes in methylation are often found in evolutionarily conserved regions of the genome (Mozhui and Pandey 2017), and certain age-dependent patterns of methylation change have been shown to be conserved between humans and mice (Spiers et al. 2016; Stubbs et al. 2017). Furthermore, Horvath's (2013) model generated robust predictions of age for chimpanzees and bonobos (the closest living evolutionary relatives of humans), but not gorillas, suggesting that some features selected by the clock come from conserved sequences but others may not. We found that the windows in the RheMacAge clock were very modestly enriched for evolutionarily conserved sequences (D = 0.09, $p = 0.007$, two-sample Kolmogorov-Smirnov test) (**Figure S3, Figure S4**).

RheMacAge Clock Predicts Age in Two Independent Test Datasets

To test the performance of our model in an independent dataset, we applied the RheMacAge clock to RRBS data generated from purified blood cells from 43 female rhesus macaques housed at Yerkes Primate National Research Center (aged 3.1 to 20.1 years). Our methylation-based age predictions were significantly correlated with chronological age (**Figure 3**) (Pearson's r = 0.69, p = 2.65 x 10^{-7}), with an MAD of 2.09 years. This indicates that the model can be used to predict age with high accuracy in independent populations of rhesus macaques.

We then tested if our clock could generalize to closely-related taxa using the Amboseli baboons. When applied to baboons, our RheMacAge clock was able to predict chronological age

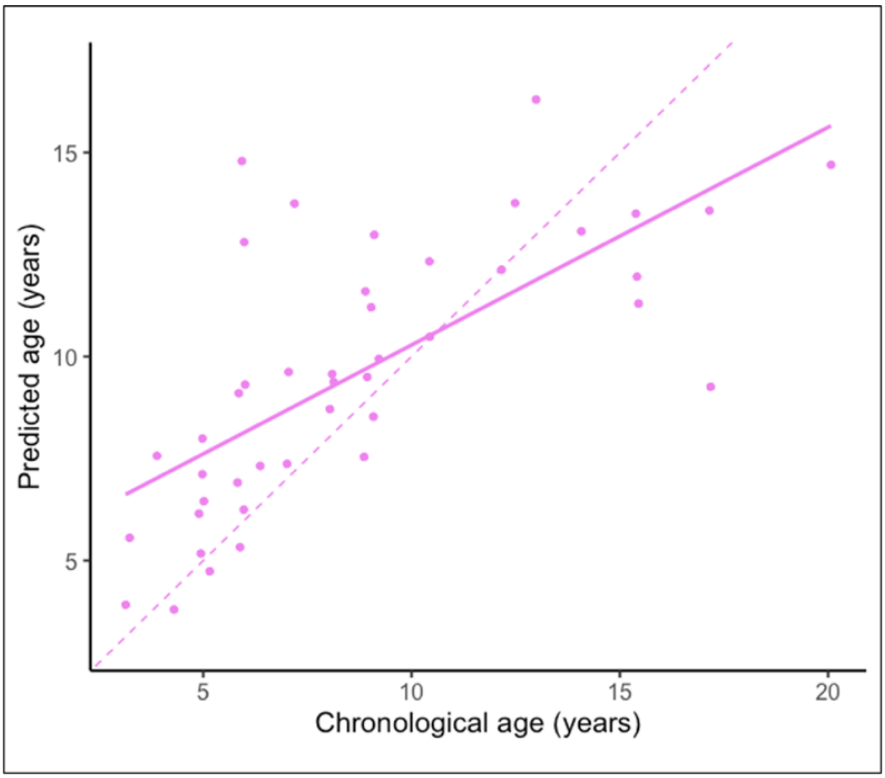


Figure 3. *Predicted DNA methylation age for Yerkes rhesus macaques is strongly correlated with chronological age (r = 0.69, MAD = 2.09 years). Solid line shows line of best fit from univariate linear regression of predicted onto chronological age. Dashed line shows x=y.*

with high accuracy and showed the same general sex-specific patterns in the rate of aging shown by Anderson et al. (2021) (male Pearson's r = 0.8, p < 2.2 x 10=16 ; MAD = 1.34 years, **Figure 4A**; female Pearson's r = 0.74, p < 2.2 x 10-16 ; MAD = 2.19 years, **Figure 4B**). Despite the modest reduction in predictive accuracy, the RheMacAge model captured a similar biological

signal to the baboon clock and residual epigenetic age calculated from both models was significantly positively correlated (males: r = 0.55, $p = 5.28 \times 10^{-12}$; females: r = 0.41, $p = 7.49 \times$

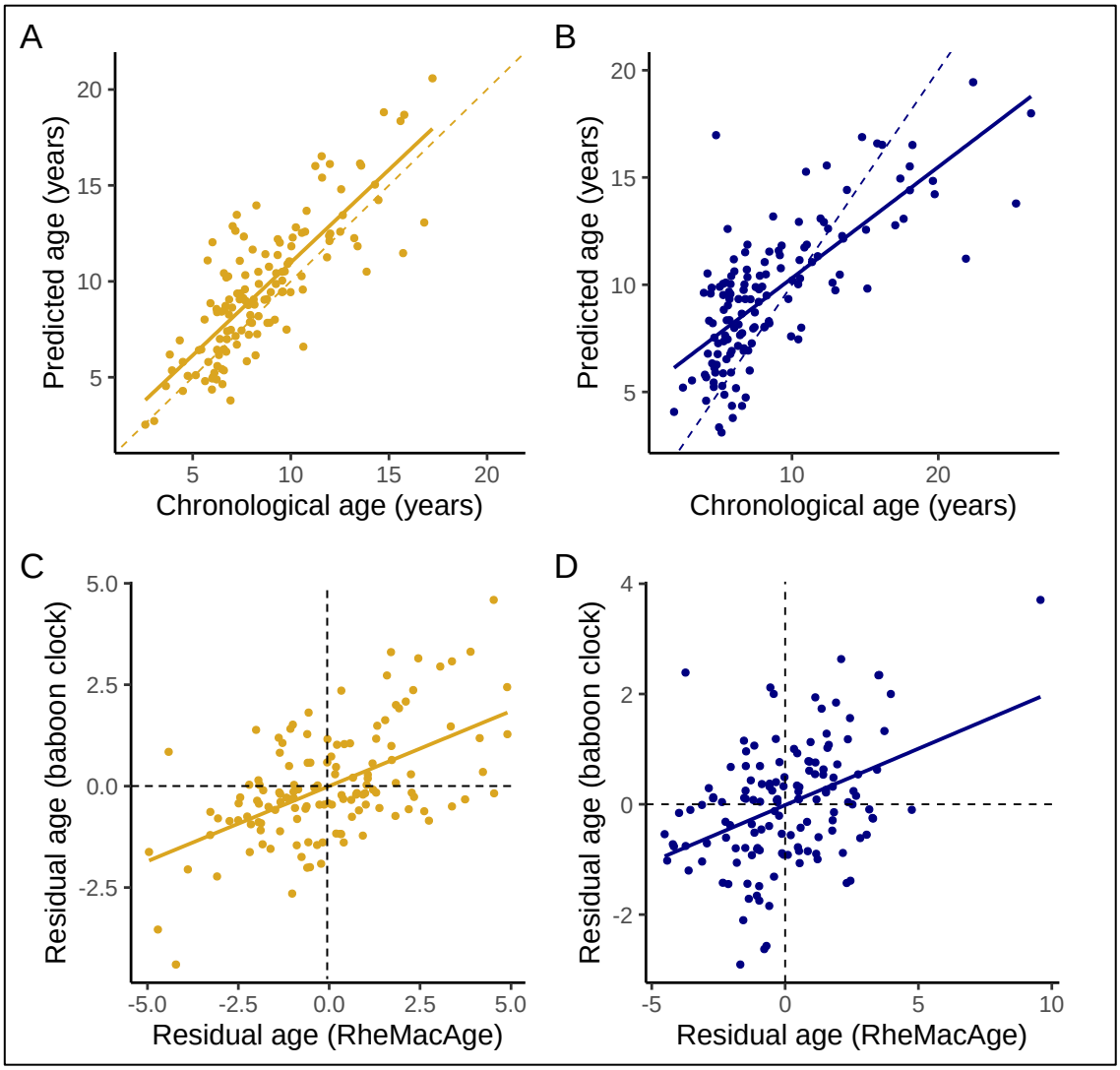


Figure 4. *RheMacAge successfully predicts interspecies epigenetic ages of baboon DNA methylation and recapitulates results from a baboon-specific methylation clock.* ***Predicted age*** *for* ***(A)*** *male and* ***(B)*** *female baboons using the RheMacAge epigenetic clock are highly correlated with known chronological age (males: r = 0.8, MAD = 1.34 years; females: r = 0.74, MAD = 2.19 years). The solid line shows the line of best fit from univariate linear regression of predicted onto chronological age. Dashed line shows x = y. Residual epigenetic age from the RheMacAge (x-axis) recapitulates residual ages from a baboon-specific clock (y-axis) for* ***(C)*** *males (r = 0.55, p = 5.28 x* 10^{-12}*) and* ***(D)*** *females (r = 0.41, p = 7.49 x* 10^{-7}*). Points in the bottom left (decelerated ages) and top right (accelerated ages) quadrants reflect concordance in residual epigenetic ages between the two clocks. Discordant predictions between the two clocks (i.e., one clock predicts accelerated age while the other predicts decelerated rate of aging) are in the top left and bottom right quadrants.*

10^{-7}; **Figures 4C, Figure 4D**).

The RheMacAge Clock and the Impact of Social Status and Adversity on the Aging Epigenome

Finally, we tested whether social status was associated with residual epigenetic age in sub-sample of rhesus macaques (n = 197) from Cayo Santiago. Higher-ranking males tended to show lower residual epigenetic age, although this relationship did not reach statistical significance (n = 81, ß = -0.345, $p = 0.06$) (**Figure 5**). Residual epigenetic age was not correlated with length of male tenure (n = 230, ß = -0.001, $p = 0.94$). Among females, we found no effect of dominance rank on residual epigenetic age (n = 116, ß = -0.03, $p = 0.80$) **(Figure 5)**. Similarly, no significant association between female rank and epigenetic age was observed in baboons by Anderson et al. (2021), despite the strong effect of rank on lifespan that previously has been observed this population of female baboons.

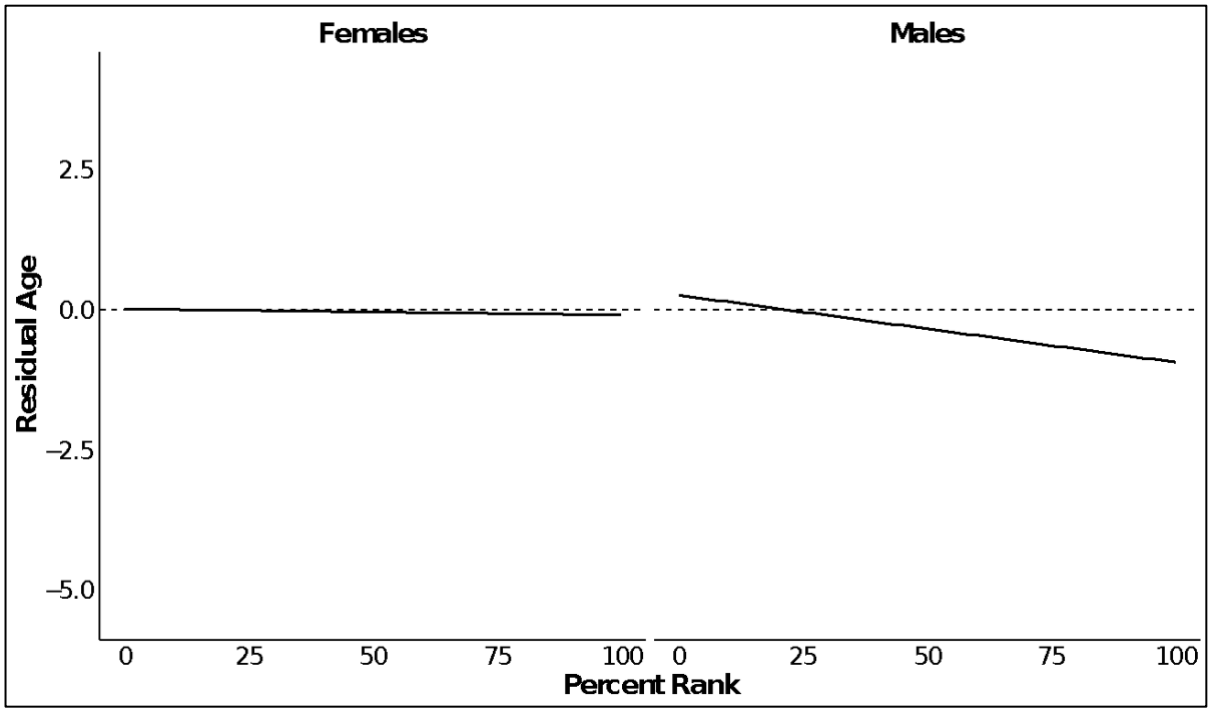


Figure 5. *Higher dominance rank was associated with lower residual age in males, although this relationship was not significant (p = 0.06). No such trend was identified in females (p = 0.80) in the Cayo Santiago dataset. Dominance rank is shown as a continuous variable that represents the percentage of same-sex animals in a social group that the focal individual outranks (thus 0% is equivalent to the lowest-ranking and 100% to the highest-ranking individual).*

For male baboons at Amboseli, estimates of residual epigenetic age from the RheMacAge model replicated the significant association between high social status (measured on an ordinal scale, where 1 is the highest rank) and greater residual epigenetic age (Pearson's r = -0.47, $p = 4.05 \times 10^{-7}$, n = 104) first reported with the publication of the original dataset (Anderson et al. 2021). Thus, using a model built with data from a different primate species, we found that higher-ranking male baboons exhibited more rapid epigenetic aging, while the pace of epigenetic aging in female baboons showed no relationship to rank ($p = 0.4$). This result underscores the suitability of our model to the interrogation of the biology of aging across social and ecological conditions in both lab and field-based primate populations.

Discussion

Rhesus macaques are an important biomedical model of human aging (Chiou et al. 2020). Despite this, there are comparatively less 'omics' resources available for rhesus macaques than humans or mice (see Meer et al. 2018). Here, we have generated genome-wide methylation data for over five hundred samples, which is the largest study of DNA methylation aging study carried out to date in this highly relevant model of human aging.

Our RheMacAge clock produced accurate age estimates from blood in an independent sample of captive female rhesus macaques and a large sample of wild baboons. We also observed a more rapid rate of epigenetic change with age in male versus female baboons, consistent with results from Anderson et al. (2021), demonstrating our approach can capture the same biological aging signatures as a model developed specifically for baboons. As such, the model serves as a reliable biomarker of aging in blood collected from these species and provides an important opportunity to test hypotheses about the aging process.

We used our model to carry out a preliminary test of the relationship between epigenetic aging and dominance rank in the Cayo Santiago rhesus macaques and found that high rank was associated with lower residual aging among males ($p = 0.06$). This contrasts with the effects seen in baboons, where high-ranking males exhibit age

acceleration (Anderson et al. 2021). Two socioecological differences between the two species likely explain this discrepancy: (1) the mechanism of male rank attainment and (2) reproductive seasonality. In baboons, male rank is determined by competitive interactions and mating occurs throughout the year. Thus, male baboon reproductive success is highly correlated with length of tenure as the alpha male (Alberts, Watts, and Altmann 2003). Maintaining alpha status is stressful (Gesquiere et al. 2011) and requires significant energy expenditure to monopolize access to estrous females and fend off competitors (Alberts, Buchan, and Altmann 2006). In contrast, male rhesus macaques obtain high social status through a less antagonistic queueing system where dominance rank is correlated with length of group tenure. Additionally, rhesus macaques are seasonal breeders and their mating season is restricted to a few months each year. We collected our blood samples during the weeks preceding the mating season, when, in anticipation of the energetically-demanding mating season, high-ranking males disproportionately increase in weight and fat compared to lower-ranking animals. Taken together, these findings suggest the RheMacAge model is sensitive to the biological effects of social status on the pace of aging and may be able to capture latent variables associated with different types of dominance hierarchies. While these specific findings require further investigation, they highlight the potential of the model to test more specific hypotheses about the effects of socio-environmental variables on the aging process.

This model can also complement and expand current and future aging research in this and other relevant primate populations. For example, our model could be used as a means of testing the effects of medical interventions aimed at delaying aging-related physiological decline, such as caloric restriction, rapamycin, or other pharmacological treatments, in two long-lived, biomedically relevant NHP species that act as important models for human aging. Our model is particularly well-suited to situations where behavior and biology intersect: it could be used to examine whether adversity experienced during the juvenile stage affects patterns of age-related DNA methylation change, or if the timing of daily feeding behavior among captive individuals (e.g., night versus daytime feeding) is associated with changes in the pace of epigenetic aging. Coupled with differential methylation and gene expression data, such research could uncover molecular mechanisms that regulate how stress becomes biologically embedded, and may help identify health or behavioral variables that contribute to increased resiliency to the effects of

such stressors. In addition, this model can be used by primate centers to evaluate an individual's suitability as a study candidate in the preliminary stage, while there is still time to make changes to a study population. This could ensure that animals in different treatment groups are matched on key physiological metrics prior to the application of any intervention, thus enabling more effective use of center resources.

Horvath and colleagues (2021) recently published an array-based epigenetic clock model for rhesus macaques that relies on a newly-designed DNA methylation array for mammals that covers 38,000 evolutionarily conserved CpG sites (Arneson et al. 2021). Because bisulfite sequencing is another very common technology used to generate DNA methylation data, our model and the array-based alternative neatly complement one another and together, should fit most methylation datasets. Together with Horvath and colleagues' array-based rhesus clock, our sequence-based model contributes to an overdue but growing analytical toolkit for biomedical and other research involving rhesus macaques. Despite these additions to the analytic arsenal, the relative dearth of analytical tools for rhesus macaques underscores the need for targeted development of tissue-specific clocks that are more sensitive and better able to detect disease-specific epigenetic changes in affected tissues, which may in turn shed light on underlying mechanisms (Bell et al. 2019).

While none of the regions in our clock model were significantly enriched for any particular GO terms or KEGG pathways, results from enrichment analyses of various epigenetic clocks are often ambiguous and very general. Given that random selections of CpG sites are able to predict age surprisingly well (see Stubbs et al. 2017), it follows that changes across the methylome may be broadly predictive of age. Often, the CpG sites automatically selected for inclusion in an epigenetic clock model have no obvious associations with aging or age-related processes but are simply good at predicting age when measured in aggregate with a particular group of CpG sites. In fact, among CpG sites included in the DNAm PhenoAge model, those with higher weights showed little or no correlation with calendar age, while those with the largest age correlation coefficients tended to have lower weights in the regression equation (Levine et al. 2018). It is possible that the lack of significant association with particular age-related genes or functions is

because in some instances, the CpG site itself has no direct association with age-related gene expression or activity. Instead, such patterns of change may reflect deterioration in epigenetic integrity and disruptions to cell signaling and regulatory networks that are characteristic of the aging process. Both global and tissue-specific changes take place in the aging epigenome, and while both are relevant to the study of health and aging, tissue-specific changes are expected to yield greater insight into the pathogenesis of specific age-related diseases and the factors that contribute to individual differences in susceptibility (Bell et al. 2019; Field et al. 2018; Levine et al. 2022).

DNA methylation-based age predictors can reliably track chronological age; accelerated aging in humans (where predicted age exceeds chronological age) has been associated with a number of socio-environmental and health variables, increased predisposition to age-related disease, and higher mortality risk (see Horvath and Raj 2018; Nwanaji-Enwerem et al. 2018). However, at present these models are more suitable as initiation points for hypothesis testing rather than as a means to an end themselves. Our exploration of the relationship between dominance rank and epigenetic aging among the macaques on Cayo Santiago is one such example of how our model is a starting point that may help shape and refine the objectives of exploratory research by providing a direction for further analysis. Nonetheless, the molecular mechanisms reflected in measures of epigenetic age have yet to be identified. Determining why and how these models work at the molecular level as well as which aspects of the aging process they capture and which they fail to detect should be a priority in aging research.

Conclusions

Here we have developed a means of overcoming an impediment to comparative analyses, independent model evaluation, and testing in the context of bisulfite sequencing data. Our sliding-window methodology is easy to implement and the generalizability of the resulting model enables cross-study comparison, as demonstrated by the successful application of our RheMacAge model to independent RRBS datasets in two species. Our model detected an inverse association between residual epigenetic age and social status in male rhesus macaques on Cayo Santiago that approached significance ($p = 0.06$) in a pilot analysis, and recapitulated the relationship between rank and epigenetic aging in wild-living baboons that was first shown by

Anderson et al. (2021). These results provide proof-of-concept for our model and its capacity to measure the influence of certain environmental elements on health, aging, disease- and mortality risk. While their mechanistic underpinnings are still largely unknown, the ongoing and increasingly widespread use of epigenetic clock models has advanced the field towards an essential goal: quantification of the impact of lived experiences on health and aging. We anticipate that this straightforward workflow will enable more frequent and robust investigation of existing but generally untested epigenetic clock models and enhance the value of existing sequence-based methylation datasets. Rather than continuing to build custom models that can be applied to a single dataset, increased attention should be directed at increasing cross-study applications and discerning the biological mechanisms that underlie the functionality of existing predictive models.

Bridge to Chapter III

Venous blood has long been used to assess systemic health in humans, and changes in the blood epigenome are likely to be systemically informative in a way that changes in other individual tissue types will not be, due the omnipresence of blood throughout the body. On the other hand, the diversity of white blood cell types present in the circulatory system may not capture more subtle physiological changes, or those that disproportionately manifest in a particular tissue or set of tissues with shared or similar functions. Additionally, it has now been established that variation in the pace of aging (as measured by rates of cell regeneration, rather than a clock model) exists not only between different tissues, but also between cells of the same organ. A study of "long-lived cells" conducted by Arrojo e Drigo and colleagues (2019) demonstrated that cell types in the liver, pancreas, and brain exhibit a phenomenon known as "age mosaicism": in the liver, hepatocytes were found to be as old as neurons, whose ages tend to correlate with the organism's actual chronological age due to a lack of neuronal regeneration, while endothelial cells in hepatic sinusoids (vascular structures with similarities to capillaries in function but a unique morphology found only in the liver) and stellate-like cells underwent major turnover events at ages 6- and 18-months in rodents. The authors posit

that hepatocytes can act as long-lived cells because they are able to remain quiescent by working in conjunction with the highly active sinusoidal vascular architecture to detoxify the blood, transport, store, and monitor levels of circulating nutrients, and maintain whole-body homeostasis (Arrojo e Drigo et al. 2019). Thus, depending upon whether changes in DNA methylation are a cause or consequence of age-related deterioration, cell types from a single organ that have originated from different stem cell progenitor populations or that have very different rates of cellular turn over may exhibit markedly different signs of cellular aging. It is therefore necessary to investigate any intra-organ epigenetic differences that might exist between cell types of a given organ.

In the following chapter, I take a more targeted approach to interrogate normal processes of age-related change in the liver methylome from rhesus macaques aged three to 33 years. The objective of this research was to characterize age-related methylation change in liver as it occurs in normal aging and under conditions of dietary modification to elucidate how different organs within the body respond and/or contribute to the larger phenotype of systemic aging.

CHAPTER III

CHARACTERIZATION OF THE RHESUS LIVER METHYLOME BY SEX, AGE, AND IN RESPONSE TO DIETARY RESTRICTION

Introduction

The liver is the central regulator of whole-body metabolism, maintaining homeostasis through regulation of energetic demands, systemic monitoring and facilitating inter-cellular crosstalk. Age-related changes in the genome and epigenome interfere with mitochondrial function and disruptions of nutrient sensing pathways (Hunt et al. 2019). This ultimately provokes cellular senescence and the persistence of a chronic but low-grade inflammatory state(López-Otín et al. 2016). Metabolic and epigenetic alterations work together to promote or delay a larger phenotype of organismal aging, and both fit mechanistically into the "early origins of adult disease hypothesis", put forth by Barker (Barker 2004), which argues that the environmental and particularly nutritional conditions experienced during perinatal life act to program fetal growth and patterns of development and may, in certain contexts, predispose individuals to metabolic and cardiovascular disease through alteration of these trajectories. Epigenetic mechanisms were quickly linked as likely facilitators of these environmentally-induced alterations (Mcmillen and Robinson 2005; Barker 2004).

Insulin sensitivity declines in normal aging but can be accelerated by the same type of high-fat, high-sugar diet that often leads to long-term cardiovascular disease and metabolic dysfunction (Morgunova, Shilovsky, and Khokhlov 2021; Younossi et al. 2016). Calorically-restricted diets, by contrast, promote extended longevity by altering the activity of sirtuins, insulin/insulin-like growth factor signaling, mTOR, and AMPK signaling (Hunt et al. 2019).

Effective inter-organ communication is essential to maintaining metabolic health and whole-body homeostasis, and the integrity of these pathways also declines with advancing age (F. Wang et al. 2021). Regulation of glucose metabolism is orchestrated through communication between intestinal lipids and the vagus nerve, which sends signals to the brain to modulate glycogen synthesis from its input in the liver (Pocai et al.

2005; Jensen, Alpini, and Glaser 2013). Disorders related to obesity and cholesterol metabolism can contribute to cognitive decline by altering the function of the gut-liver-brain axis (F. Wang et al. 2021; Palmisano, Zhu, and Stafford 2017). The liver's involvement in the function of a diverse array of other organs and tissues to maintain homeostasis underscores the importance of understanding how disruptions to this organ's functionality, structural integrity, signaling mechanisms, and communication networks alter disease risk and modulate the shape of the aging trajectory at the level of the whole organism.

The regulation of energy metabolism has long been a promising avenue for interrogating the mechanisms that underlie the aging process. Deregulated nutrient sensing occurs by way of several pathways (López-Otín et al., 2013). The highly conserved nutrient-sensing mechanistic target of rapamycin (mTOR) signaling pathway interacts with a number of physiological networks that have been implicated in the aging process (Kapahi et al. 2010). Importantly, mTOR inhibition is associated with two pro-longevity processes: enhancement of the regenerative capacity of hematopoietic stem cells and the suppression of pro-inflammatory cytokines produced by circulating senescent cells (H. Pan and Finkel 2017).

Nutrient-sensing pathways are typically involved in epigenetically-mediated cellular responses to changes in energy availability. mTOR acts to dynamically regulate cell growth, proliferation, and homeostasis (Balistreri et al. 2013; Kapahi et al. 2010). mTOR is a serine/threonine kinase that functions as part of two central signaling complexes, mTORC1 and mTORC2 (H. Pan and Finkel 2017; Müller et al. 2018). When stimulated by growth factors or high nutrient availability, mTORC1 augments anabolic processes like lysosome biogenesis, mitochondrial metabolism and protein translation to facilitate cell growth and proliferation while inhibiting catabolic processes like autophagy through repression of FOXO transcriptional activity (Pan and Finkel 2017). Insulin/insulin-like growth factor signaling, mTOR, and the activity of sirtuins are all involved in autophagic activity, a crucial mechanism of maintenance and self-preservation at the cellular level for cell types that rarely regenerate, such as most hepatocytes and neurons (Bellanti et al. 2020; Arrojo e Drigo et al. 2019). Stem cell functionality declines with age and contributes to age-related organ dysfunction (Wang et al. 2021), while the senescence-associated secretory phenotype (SASP) of circulating senescent cells promotes the

accumulation of tissue damage that eventually outpaces the rate at which it can be repaired (López-Otín et al. 2013; Benayoun et al. 2015).

Dietary restriction (DR, also referred to as calorie restriction, or CR) is defined as reduced caloric intake without malnutrition. It is the only behavioral modification shown to have a demonstrable, positive impact on health, longevity, and the onset of age-related disease (Greer et al. 2007; Gensous et al. 2019). Studies in yeast, nematodes, and fruit flies have confirmed the longevity-promoting effects of this intervention across short-lived taxa but understanding the impact of caloric restriction on longevity in long-lived species has been a more difficult, time- and resource intensive task (Bitto et al. 2015; Mitchell et al. 2015; Mattison et al. 2017). The primary molecular mechanisms that underlie variation in the aging trajectory have yet to be elucidated, and it is possible that the processes that confer such benefits in the context of shorter lifespans differ in activity or functional identity between long- and short-lived organisms. Captive rhesus macaques can develop many of the same chronic, age-related conditions that manifest over the course of human aging, such as cardiovascular disease, diabetes, and other age-related disorders, highlighting their importance as a model for understanding human disease (Colman et al. 2009). A 20-year longitudinal study of calorically-restricted rhesus macaques was undertaken at the Wisconsin National Primate Research Center (WNPRC) to determine whether rhesus monkeys fed a calorically-restricted diet exhibited delays in mortality and the onset of "classic" age-related pathologies as compared to a control group. Results from this study show a strong positive effect on survival and healthspan. Calorically-restricted monkeys in the WNPRC experimental group showed no signs of brain atrophy or glucoregulatory impairment, and had a 50% lower incidence of neoplasia and cardiovascular disease (Colman et al. 2009). By contrast, monkeys in the control group died from age-related causes at 3x the rate of the experimental group and also showed similar increases in the incidence of age-related disease (Colman et al. 2009).

Intriguingly, a second report was published part way through (23 years after the initial start) from the only other study of long-term DR in rhesus macaques, carried out on a separate population of rhesus macaques at the National Institute on Aging (NIA).

This study found that while dietary restriction resulted in improvements in overall physiologic function, it had no measurable impact on survival (Mattison et al. 2012, 2017). However, two key differences existed between the WNPRC and NIA studies that are of note: first, the WNPRC diet contained 6X the sucrose and twice the fat of the NIA diet, which may have contributed to the more apparent health and longevity outcomes observed at WNPRC at the time they were analyzed; the NIA diet was more nutrient-rich, and apart from the difference in total calories, was the same in macronutrient composition for experimental and control individuals. Second, the NIA experimental design was relatively unique in that individuals entered the study at varying ages, rather than beginning and ending the diet at a uniform time in adulthood across all animals.

Due to the resource-intensive nature of this type of research, the WNPRC and NIA studies represent the only long-term studies of dietary restriction in a long-lived non-human primate conducted to date. Here, I analyzed DNA methylation data from the NIA study. I examine how differences in an individual's developmental stage "at diet start" interact with this putative pro-longevity intervention to differentially moderate later-life health outcomes in a long-lived primate. The question of how DR influences longevity in species whose life histories are already "long and slow" (Jones 2011) has remained largely unresolved, as the explanation for the physiological response to restricted nutritional resources was initially proposed as a means for shorter-lived organisms like mice to delay reproduction during periods of famine and/or to reduce juvenile mortality rates (Shanley and Kirkwood 2000). Given that many primates already exhibit life history characteristics that have been shaped by past resource scarcity or environmental instability to favor increased parental investment (i.e., protracted period of pre-adulthood, a later age at first reproduction, smaller litter size, and longer inter-birth intervals), there are likely disparities in the physiological response that manifest in relation to longevity.

This research had three main objectives. The first objective was to develop a liver tissue-specific epigenetic clock for rhesus macaques, as no tissue-specific age predictor model for rhesus liver currently exists. The second objective was to apply the liver-specific epigenetic clock model to determine whether I could detect a delayed aging effect as a result of restricted nutritional resources in this rhesus macaque population. In theory, this would provide additional evidence for the functional role of the clock as a reliable biomarker of aging and further support dietary restriction as a successful pro-longevity intervention in a long-lived primate species.

Finally, I sought to compare patterns of change in the aging DNA methylome under DR against those of age- and sex-matched individuals fed a typical research colony diet, and to examine how the effects of DR on the aging methylome varied by developmental stage at start of the diet (Objective 3). I found that long-term DR had a profound impact on the aging methylome and seemingly delayed the stochastic deterioration that characterizes the "normal aging" trajectory in mid- and late adulthood. I also identified significant differences in the extent of global hypomethylation change by sex and developmental stage. The effect of dietary restriction on the aging methylome varied markedly along these two variables, as did levels of circulating blood glucose and lipids.

Methods

"Normal" Samples from the ONPRC Biobank

Samples used for the initial phase of this study ("normal aging" cohort) consisted of 96 liver samples from the Oregon National Primate Research Center's (ONPRC) Tissue Archive. I refer to them as the ONPRC dataset or the "normal aging" cohort throughout the text. I selected samples with the primary objective of covering the widest possible age range. The ratio of males to females in the normal aging cohort was approximately 2:3, with 59 females and 37 males included in the dataset. I used a set of pre-determined selection criteria to exclude tissue samples from individuals who had prior involvement in studies requiring diet modification, frequent invasive surgeries, fatal injury or severe illness near time of death, or were involved in more than three different experimental studies (excluding strictly observational research) in the ten years prior to the animal's death.

Samples from the National Institute on Aging

The second dataset include in this analysis came from storage at the NIA biobank from the aforementioned study of DR in rhesus macaques carried out over more than three decades. I began this analysis with 68 samples and removed five samples during quality control: four were removed due to poor quality of the raw tissue, and one sample

due to insufficient coverage post-sequencing. This left 63 individuals in the dataset. There were 23 females ranging from 17 to 42 years of age at their time of death; and female animals began the study between the ages of 1.67 and 18.75 years. The proportion of a female monkey's lifetime during which she was involved in the DR study ranged from 44% to 92.6%. There were 40 males ranging from 21.5 to 44.2 years of age at time of death included in the study. The proportion of a male monkey's life during which he was under DR ranged from 2.7% to 97.6%.

All animals were initially categorized as control (n = 29) or experimental (n = 34) and matched as closely as possible for age and sex. Each group was fed the same diet apart from 30% fewer calories in the diet for the experimental group. In lieu of the monkey chow that is typical for captive research colonies, the diet fed to the monkeys in both groups was enriched to help prevent malnutrition in the experimental group. While the control monkeys did not indicate a desire for more food than they were given (some amount of food was generally left uneaten at the end of each feeding period), as this study progressed, the individuals in the control and experimental groups increasingly converged in weight, body fat percentage, and to a lesser degree, in caloric intake, although control monkeys did still typically eat between 10 -20% more calories than DR group individuals (Mattison et al. 2012). Thus, physical measurements from the NIA *control* group were consequently more similar to those from the WNPRC *experimental* DR group. Both NIA and the WNPRC experimental groups stand in stark contrast to the WNPRC study's control monkeys, who were heavier, had more body fat, and exhibited higher rates of chronic disease compared to the WNPRC experimental group or either NIA group considered.

To more accurately reflect the characteristics of the dataset, I reclassified the control group as the "healthy diet" (HD) group, and the experimental group as the "healthy diet + dietary restricted" (HD+DR) group; the HD+DR group received the same diet but with 30% fewer calories than the HD group.

Library Preparation and Sequencing

Genomic DNA was isolated and quantified at the ONPRC Primate Genetics Core using standard approaches. RRBS libraries were generated by the KCVI Epigenetics Consortium using established protocols (Carbone et al. 2019). For the 96 ONPRC liver samples, libraries were subjected to quality control and normalized using qPCR and sequenced on an Illumina

HiSeq4000 by the Genomics & Cell Characterization Core Facility (GC3F) to obtain single-end 75bp reads. For the 68 NIA liver samples, the same protocol was followed but libraries were sequencing on a NovaSeq S4 flowcell to produce paired-end 150bp reads.

Data Pre-Processing and Quality Control

I used TrimGalore! (Martin 2011) with the "--rrbs" parameter to remove low-quality bases and adaptor contamination from raw sequence reads. I used FastQC and MultiQC (Ewels et al. 2016). to quality-check trimmed reads. Mean Phred score at each base position within a given read was > 30 for all samples that were retained in both datasets.

Alignment and Methylation Calling

I used Bismark v0.20.0 (Kreuger and Andrews 2011) to perform *in silico* bisulfite conversion of the rhesus macaque reference genome (Mmul10), and aligned trimmed reads to the reference using "–score_min -L,0,-0.6" and default settings for all other alignment parameters. I performed methylation calling using Bismark's methylation extractor script and included the parameter flags "--merge_non_CpG, --comprehensive, --bedGraph".

Data Filtering

BedTools (v2.24.0) (Quinlan and Hall, 2010) was used for data filtering procedures. I used a custom shell script to discard CpG sites missing in more than 10% of samples. I then split reads in each file into 20 distinct files, one file per autosome per sample, discarding reads mapping to sex chromosomes. Next, I generated 20 BED 'filter' files, one per autosome, containing all CpG sites located on a given chromosome that were present in at least 90% of the samples. I removed any row containing a missing value and those with less than 10X median coverage, and retained only sites with median percent methylation between 10 and 90%, to exclude CpG sites that show little variation across the age spectrum.

Model Training and Calibration

To confirm the suitability of these data for modeling variation in epigenetic aging, I used leave-one-out cross-validation (LOOCV) to train 96 proto-models using glmnet (v4.0.2) (Friedman, Hastie, and Tibshirani 2010) with an inner 10-fold cross-validation loop. I did not perform any normalization or pre-selection steps, did not tune the alpha hyperparameter (*a priori* default: alpha=0.5) or modify the range of lambda values examined by glmnet.

As in Chapter II, I took the residuals of a loess regression of predicted onto chronological age to adjust for the effects of the modeling process on the defined measure of age acceleration ("residual age" or "residual epigenetic age").

Optimization and Construction of the Site-Based Clock Model

I followed the same optimization protocol detailed in Chapter II under "Model Training and Optimization via Cross Validation". I performed 10-fold cross-validation on the entire dataset of 96 samples across a grid of alpha and lambda values. I input these optimized hyperparameter values into a single run of the glmnet command to define the 300 CpG sites and associated coefficients which compose the final site-based model. Together, these sites make up the model that predicts age with the lowest median error in liver.

Gene Ontology and KEGG pathway analysis for Site-Based Model

As with the features in the blood-based model, I used gProfiler to test the 300 clock sites automatically selected through the model training process for enrichment against a custom background of all CpG sites included in our dataset (n = 216,542 CpG sites). I restricted the output of the analysis to gene ontology terms to which ≤ 500 genes were annotated.

Optimizing the Generalizable Model

I used the sliding-window approach described in Chapter II to build a second epigenetic clock model for liver that could easily be generalized and applied to independent data, such as the second dataset analyzed in this chapter (see below). For a detailed description of the "sliding-window" method I developed for increasing inter-study comparability using independently-generated RRBS datasets, see Chapter I Methods. Briefly, I binned CpG sites into adjacent, non-

overlapping 1,000bp windows and trained and optimized the final model on the full dataset to obtain the best hyperparameter values and automatically select model features. In total, 133 windows were automatically selected, of which 79 were hypomethylated with age and 54 of which gained methylation with age.

Application of the Liver Clock to the Dietary Restriction Study

I used the sliding window-based model to generate age predictions for the test dataset from the NIA study of long-term dietary restriction (N=63; 40 males; 23 females). To determine if a relevant biological or demographic variable was a significant predictor of the rate of epigenetic aging, I regressed epigenetic age onto diet type (HD or HD+DR), age-at-start, sex, and blood-based measures of glucose, triglycerides, cholesterol, total protein, albumin, alanine aminotransferase (ALT) and aspartame aminotransferase (AST) levels.

Differential Methylation Analysis of Normal Liver Aging

The same filtered set of 216,542 CpG sites that were used to train the clock were similarly used for differential methylation analysis. I identified significantly differentially methylated CpG sites (DMCs) using the PQLseq package (Sun et al., 2018). Probabilities (p-values) were corrected for multiple testing using the Benjamini-Hochberg False Discovery Rate (FDR) implemented using the package q-value (v 2.26.0) (Storey, 2003).

After identifying CpG sites showing significant differential methylation with increasing age, I divided them into two groups. The first consisted of those that lost methylation with age (hypo-age DMCs) and the second those that gained methylation with age (hyper-age DMCs).

Analysis Of Sex-Specific Trends In The Aging Liver Methylome

I used the same data and analytical methodology described above but divided the samples into females and males prior to running the differential methylation analysis. I employed k-means hierarchical clustering to examine patterns of similarity and patterns

of change across CpG sites showing significant differences in methylation with age within sex and between study populations (NIA and ONPRC).

Age- and Sex-matched Differential Methylation Analysis With or Without Dietary Modification

Age-matched females (n = 20, n = 23 for NIA and ONPRC, respectively) and age-matched males (n = 24, n = 15) were examined separately. Ages of individuals ranged from 17 to 33 years of age at time of death. Within each sex, CpG sites analyzed for differential methylation were filtered to include sites that were present across all females (or across all males) at a minimum of 5X median coverage and with 10 - 90% median methylation. I determined the number of significantly differentially methylated sites at a pre-defined False Discovery Rate (Benjamini-Hochberg method) and employed a custom R script to filter out sites that failed to converge or were not significant at the specified FDR. I performed two-sided Fisher's exact tests to determine whether individuals whose diets had not been manipulated ("normal agers") showed a larger number of significant changes in DNA methylation, as compared to individuals in the group from the National Institute on Aging. As healthy, calorically-restricted diets that avoid malnutrition of the individual are associated with increased longevity, I hypothesized that the rate of age-related epigenetic change would be less rapid, with dietary restriction acting to protect against or at least delay stochastic and age-dependent deterioration of canonical epigenetic mechanisms. I used the PQLseq package *(Sun et al. 2019)* to test for differential methylation at each CpG for 175,889 shared sites in females and 182,592 sites in males to determine whether individuals subject to dietary modification showed fewer significant changes in methylation with age, which would suggest lower levels of the type of stochastic change that contributes to age-related decline. I retained only sites that were significant at FDR< 0.05 after testing for differential methylation with age across the lifespan.

Differential Methylation Analysis of "Juvenile Start" Diet Group

To examine the effects of dietary restriction specifically on animals who entered the study under the age of three years, I ran a differential methylation analysis using the interaction between age-at-start (in years) and diet type (healthy or dietary restricted) as the predictor

variable of interest, with sex as a covariate. I used the Bioconductor AnnotationHub (v3.2.2) and GenomicRanges (v1.46.1) packages to retrieve annotation data, and the Ensembldb (v2.18.3) package to identify genes directly overlapping significant age-DMCs.

I performed GO and KEGG Pathway analysis using gProfiler (Raudvere et al. 2019) with a custom gene background; genes were included as background if they could be annotated to a site in our dataset, irrespective of significance, and excluded if they had no potential connection or relationship. I restricted output of significant GO terms and KEGG pathways to include genes to which at most 500 terms were annotated at a pre-defined FDR. I used Reactome Pathway Analysis (Gillespie et al. 2022) to perform exploration and visualization of relevant and interrelated pathways, examining only those genes that directly overlapped a significant age-DMC at a pre-defined FDR. I used Enrichr (Kuleshov et al. 2016) to examine the Mammalian Phenotypes (Smith, Goldsmith, and Eppig 2004) associated with genes overlapping significant DMCs.

Differential Methylation Analysis of Super Ager Males

Because males in the old-start group on both the HD and HD+DR diets lived remarkably long lives, I sought to identify some of the molecular mechanisms involved in this "Super Ager" phenotype. I performed differential methylation analysis with PQLseq using age at death as the main predictor variable, and precise age at start (in years) and diet type (healthy diet [HD] or healthy diet + dietary restriction [HD+DR]) as covariates. I extracted significant age-DMCs (FDR<0.05) and divided them into hypo- and hyper-age DMCs. I performed hierarchical k-means clustering using the pheatmap package (v1.0.12) (https://github.com/raivokolde/pheatmap), and used gProfiler with a custom background of genes only present in the "old start" male dataset to identify GO terms and KEGG pathways for which the age-DMCs were significantly enriched (FDR<0.001).

Results

Annotation and Enrichmentof the 300 Liver Clock Sites

Of the 300 sites included in this liver-specific age predictor model, 164 gained methylation and 136 lost methylation with advancing age. More than half of hypermethylated sites in the clock fell within a CpG island (CGI) (two-sided Fisher's Exact Test [FET], $p < 2.2e-16$, OR = 5.26, 95% CI: 3.81, 7.27), indicating significant enrichment in this genomic compartment. By contrast, hypomethylated sites were significantly underrepresented in CGIs (two-sided FET, $p < 1.03e-05$, OR = 0.27, 95% CI: 0.12, 0.53).

Among the genes tested, two were significantly overrepresented among liver-specific clock sites: cyclin-dependent kinase inhibitor 2B (*CDKN2B)* and SRC kinase signaling inhibitor 1 (*SRCIN1*). Twelve of the 300 clock sites mapped to *CDKN2B* (two-sided FET, $p = 5.235e-07$, OR = 7.17, 95% CI: 3.52, 13.41) and eight of 300 mapped to *SRCIN1* (two-sided FET, $p = 0.007$, OR = 3.02, 95% CI: 1.27, 6.22).

Liver Model Performance During Training

Predicted ages generated using the window-based model with LOOCV were strongly correlated with chronological age in the ONPRC cohort (**Figure 6**) (Pearson's r = 0.94 ; median absolute difference [MAD] = 1.74, slope of age = 0.8). There was no significant difference in predictive accuracy of the liver epigenetic clock for females and males in the normal aging cohort. Weight also showed no correlation with residual epigenetic age in the ONPRC sample population**.**

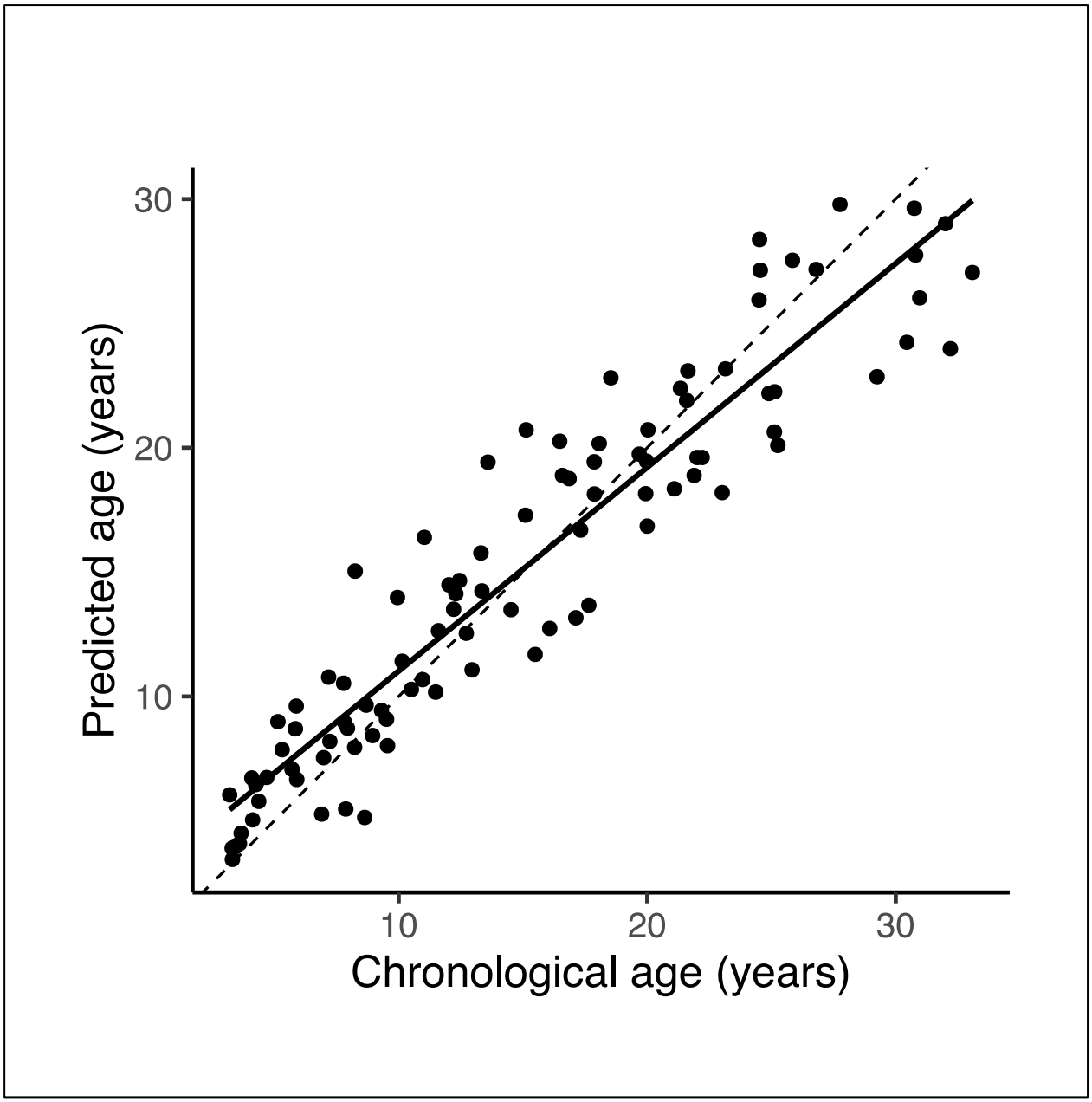


Figure 6. *Univariate regression of predicted onto chronological age for 96 liver samples used to build the liver clock. Estimates per animal were generated by elastic net regression using LOOCV.*

Age Predictions from NIA Study Correlate with Calendar Age

Using the window-based model on the NIA cohort as a whole, predicted and chronological age showed a strong positive correlation (Pearson's r = 0.69, [95% CI: 0.53, 0.8], $p = 4.35 \times 10^{-10}$) (**Figure 7)**.

The interaction between dietary restriction and juvenile-start was significantly predictive of a lower rate of epigenetic aging (t = -2.46, p = 0.02) (see **Figure 8**).

Later Age-at-Diet-Start Correlated with Longer Lifespan in NIA Monkeys

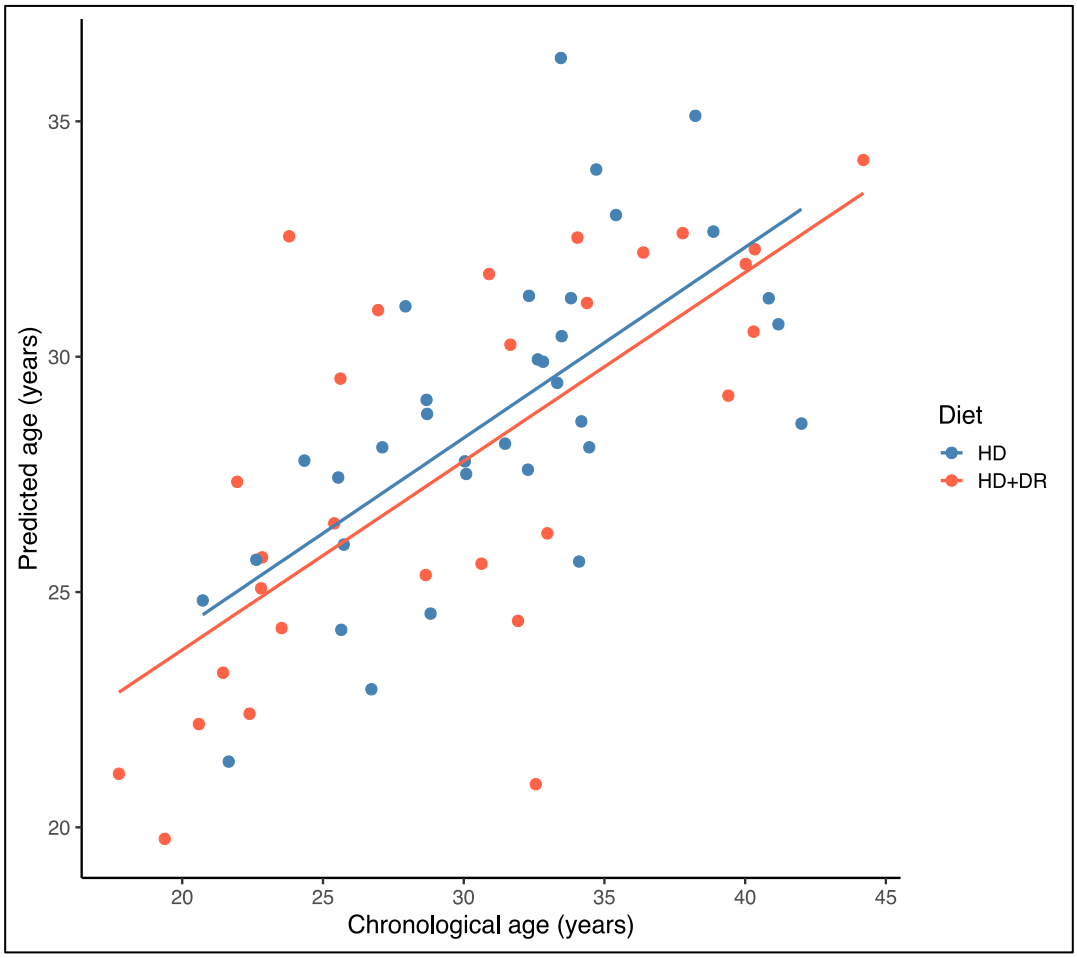


Figure 7*. Univariate regression of predicted age onto chronological age in the NIA dataset. HD, healthy diet. HD+DR, healthy diet with reduced caloric intake (dietary restriction).*

In both males and females, age at the start of the diet (in years) was significantly positively correlated with age at death (males: Pearson's r = 0.74 [95% CI: 0.56, 0.86], p = 3.88 x 10^{-8}; females: r = 0.65 [95% CI: 0.33, 0.84], p = 0.0008).

Aggregate Analyses of Differential Methylation in Normal Liver Maturation and Aging

Figure 8. *Residual age distribution among individuals in the control group by developmental stage at start (left). Residual age distribution for individuals in the dietary restricted (HD+DR) group by developmental stage at start (right).*

I identified 16,161 CpG sites that were significantly differentially methylated with age (FDR < 0.001). Of these, 9,923 and 6,238 were hypo- and hypermethylated with age, respectively. There were significantly more sites that lost rather than gained methylation with advancing age (OR: 1.73 [95% CI: 1.12, 2.7], p = 0.009), consistent with known trends of global hypomethylation coupled with more targeted hypermethylation in specific regions. This finding also aligns with a previously identified trend in the liver of widespread DNA demethylation between the perinatal period and adulthood in mice (Liang et al. 2011). Enriched pathways for hypomethylated sites included protein-protein interactions at the synapse, collagen chain trimerization, and extracellular matrix organization (FDR<0.1).

Sex Differences in Methylation in Adult Liver Aging

Adult females showed a greater number of CpG sites with significant hypomethylation (OR: 3.18 [95% CI: 2.68, 3.78], $p < 2.2 \times 10^{-16}$, two-sided FET) and hypermethylation (OR: 1.62 [95% CI: 1.23, 2.14], $p < 0.001$, two-sided FET) compared to adult males.

Sex-Specific Differences in Methylation by Age and Diet

Normal aging adult females showed significantly more hypomethylation (but not more hypermethylation) compared to their age-matched counterparts in the NIA group (OR: 7.12 [95% CI: 5.65, 9.07], $p < 2.2$ x 10-16, two-sided FET). A similar trend was observed for males, with normal aging males showing significantly more hypomethylation with age (but not more hypermethylation) compared to their age-matched NIA counterparts (OR: 12.74 [95% CI: 7.39, 23.78], $p < 2.2$ x 10-16, two-sided FET).

Differential Methylation in DR Juveniles Occurred at Genes Involved Growth and Development

Among juvenile-start individuals, there were 256 CpG sites that were significantly differentially methylated by the interaction between diet (HD or HD+DR) and precise age at start (in years). Genes overlapping these significant DMCs were related to the cellular response to DNA damage, as well as to several Mammalian Phenotypes (MPs) that pertain to abnormal or altered patterns of growth and/or age-specific mortality risk (**Figure 9**). The MP to which the greatest number of genes (15, more than 3x as many as any other MP apart from decreased grip strength [7 genes]) were associated was “preweaning lethality”. Other relevant associated MPs included liver hypoplasia, abnormal myelopoiesis, lethality throughout fetal growth and development, insulitis, decreased skeletal muscle mass and decreased skeletal muscle fiber.

Differential Methylation in Super Agers Reflects Processes Distinct from the Epigenetic Clock

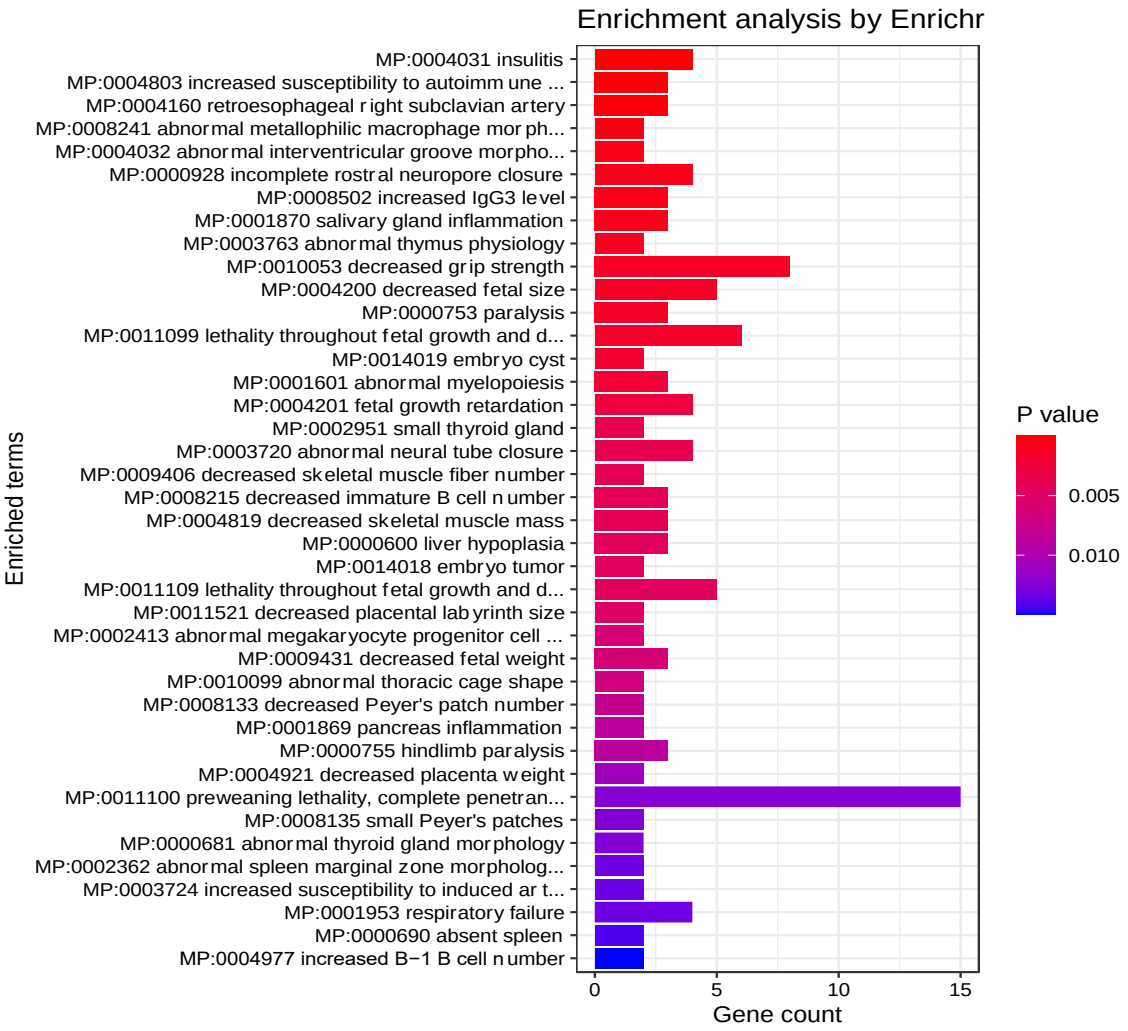


Figure 9*. Mammalian phenotypes associated with the interaction between diet and age-at-start in juvenile-starts.*

Males who entered the NIA study between the ages of 14.8 and 23 ("old start" males) lived exceptionally long lives. However, these "Super Agers" did not show consistently lower rates of epigenetic aging (residual epiegentic age) as measured by the epigenetic clock compared to the rest of the individuals in the dataset (**Figure 10**). Of the 3,548 CpG sites that were significantly differentially methylated in super-agers, 79% of them (n= 2,803) showed *gains* of methylation with age and clustered discretely by diet (**Figure 11**). Hypo-age DMCs (n=745) did not cluster neatly by any of the variables tested (**Figure S5, Appendix A**). Hyper-age DMCs were significantly enriched for nervous system development terms (**Figure S6, Appendix A**). Hypo-age DMCs did not show any significant enrichment for particular GO terms or KEGG pathways.

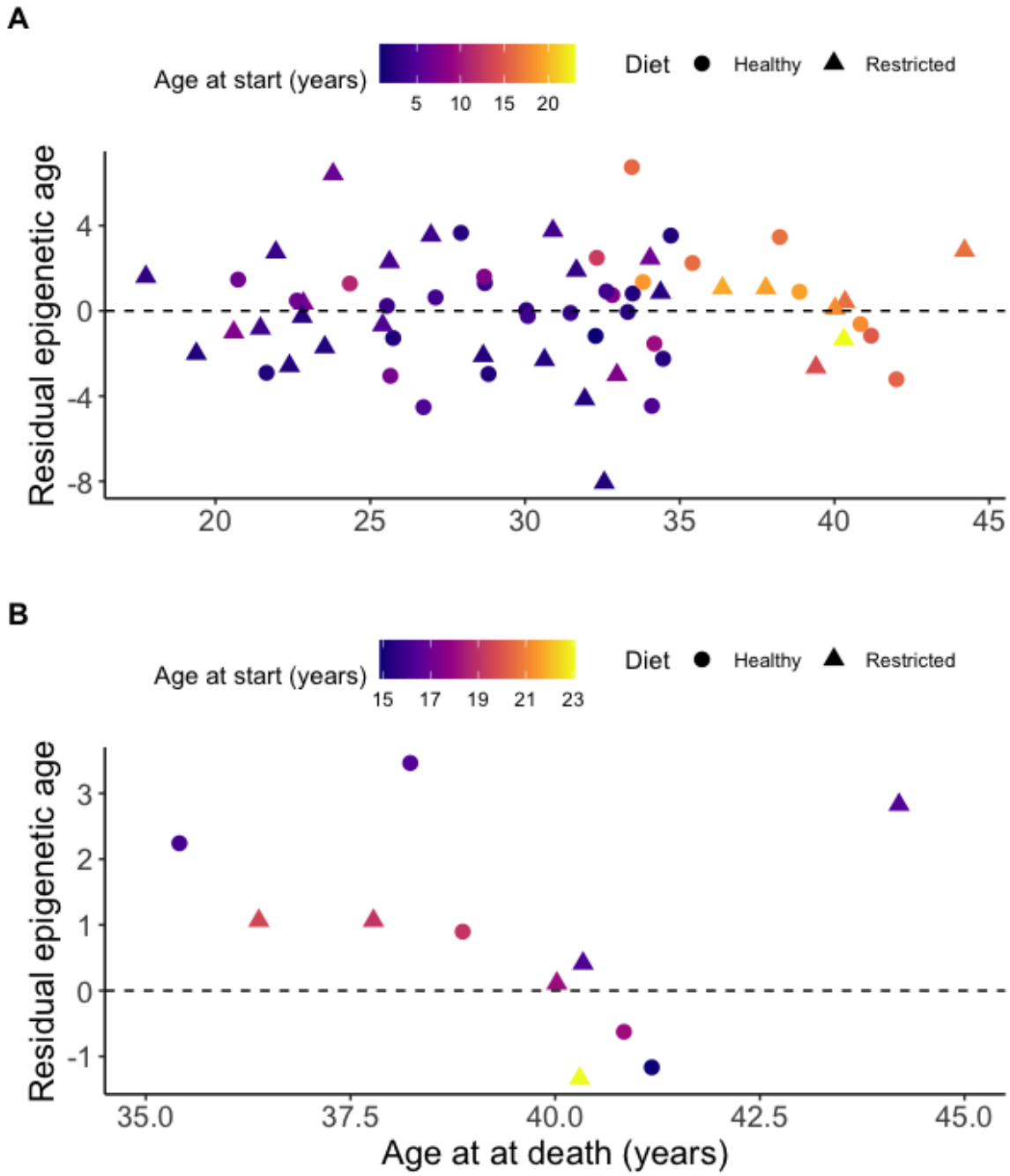


Figure 10. *Residual age plotted against chronological age for A) all individuals in the NIA study, B) only long-lived males (n=11). Long-lived males do not show consistently lower residual age as compared to all animals in the study. Circles correspond to HD = healthy diet; triangles correspond to HD+DR = healthy diet + dietary restricted. Color shows age at which an individual entered the study.*

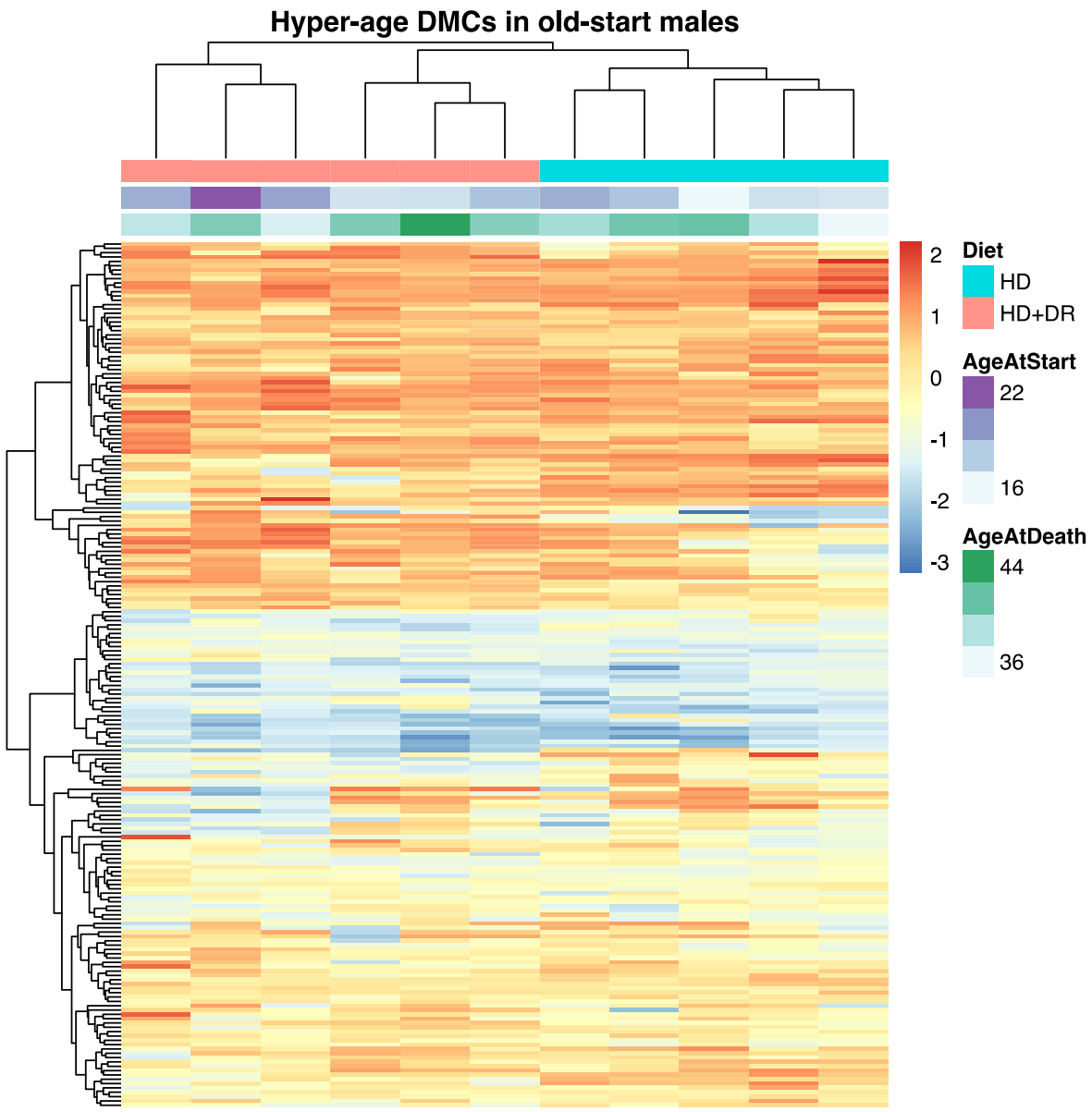


Figure 11. *Heatmap of significantly hypermethylated sites with age in long-lived males. Note samples cluster first by diet type (healthy versus healthy+dietary restricted).*

Discussion

The liver acts as the body's metabolic conductor, monitoring and coordinating the activity of numerous molecular networks by integrating and sending signals across cells and tissues, allocating energetic resources, and maintaining tissue homeostasis. An essential role of DNA methylation is the dynamic regulation and timing of activities that

are part of the complex, coordinated program of whole-body metabolism. A key aspect of the liver is the constancy with which it works—its pursuit of homeostasis is arguably its defining feature. The DNA methylome can also be characterized by its dynamic nature and capacity to facilitate reversible changes in gene regulation, and this makes it a natural complement to molecular analyses in the liver. Studies targeting the mechanics of DNA methylation change in the liver across the lifespan have been slow to emerge and are very rarely performed outside of humans, mice and rats.

Here, I endeavored to characterize molecular aging in the liver of rhesus macaques through the lens of the DNA methylome. I developed a tissue-specific epigenetic clock and subsequently evaluated its sensitivity to a long-term pro-longevity intervention (dietary restriction). I complemented the creation of this predictive model with differential methylation analyses across ages, by sex, and under distinct behavioral/dietary conditions with the objective of understanding how changes in the liver methylome may contribute to the larger phenotype of organismal aging. Below, I highlight three key findings that have emerged thus far from this research.

Sex-Specific Trends in Methylation Aging

I observed significant differences in the relative amount of age-related change in the female versus the male liver DNA methylome in rhesus macaques. In terms of the number of CpG sites that gain or lose methylation with age, females significantly outpaced males. While it is tempting to speculate on theoretical causes for this disparity, further research is required to understand the consequences of change in methylation at different genomic elements (e.g., 5' UTRs, intergenic regions, exons, etc.), which can up- or downregulate gene expression depending on the genomic element at which methylation occurs. Such research will contribute to the developing portrait of the rhesus liver methylome that is just starting to come into focus. Ideally, such a reference will help to elucidate the underlying biology of sex differences in the liver as well as how changes in this organ are connected to both external and internal mechanisms of aging.

A major cause of non-age related death in the NIA female population was complications from endometriosis (J. Mattison, *personal communication*), which resulted in very few females reaching very old age. The relationship between liver aging, fibrosis, endometriosis, and endometrial and other female reproductive cancer demands further investigation. I speculate that the effects of DR may be particularly detrimental in young and reproductive-age females who are predisposed to endometriosis, due to association of endometriosis with undesirable weight loss, difficulties in nutrient absorption, and low BMI. This emphasizes the fact that DR is only likely to exert a pro-longevity effect under conditions of robust health, and could otherwise be potentially lethal.

A trend of global hypomethylation is commonly observed with advancing age. While hypermethylation with age occurs in a seemingly less random manner (often at proto-oncogenes), age-related hypomethylation is more stochastic (Vandiver et al. 2015). Rather than being a cause or direct consequence of mechanistic aging, it is likely the result of an increasingly error prone molecular architecture that promotes the loss of genomic integrity with age. CpG sites showing hypomethylation with age in the normal aging (ONPRC) cohort were enriched for broad pathways that are not inherently pathological but can easily induce a pathogenic cascade that snowballs into an adverse aging phenotype. First, collagen chain trimerization is critical for proper protein folding, and the accumulation of misfolded proteins is considered to be one of the most conserved molecular features of aging, affecting both unicellular and multicellular organisms (Lemoine 2021). Second, altered activity of protein-protein interactions at the synapse may disrupt patterns of inter-cellular adhesion and binding of transmembrane proteins, thereby degrading the ability of these highly specialized sites to engage in proper communication. Third, extracellular matrix elements found in the perivascular space of the central and portal veins of the liver are thought to contribute to the liver's longevity (Arrojo e Drigo et al. 2019), and disorganization of the extracellular matrix is a harbinger of perturbed cellular communication and migratory networks. Modifications to the tissue microenvironment that can lead to proto-oncogenic changes in stem cell niches, increased cell proliferation, loss of stem cell differentiation capacity, and promote the pathogenesis of various types of cancer and hepatic fibrosis. In captivity, the rhesus macaque liver is

very susceptible to the condition of fibrosis (excessive wound-healing that replaces parenchymal with connective scar tissue) (Kim et al., 2016). Among females in the NIA study, the main cause of non-age related death was due to complications from endometriosis, highlighting the potential role for dysregulated wound repair mechanisms in driving pathologies of aging (Taylor, Kotlyar, and Flores 2021).

Developmental Stage is a Key Determinant of the Organismal Response to Dietary Restriction

Through the application and testing of this tissue-specific epigenetic clock in the context of a known pro-longevity intervention, I found that the epigenetic clock does indeed capture changes with age that are relevant to organismal health. That the rate of epigenetic aging in the juvenile-start group was significantly slower but showed no corresponding increases in life expectancy provides support for a trade-off that sacrifices later-life survival for the processes of growth, maintenance/repair, and potentially reproduction. Alternatively, this slowed rate of epigenetic change could be the result of programming in the early-life epigenome in anticipation of a nutritionally-scarce environment (Hanley et al. 2010), but not necessarily a component of a particular evolutionary trade-off. Under either scenario, the results from this study indicate that the developmental stage at which an individual starts a healthy diet with or without dietary restriction may be predictive of life expectancy in primates and other long-lived species, but not necessarily in the expected direction. Dietary restriction in young or reproductive-aged primates may thus be counterproductive due to a lack of energetic capital that impairs normal processes of growth and thus increase the risk of premature mortality. This is an important finding as this is the only study to date that has examined the effects of DR beginning at a very young age in a long-lived primate species.

In contrast, for the old-start males, who need not have shouldered the energetic demands of growth or reproduction at the time they began DR, I suggest that the mechanism underlying the extended longevity phenotype may be related to the preservation and/or rejuvenation of healthy levels of reactive oxygen species (ROS). (Ristow and Schmeisser 2014). The body's response to ROS has been shown to be dose-dependent: while high levels of cause damage and cellular aging, low levels enable ROS to act as signaling molecules that maintain the functional

integrity of cellular capacity to effectively remove waste, repair damage, and thus extend cellular lifespan (Sohal and Orr 2012; Pickering et al. 2017; Barja 2004).

Surprisingly, I found exceptionally long-lived individuals showed relatively high rates of epigenetic aging despite their remarkable longevity. I posit that the pace of biological aging as reflected in the epigenetic clock may be in large part programmed by the early-life nutritional environment, and that predictions of the epigenetic clock grow increasingly decoupled from an individual's mortality risk over time in certain long-lived species. In the context of liver and quite possibly in other tissues too, the epigenetic clock appears to capture at least two age-related phenomena, one of which is active and the other passive. The "active" stage is defined by experiences and exposures in early life: the cells of the body are "listening" for cues from the environment most intently at this stage, and this heightened sensitivity enables external signals to more readily "get under the skin" and directly provoke epigenetic activity that sets the pace of an aging trajectory, and may predispose (or protect) an individual from chronic non-communicable diseases later in life (Barker 2004; Kohil et al. 2021; Dumolt, Patel, and Rideout 2021; Gluckman and Hanson 2004).

The second, "passive" component of this mechanical framework does not mold the shape of the aging trajectory directly, as I posit it does in early life. Instead, it acts more as a distracted stenographer, recording stochastic deterioration as it pertains to the operational integrity of the aging methylome, but with varying levels of attention depending on the magnitude of the effect. Furthermore, it is not clear that there is necessarily a causal relationship between randomly-accumulating damage and disease and mortality risk, which may require reconceptualization of this biomarker and how it is used.

Additionally, dysregulation of intra- and inter-cellular communication networks may contribute to intrinsic age-related physiological deterioration. This type of molecular change can still contribute to variation in rates of aging, but may largely be caused by random error as genomic stability and epigenomic integrity decline with age. Thus, I suggest that the activity of these "pro-aging" forces are likely to be more responsible for

the gradual deterioration that characterizes the aging process at the cellular level than the types of environmental factors that play a more substantial role in early life.

Under my proposed theory of epigenetic aging and the mechanics of the epigenetic clock, the perinatal methylome facilitates direct responses to cues from the external environment in an effort to acclimate and maximize prospective fitness in a newly discovered environment, before more sophisticated networks (e.g., immune, metabolic, endocrine) take over. Following this early-life period, developmental trajectories may become increasingly canalized and thus more difficult to perturb, as an extended period of heightened sensitivity to the external world is unlikely to have a net positive effect on the fitness of an organism, and instead may simply interfere with development or delay reproductive success. It follows that DNA methylation in adults would be less susceptible to direct signals from the environment. However, this pathway could still contribute to variation in aging indirectly, by modulating the impact of factors that promote DNA damage, epigenetic or metabolic alterations, and/or more (or less) rapid rates of age-related molecular change. Thus, in early life, the pace of epigenetic aging is primed to respond to factors that set the body on a particular trajectory (i.e., it is developmentally plastic), along which it proceeds through the rest of life with possible periodic spikes in environmental sensitivity throughout the juvenile stage and adulthood.

Related to this proposed framework, it is notable the Mammalian Phenotypes associated with the interaction term of age-at-start and diet in juveniles suggest potential alterations in patterns of growth. Many of these phenotypes are similar to changes that result from malnutrition *in utero* in human fetuses (Gluckman and Hanson 2004). This suggests exposure to a calorically-restricted diet among young rhesus macaques may alter life-long metabolic function through altered programming of the growth trajectory and negatively impact later-life survival.

In non-human animals, earlier age at first reproduction has been associated with increased age-specific fecundity but reduced overall longevity in multiple taxa. In a study of female rhesus macaques, earlier age at first reproduction was negatively associated with survival to ages 11 and 16, but showed no significant association with survival to 21 and 26 years of age (Blomquist 2009), suggesting some kind of physiological shift occurs between middle- and old age in females of this species. One possible explanation is that natural age-related declines in fertility automatically shifts resource allocation away from reproduction to somatic maintenance.

Relatedly, individuals who live to age 21 despite an early age at first reproduction may be more resilient to the effects of resource-related stress, and may be more likely to continue to survive if they successfully make it through the period of peak reproductive activity. In Seychelles warblers (*Acrocephalus sechellensis*), a small but relatively long-lived avian species with a life expectancy at fledging of 5.5 and a maximum lifespan of 17 years, environmental quality and greater food abundance was associated with a later age at first reproduction, which itself was significantly positively correlated with better later-life survival (Hammers et al. 2013). Similarly, in female Asian elephants (*Elephas maximus*) earlier age at first reproduction was associated with a significantly reduced likelihood of survival in later-life (Hayward et al. 2014).

A study of the effects of age at first reproduction in multiple zoo-housed avian and mammalian species on later-life survival found no significant association, arguably due to the absence of resource stress common to life in the wild (Ricklefs and Cadena 2007). While the macaques in our study were captive-housed and thus not subject to the same ecological pressures as wild populations, individuals under DR were subject to a type of resource stress (i.e., caloric restriction). In rhesus macaques, the sex of an individual as well as the developmental time point at which they begin a calorically restricted diet may be a critical determinant of whether they are able to reap the putative pro-longevity benefits from such a dietary program.

Dietary Restriction Delayed Age-Related Deterioration of the Methylome

Finally, dietary restriction appears to delay the rate of stochastic deterioration that accompanies aging in the rhesus liver methylome. The known trend of global hypomethylation that portends dysfunction in the epigenetic landscape happens at a slower rate in both females and males under DR. Intriguingly, sites that were significantly hypermethylated with age in the Super Age males showed several notable features. As mentioned earlier, 79% of significant DMCs showed *gains* in methylation with age, despite the known trend of widespread hypomethylation that typically occurs during aging. This finding aligns with previous work in mice, who show similarly

disproportionate gains and relatively little loss of methylation in response to DR (Hahn et al. 2017). Second, hierarchical clustering analysis revealed that patterns of methylation at these sites showed greater similarity by diet type, rather than by age at start or death (**Figure 11**). Third, the hyper-age DMCs in the "old-start" males were significantly enriched for specific GO terms pertaining to the extracellular matrix, and in particular the molecular processes and cellular components involved in synaptic signaling and other forms of cellular crosstalk, whose integrity may be better preserved under dietary restriction.

I further suggest that a major component of pathological aging in the liver is the overactivation of wound-healing mechanisms leading to fibrosis, which can alter the structural anatomy and thus degrade the functional integrity of the liver (Jensen, Alpini, and Glaser 2013). Fibrosis causes infiltration of the sinusoidal space by scar tissue, which redirects the flow of blood carrying potentially infectious agents; as a result, it may act as part of a positive feedback loop that promotes disruptions to cellular crosstalk and further pathogenic alterations to the liver physiology (Jensen, Alpini, and Glaser 2013). An increase in dense connective tissue due to the overactivity of collagen-producing genes is related to alterations of the extracellular matrix (Karsdal et al. 2020), and thus I propose this may progressively weaken the robustness of cellular signaling networks. Much like in games of telephone, the more points of transfer that exist between the original message and final recipient, the more likely it is that the message and its meaning will be corrupted. This however is merely one hypothesis that remains to be thoroughly interrogated.

Conclusions

The liver has a remarkably diverse set of functional capabilities, acting as the body's master metabolic regulator, with additional roles in the immune and endocrine systems, and is of critical importance to the maintenance of health. While hepatocytes make up approximately 80% of the constituent cell types in liver, the liver nonetheless hosts a wide range of highly localized cells that assist in the transport of nutrients, detoxification and other critical physiological processes. The liver shares several active pathways involved in photoaging of skin, particularly among the activity of genes related to elastic fiber and collagen production and the extracellular matrix (McCabe et al. 2020). Many of these shared aspects are themselves shared features of the

chronic inflammation that is associated with “intrinsic aging”. McCabe and colleagues (2020) found metalloproteinase inhibitor 3 (*TIMP3*) expression was significantly increased in aged skin (>80 years), complementing a finding of significant hypomethylation of *TIMP3* with age in our normal liver aging dataset. The relationship between extracellular matrix degradation and the dysregulation of inter-cellular signaling networks across cells and tissue types is worthy of further investigation to elucidate how deregulated cell communication and signaling promote the progression of age-related deterioration.

While the notion of a multi-tissue biomarker of aging has excited the geroscience community, this study and other research strongly suggest that the liver may exhibit subtle disease-associated changes that are not readily apparent in other tissues or organs, especially those with disparate functional roles in the body. For this reason, future research that causally links observable mechanisms to known anatomical and physiological networks (such as changes in the liver, pancreas or gastrointestinal tract that operate through the gut-liver-brain-axis) is likely to enhance our understanding of the role of this organ within the larger organism and processes of phenotypic aging.

Bridge to Chapter IV

In the final chapter, I summarize key intellectual and material contributions that have emerged from the research I have presented here. I also suggest avenues of investigation that I believe will continue to advance our knowledge of the aging process.

CHAPTER IV

CONCLUDING SUMMARY

It is critical that we seek to unify scientific findings from functional genomics experiments with evolutionary theory to deepen our understanding of the intricacies of the aging process. To treat mice and humans as equivalent taxa in the context of aging science ignores a force (i.e., the passage of time) that has fundamentally shaped the different ways in which long- and short-lived species have evolved to respond to environmental conditions. Non-human primates, particularly great apes and cercopithecoid monkeys, are well-suited to bridge the gap between organisms with very short life expectancies and the exceptional longevity of humans. Filling this void in knowledge enables meaningful advancement towards a grounded, empirical theory of aging.

The ability to quantify biological age and determine the circumstances under which accelerated aging occurs will help deconstruct the complex, multifaceted nature of the aging process. Accelerated aging has been associated with chronic disease, psychosocial stress and has been shown to be predictive of mortality in humans (see Horvath and Raj 2018 for review). How epigenetic changes contribute to variation in the rate and severity of age-related decline is still unclear because it remains difficult to determine how specific environmental factors impact the progression of aging in humans due to the inherent lack of control over the highly variable environment. Few molecular biomarkers of physiological aging have historically been available for use in epidemiological and population- based research. However, the study of molecular aging has been bolstered by the advent of high-throughput, genome-wide techniques that have enabled the development and testing of a new generation of molecular biomarkers of the aging process. While several promising biomarkers of aging have been developed, no one marker may be truly comprehensive at present.

Changes in DNA methylation exhibit predictable patterns of change over time but are also responsive to environmental factors that contribute to variation in age-related disease pathogenesis as well as differences in the relative pace of cellular aging (E. Li and Zhang 2014). Unlike chronological age, biological age accounts for interindividual variation in the progression of age- related phenotypic change, providing a more accurate picture of overall health. The

effective treatment of age-related disease is one the great challenges faced by modern medicine; while tremendous strides have been made in aging science, the question of how the underlying molecular mechanisms of biological aging interact with parameters of the human environment to produce such marked variation in health and disease risk has not been answered. Deciphering the effects of individual environmental and behavioral variables on the phenotype of whole-organism aging is a key step towards understanding the variation we observe in the progression of biological aging.

To enable ongoing advancement in the field of aging biomarker research, distinguishing the molecular changes that reflect the normal course of human aging from those which are indicative of disease and/or disease risk is essential. A logical place to start is the examination of cell type- and tissue-specific variation, starting at a fine scale and moving progressively towards a systems-based perspective that can unify the processes of aging across the multiple scales at which it occurs. Here, I have characterized age-related change in the methylome as it manifests in specific tissues in rhesus macaques and developed and applied optimized epigenetic clock models to test hypotheses surrounding sources of variation in the aging process.

Intellectual Contributions

Studies of the methylome using both epigenetic clocks and differential methylation analyses across tissue types are well-suited to testing a range of hypotheses and can be flexibly applied to suit the needs of different disciplines. The research I have conducted for this dissertation makes several impactful scientific contributions to biological anthropology, the discipline of geroscience, and the study of aging epigenomics.

As part of this project, methylation data were generated from over 800 banked rhesus macaque tissue samples. This is the largest amount of methylation data produced for this species to date (despite being quite modest in comparison to what is available for mice and for humans). Moreover, these data come exclusively from tissues previously collected and stored in biobanks, thus making use of the rich tissue resources that exist at research centers across the country without any additional sample collection. Only a

small number of methylation datasets have historically been available for rhesus macaques, despite the prevalence and involvement of this species across many types of biomedical research (Didier et al. 2016; Mitchell et al. 2015; Phillips et al. 2014). In addition to the size of the dataset, the ample numbers of both females and males as well as the breadth of the age range covered are also exceptional assets. Most publicly available datasets for rhesus macaques are limited in age range, and some do not provide information regarding the age of the individual from which samples came. Therefore, the methylation datasets generated from blood, liver, and brain for this dissertation will facilitate novel investigations into the epigenetics of aging in a long-lived primate. Many of the factors that negatively impact human health manifest in what appears to be acceleration in the course of natural aging, but this can only be confirmed in species whose life expectancy is comparable to that of humans. Such investigations are an essential next step in deepening our understanding of the phenotype of human longevity.

To understand how variation in the epigenome interacts with the environment to differentially affect healthspan, I characterized molecular signatures of aging in rhesus macaques and developed generalizable predictive models to enable testing of hypotheses surrounding the mechanics of aging at the molecular level and how they manifest differently across tissues, individuals, environments, and species. I performed rigorous comparative analyses of the aging methylome by sex, age, and in response to dietary restriction in the liver. I additionally developed three targeted, tissue-specific epigenetic clocks for rhesus macaques and tested two of these models under different environmental conditions expected to influence health and the aging process.

To the best of my knowledge, the single-tissue clocks I have developed are the first of their kind for use in rhesus macaques, and are expected to have broad utility to research being conducted at the ONPRC and similar research institutes. While the idea of a multi-tissue clock holds clear appeal, single-tissue clocks are more sensitive to more subtle age-relevant change that occur exclusively in or disproportionately affect a particular tissue type (Bell et al. 2019). I first developed a blood-based epigenetic clock using venous blood samples previously collected from over five hundred free-ranging rhesus macaques, aged 1.32 months to 28.82 years, living on the island of Cayo Santiago (Chapter II). These monkeys are provisioned with food and water but otherwise range freely across the island. While not formally included as part of this

dissertation, I have also explored differential methylation by age, sex, in response to environmental disruption, and according to different reproductive characteristics in this dataset. While these specific analyses are ongoing, this work speaks to the diversity of hypotheses that can be tested with the Cayo Santiago methylation dataset and demonstrates the tremendous potential of this resource for investigating the methylome in the context of physiological, health or age-related change.

While blood is a sound choice for assessments of general systemic health in humans and other animals, certain tissues are better suited for addressing some questions over others. One very salient and obvious example is the study of dietary intervention, where metabolically active tissues like liver or pancreas are likely to be more illustrative of underlying change. As an example, in one study of people with chronic obesity preparing to undergo bariatric surgery, epigenetic age was shown in to increase by 3.3 years for every 10-unit increase in BMI in liver, but not blood, muscle, or adipose tissue (Horvath et al. 2014). This underscores the likelihood of unique, tissue-specific phenomena that warrant further inquiry. Other studies have shown that different organs and tissues (e.g., hippocampus, Levine et al. 2015) appear to age at distinct rates, which could ultimately mask changes in the pace of aging that occur first or only in a single tissue. A study that systematically compared tissue-specific to pan-tissue clocks found that the models varied both in their predictive capacity and underlying genomic features: while both tissue-specific and multi-tissue models were able to predict age with high accuracy, tissue-specific clocks showed better performance in independent testing, and the CpG sites that constituted the tissue-specific models were more likely to be located in CpG shores (as opposed to CpG islands for the multi-tissue model), and less likely to be found in evolutionarily conserved regions (Choi, Joe, and Nam 2019). This again suggests that tissue-specific clocks are better suited to monitoring and deciphering the etiology of certain diseases. Studies of how the methylome changes in response to dietary modification and aging in rats have demonstrated consistent distinctions in patterns of methylation between tissue types, with liver often showing anomalous patterns of methylation change in comparison to other organs (Guarasci et al. 2018). As an organ, the liver is the master regulator of whole-body metabolism, and metabolic dysregulation

is a common feature of premature aging. The liver may exhibit sub-clinical signs of metabolic dysfunction much earlier than blood or other tissues, highlighting the value of a more targeted approach in the context of diseases such as diabetes, non-alcoholic fatty liver disease, or other components of Metabolic Syndrome.

This is the first research to investigate sex differences in the liver epigenome of healthy macaques, to characterize epigenetic age across the lifespan in this species in multiple tissue types, and to evaluate the effects of a pro-longevity intervention using a custom, tissue-specific epigenetic clock (Chapter III). Results from this research underscore the necessity of elucidating the role of the liver within the larger process of physiological aging and the role it plays in disease and mortality risk in long-lived species. It is worth noting one relevant difference in the neural anatomy of the liver between mice and rats compared to humans: humans and cercopithecoid monkeys show liver tissue that is deeply innervated (Nobin et al. 1978) and contains direct lines of communication to the brain, specifically the hypothalamus (Jensen et al. 2013). The hypothalamus can activate hepatic metabolism through the vagus nerve, which innervates the liver (Pocai et al. 2005). By contrast, the livers of mice and rats do not show this deep level of innervation, and instead have a greater concentration of cell-cell gap junctions between hepatocytes that occur in inverse proportions to the level of innervation (Seseke, Gardemann, and Jungermann 1992; Hertzberg and Gilula 1979). Additionally, in rats and mice, the liver has no direct connection to the brain and communicates only indirectly with the nervous system (Yi et al. 2010). These differences are salient given the heavy (albeit incompletely understood) involvement of the nervous system in the progression of aging in humans.

It is notable that gene expression patterns in the liver are generally similar across a range of mammalian taxa, namely mice, cynomolgus macaques, pigs, and humans (Wagenaar et al. 1994). Nonetheless, the effects of DNA methylation are not limited to gene expression. These trends towards heterogeneity between species emphasizes the value of targeted models for this (and other) tissue types for elucidating the etiology of disease. Both species- and tissue-specific studies will yield novel discoveries about age-related change in the context of the hepatic epigenome.

Findings from this project also highlight the importance of age as it relates to developmental stage and to sex in determining the likelihood of reaping the pro-longevity

benefits of DR or a pharmacological DR mimetic. Given this finding contrasts with studies in mice that show increased longevity when caloric restriction is started at earlier ages, it is important to further explore the underpinnings of this finding within an evolutionary and life history-oriented framework. These analyses have also highlighted potential roles of the nervous system in the modulation of age-related change in the methylome of rhesus macaques, a relationship previously reported by an investigation as part of the WNPRC study of long-term dietary restriction that looked at changes in gene expression in response to DR (Kayo et al. 2001). This is an intriguing finding that warrants further investigation, given the dearth of knowledge regarding how the nervous system affects tissues and tissue-specific processes throughout the body, and would benefit from incorporating additional data from other tissues. The central nervous system plays an essential role in the sensing and regulation of both glucose and lipids in the hepatic portal system, and this line of communication between the brain and liver to regulate metabolism has been suggested as a therapeutic target for obesity (Jensen et al. 2013).

While not a part of my dissertation project, I also developed a hippocampus-

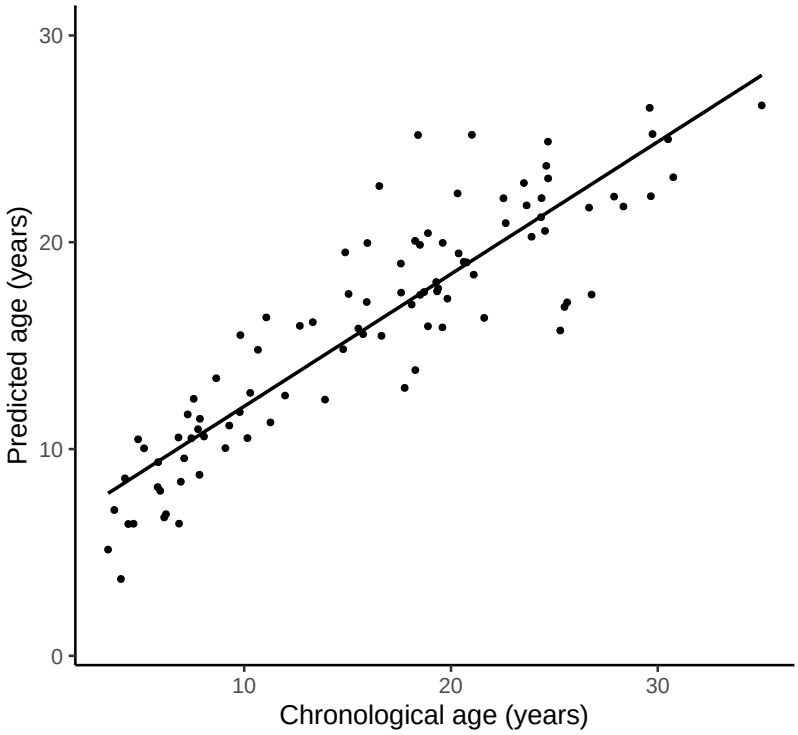


Figure 12*. Predicted ages from hippocampus samples (n=96).*

specific clock for rhesus macaques that predicts age with high accuracy (Pearson's r = 0.9; median absolute difference = 2.44 years; **Figure 12**). In addition to the DNA methylation data used to build this model, we recently obtained matched RNA-seq data for these same samples. These matched datasets are well suited to interrogate how the nervous system and associated changes in the methylome contribute to broader patterns of age-related phenotypic change.

Future Directions

The contemporary field of aging research is pivoting away from the identification of genes associated with longevity in ways that remain largely mysterious (Wensink and Cohen 2022) to the characterization of the molecular mechanisms by which the external world "gets under the skin" to promote such variation in longevity. With the advent of multiple molecular biomarkers that purportedly capture systemic biological aging (e.g., Levine et al. 2018; Lu et al. 2019), understanding the underlying mechanisms should be prioritized to maximize their translational potential to the clinic. This necessitates the thorough characterization of *intra*-individual aging variation to evaluate the sensitivity of different organs to putative pro- or anti-aging factors, as well as their relative contribution to the phenomenon of aging in other tissues and for an organism as a whole.

There are a remarkable number of directions in which any part of this research can be taken. The work presented in this dissertation forms a foundation for future empirical analyses involving complementary components of the aging process, such as epigenetic drift, whereby the epigenome becomes increasingly unique and more prone to errors with advancing age (Issa 2014; Y. Li and Tollefsbol 2016). Drift can be elegantly measured using the information theoretic concept of entropy (Jenkinson et al. 2017). Entropy is a measure of predictability or disorder in a system, and has been used in the past to capture the phenomenon of epigenetic drift (e.g., Maegawa et al. 2017). Future research should aim to elucidate epigenetic drift in different tissues and develop a clearer understanding of how this phenomenon fits within the larger network of age-related changes that occur within the epigenome. Additionally, these data can be used to identify putative methylation quantitative trait loci (meQTLs). Incorporating chromatin accessibility and other types of epigenomic data will help to disentangle the effects of genes versus environment on methylation change across the lifespan. Furthermore, multi-omics studies

may identify genes and/or genetic mechanisms with active roles in the aging process that were previously unconfirmed due to insufficient data or methodological resources. The field of aging science is at an inflection point, and there is enormous potential for epigenetic and comparative evolutionary studies to make meaningful contributions to emerging science.

Future directions for pro-longevity dietary interventions should include research aimed at completing the diverse molecular portrait of the aging process. For the long-term dietary restriction study carried out at the NIA, it would be informative to examine methylome profiles in blood longitudinally, to see how the methylome of dietary-restricted individuals changed across the lifespan and in accordance with length of time on the diet. The liver datasets analyzed in this dissertation are rare resources due to the lack of available samples from healthy or normal liver tissue from any primate (including humans) at young, middle, and early adult ages. Blood is advantageous in that is relatively easy to collect and can be sampled repeatedly over the lifetime of an individual. By examining longitudinal change in these animals, we can see discern whether the effect of DR is tissue-specific or more systemic, determine whether DR leaves a "signature" of energetic restriction in the methylome, and whether this signature accurately portends extensions in lifespan and healthspan. Furthermore, the absence of a lifespan-extending effect in the "adult start" group warrants attention. This is a notable distinction compared to mice, for whom starting DR earlier in adulthood correlates with more beneficial longevity outcomes (Cole et al. 2017). Additionally, the changes in the methylome that occur with age and in response to DR in this rhesus macaque population should be comparatively analyzed to see if an independent signature of DR is discernable, and if such a signature, assuming it exists, is functionally distinct from predictable patterns of age-related change. Functional characterization of the differentially methylated sites (particularly those showing significant hypermethylation with age) in the Super Agers is one potentially fruitful avenue for this pursuit.

Characterizing the genes and genomic elements to which CpG sites showing significant differential methylation with age between the normal and NIA datasets will help shed light on the delayed aging effect of DR supported by findings from this

research. Additionally, identifying the mechanisms which underlie the difference in magnitude of age-related change in female versus male rhesus macaques will be important to furthering our understanding of how chromosomal sex impacts aging in different tissues and in response to different pro-longevity interventions. Future research on DR in primates should consider whether it is worthwhile to include very young non-human primates in such studies, given that trade-offs with growth appear to interfere with any substantial longevity benefit that might be reaped.

Lastly, while this dissertation has made important contributions, it too has limitations. It will be worthwhile to explore how cell type and cellular age (based on regeneration rates) contributes to age-related methylation change using single-cell approaches. It is also important to recognize that while the two species' evolutionary similarity is a substantial asset, macaques and humans are nonetheless very distinct species. While we can isolate variables in the laboratory, ultimately, removing the effects of the environment may be akin to introducing a different type of confounding variable when it comes to the study of epigenomics and gene-environment interactions. The development of approaches that can capture and quantify the complexity of interaction between different environmental forces in real time will be challenging, but may hold the final answers to a theoretically diminishing number of outstanding questions about aging. Moving forward, it will be advantageous to employ and unify findings from both targeted and system-wide approaches to identify mechanisms that underlie relationships between accelerated (or decelerated) aging and specific age-related diseases. This in turn may enable our understanding of the larger phenomenon of biological aging to come full circle.

APPENDIX A

SUPPLEMENTAL FIGURES

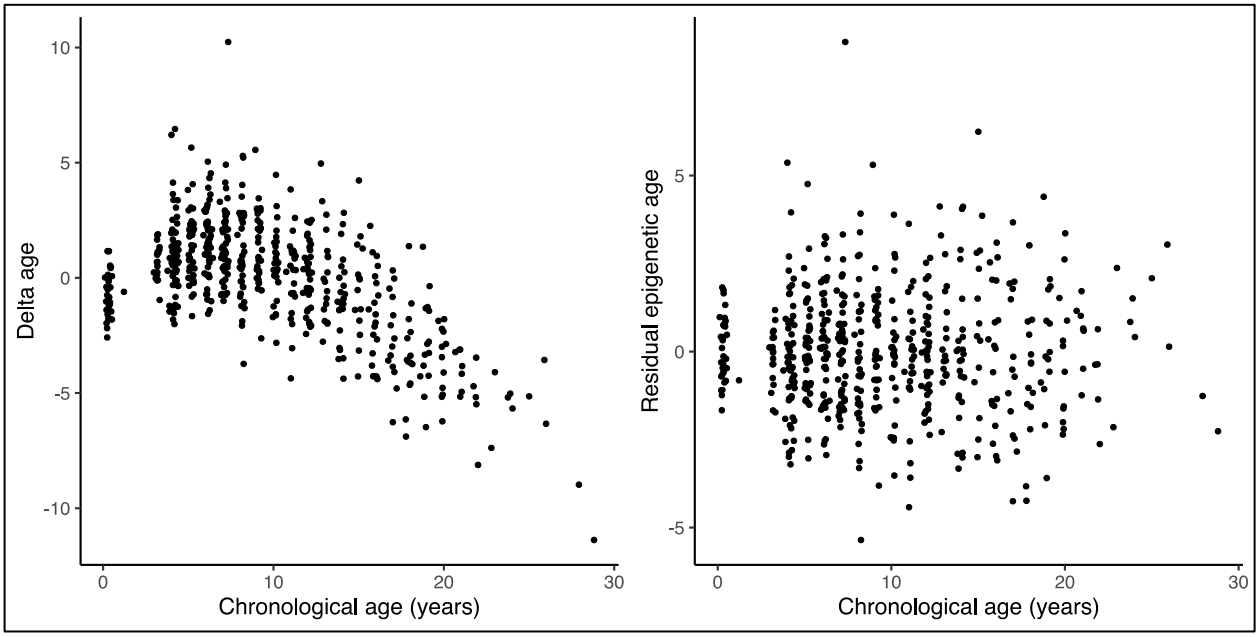


Figure S1. *Plot on the left shows uncorrected measure of age acceleration, "delta age", which displays systematic bias towards under prediction at older ages. Once we regress out the effects of the modeling process, the measure is much more evenly scattered across age ranges and no longer reflects any apparent technical confounders.*

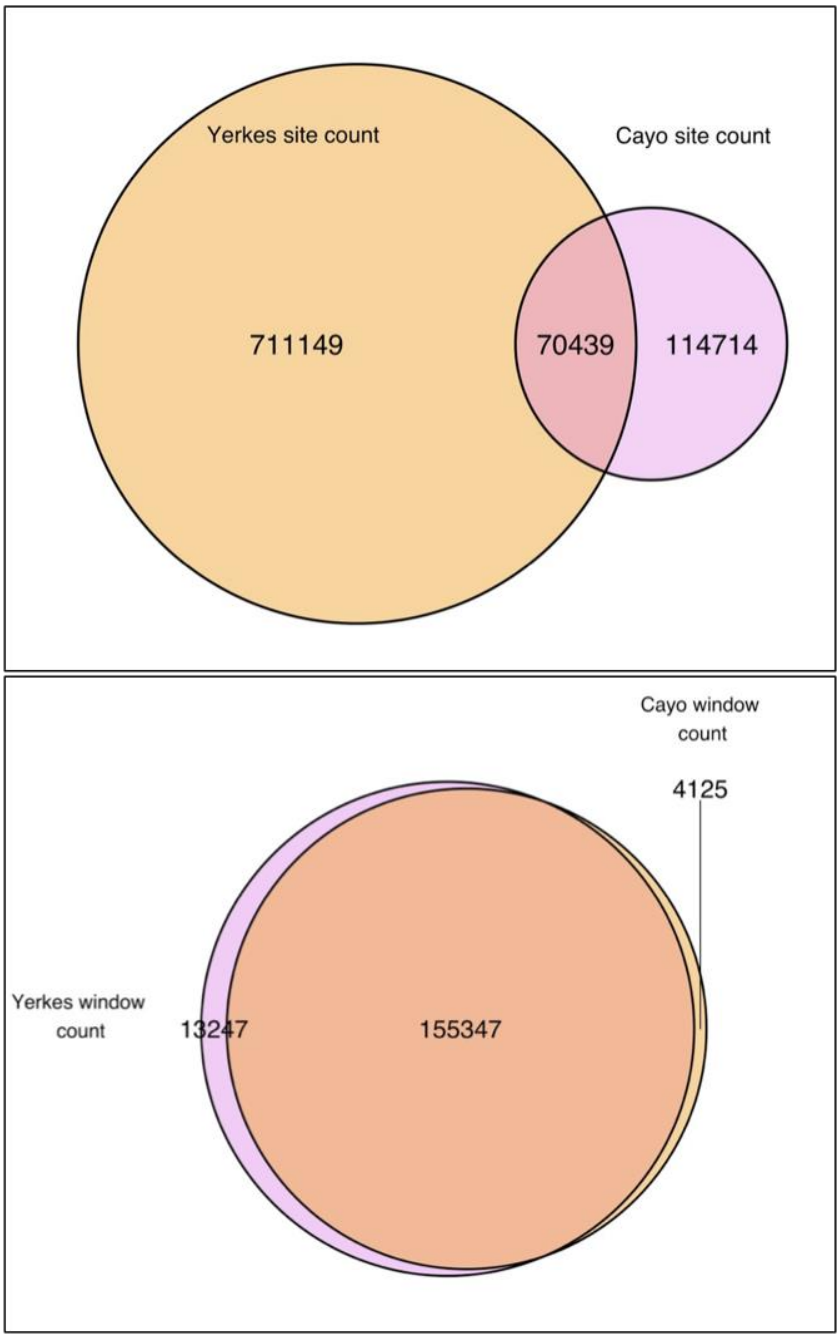


Figure S2. *The proportion of shared coverage between our rhesus training and test datasets increased from 38% for the site-based approach (top) to 97% for the window-based approach (bottom). Numbers on the left of each venn diagram show the number of features (sites or windows) unique to the Yerkes dataset, while numbers on the right-hand side show those unique to the Cayo Santiago dataset. The numbers shown where the two circles overlap refer to the count of shared features between the two datasets when either approach was used.*

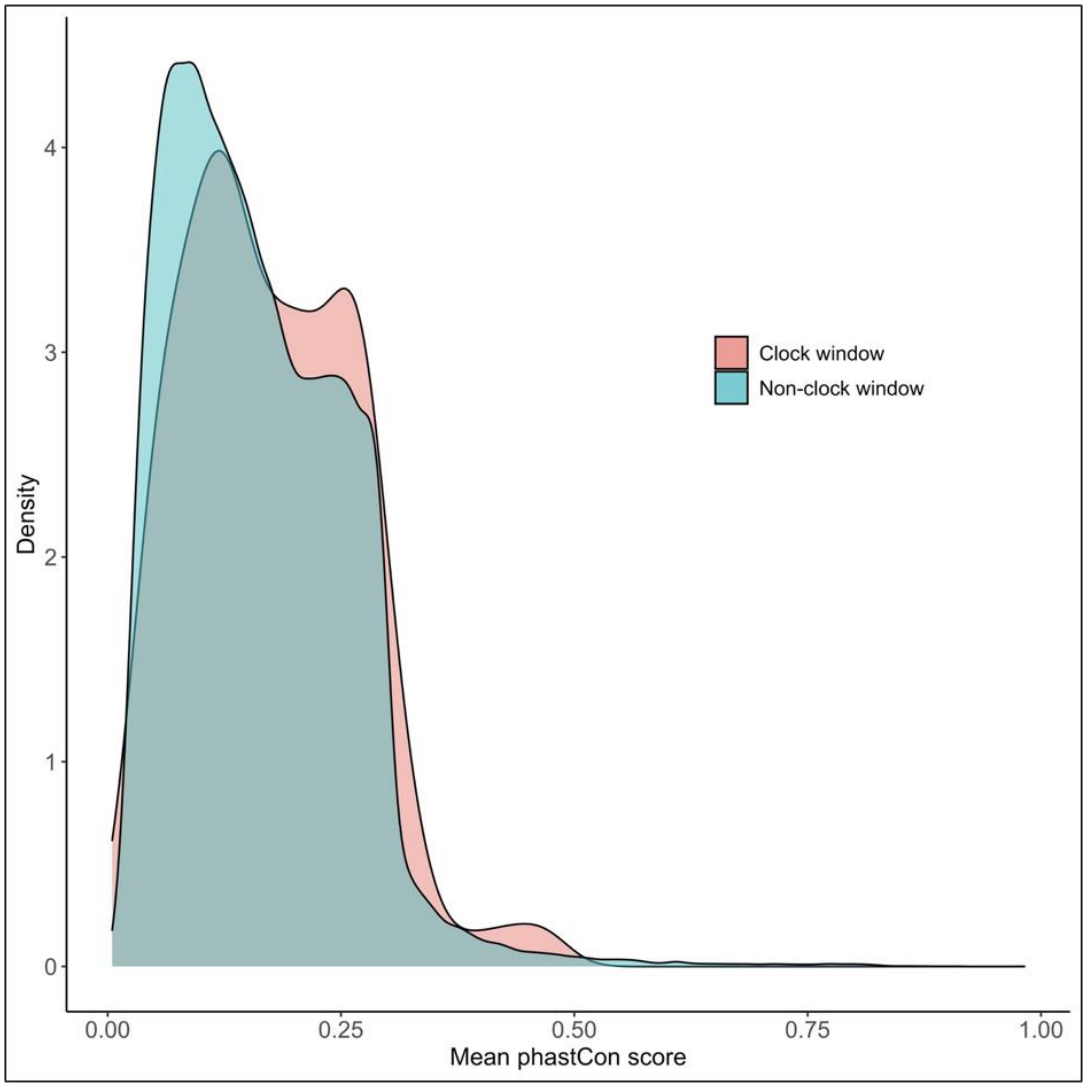


Figure S3. *Clock windows are modestly but significantly enriched for evolutionarily conserved sequences (two-sample Kolmogorov-Smirnov test, D = 0.09, p = 0.007).*

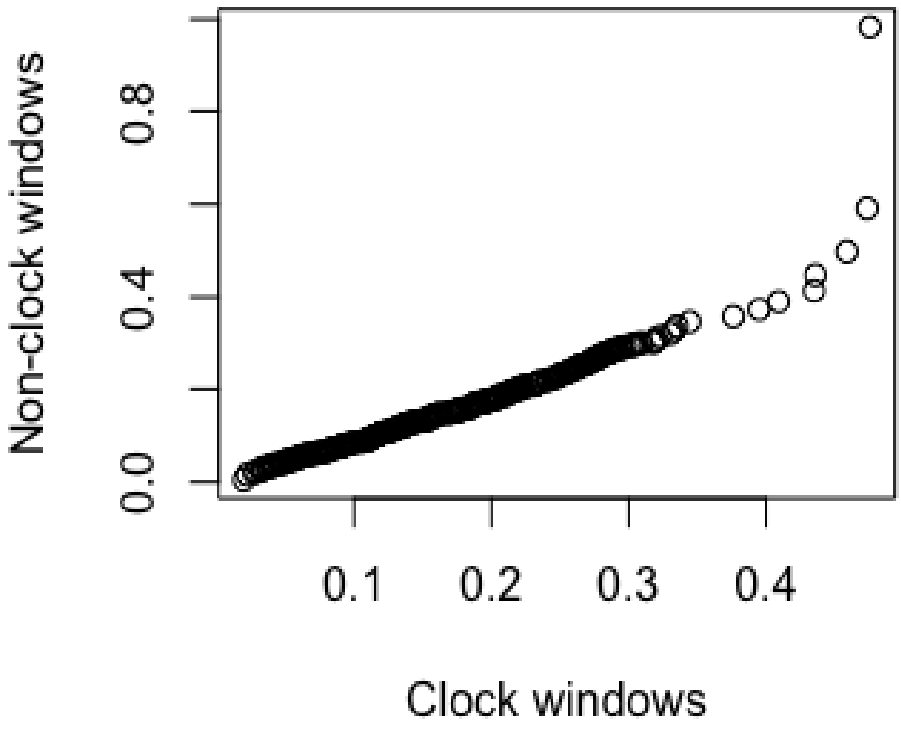


Figure S4. *Distribution of mean phastCon scores for clock windows (those automatically selected for inclusion in the model) and non-clock windows. Clock windows show a modest trend towards sequence conservation as compared to the windows that were not selected.*

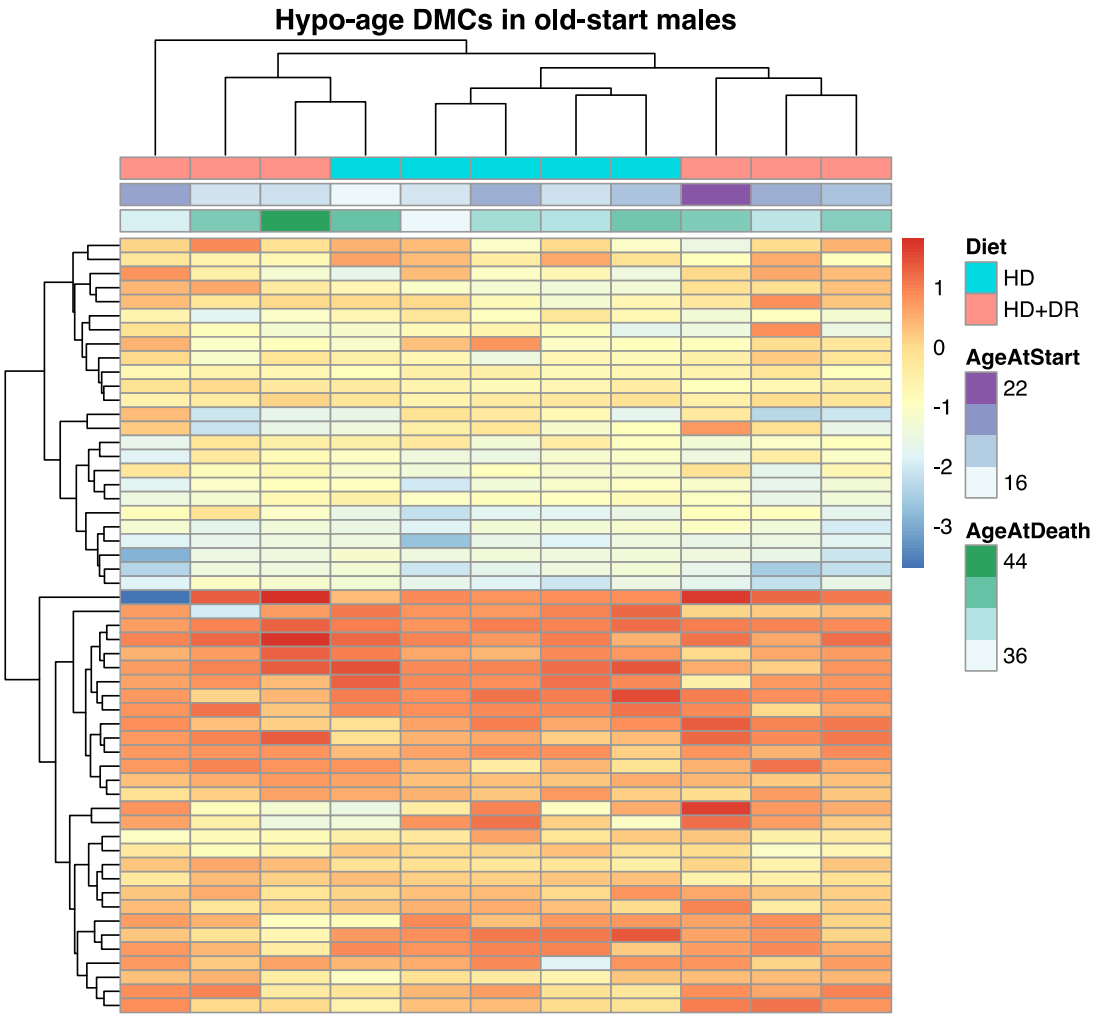
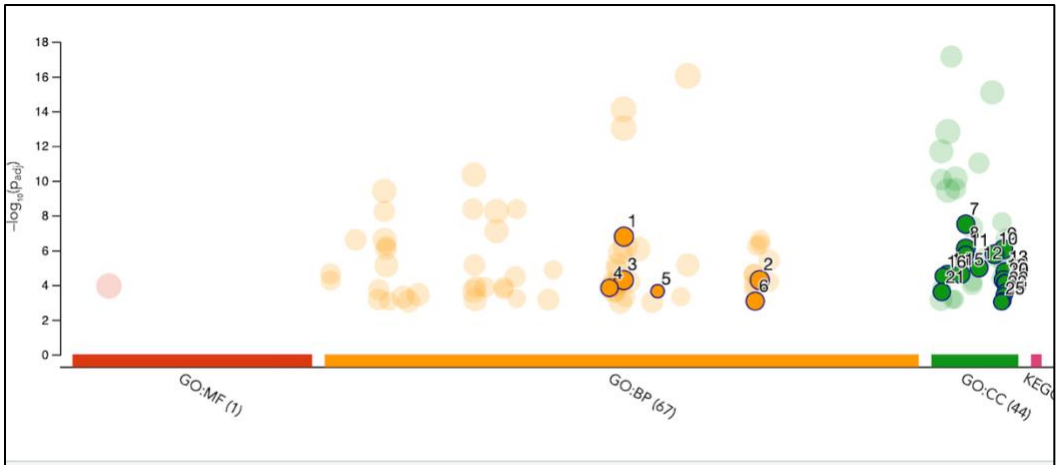


Figure S5. *CpG sites showing significant loss of methylation with age in old-start males on HD and HD+DR diets.*



ID	Source	Term ID	Term Name	p_{adj} (query_1)
1	GO:BP	GO:0050808	synapse organization	1.654×10^{-7}
2	GO:BP	GO:0099177	regulation of trans-synaptic signaling	5.238×10^{-5}
3	GO:BP	GO:0050804	modulation of chemical synaptic transmission	5.238×10^{-5}
4	GO:BP	GO:0048167	regulation of synaptic plasticity	1.475×10^{-4}
5	GO:BP	GO:0060292	long-term synaptic depression	2.276×10^{-4}
6	GO:BP	GO:0098742	cell-cell adhesion via plasma-membrane adhes...	8.511×10^{-4}
7	GO:CC	GO:0034703	cation channel complex	3.147×10^{-8}
8	GO:CC	GO:0034702	ion channel complex	8.076×10^{-7}
9	GO:CC	GO:0098978	glutamatergic synapse	8.890×10^{-7}
10	GO:CC	GO:0097060	synaptic membrane	1.682×10^{-6}
11	GO:CC	GO:0034705	potassium channel complex	1.682×10^{-6}
12	GO:CC	GO:0045211	postsynaptic membrane	1.036×10^{-5}
13	GO:CC	GO:0099572	postsynaptic specialization	1.769×10^{-5}
14	GO:CC	GO:0014069	postsynaptic density	2.464×10^{-5}
15	GO:CC	GO:0032279	asymmetric synapse	2.464×10^{-5}
16	GO:CC	GO:0008076	voltage-gated potassium channel complex	3.066×10^{-5}
17	GO:CC	GO:0098839	postsynaptic density membrane	5.105×10^{-5}
18	GO:CC	GO:0098984	neuron to neuron synapse	5.105×10^{-5}
19	GO:CC	GO:0099240	intrinsic component of synaptic membrane	7.545×10^{-5}
20	GO:CC	GO:0099634	postsynaptic specialization membrane	7.604×10^{-5}
21	GO:CC	GO:0005912	adherens junction	2.537×10^{-4}
22	GO:CC	GO:0099699	integral component of synaptic membrane	2.641×10^{-4}
23	GO:CC	GO:0099055	integral component of postsynaptic membrane	4.051×10^{-4}
24	GO:CC	GO:0098936	intrinsic component of postsynaptic membrane	7.896×10^{-4}
25	GO:CC	GO:0098685	Schaffer collateral - CA1 synapse	9.530×10^{-4}

Figure S6*. Significantly enriched Gene Ontology (GO) terms for Super Ager males (FDR < 0.05). Numbered (solid color) circles indicate terms that were significantly overrepresented among the age-DMCs for the Super Ager males and to which fewer than 500 genes were annotated. These terms are identified by number in the table below the Manhattan plot. Transparent circles represent terms that are significantly overrepresented but to which more than 500 genes were annotated (meaning they are more general and less informative regarding specific functions or mechanisms). The y-axis shows the (negative log) p-value for tests of enrichment per GO term, with higher values indicating greater significance. MF: Molecular Function; BP: Biological process; CC: Cellular component.*

APPENDIX B

SUPPLEMENTAL METHODS AND RESULTS

Bismark Parameter Settings

When aligning the reads to the reference genome using Bismark, we made two modifications to the alignment default parameters to reduce the number of ambiguously mapped reads (which results in data loss because these reads are discarded due to the inability to determine a unique best alignment). We relaxed the minimum alignment score to allow approximately 3 mismatches or gaps in the alignment of 1-2 bp each (this is the "--score_min" parameter; note that the maximum alignment score is zero, corresponding to perfect alignment with no mismatches or gaps). We also increased the number of times Bismark attempted to reseed a repetitive (low complexity) read before marking it as invalid, from a default of 2 to 8 times ("-R" parameter).

Criteria Used to Eliminate Samples from the Site-Based Dataset

Our initial dataset contained 631 genomic libraries. We removed 21 low coverage libraries following alignment to the reference genome. We combined data from duplicate libraries (those derived from samples collected from the same individual on the same day, n = 29), leaving 581 samples. We removed eight samples after plotting the ratio of X-chromosome to chromosome 19-mapping sites by sex and finding four samples labeled as female that clustered with the males, and four labeled as male that clustered with the females, suggesting they had been mislabeled. Finally, we removed 24 samples that were missing > 25% of their data in the final filtered dataset.

Considerations for Training, Optimizing, and Implementing Epigenetic Clock Models

We used an elastic net penalized regression algorithm that automatically selects different subsets of CpG sites (or 1 kb windows) that together generate the most accurate and precise age predictions. We used a nested loop structure to train and optimize our penalized regression model, with a leave-one-out cross validation (LOOCV) outer loop to

tune model hyperparameters and an inner 10-fold cross validation loop to fit the model to training data (which determines the model's coefficients).

In the case of DNA methylation-based epigenetic clocks, our goal is to model the relationship between methylation at CpG sites or windows (independent variables) and calendar age (dependent variable).

We first fit a model to our training dataset. During the training stage, the algorithm is given both the methylation ratios and the chronological age of each sample. The algorithm then uses N-1 samples to train "proto-models" by dividing the data into 10 folds and running an internal cross-validation loop to train and validate on the inner folds. It determines which combination of features predict calendar age while minimizing the mean squared error. Hyperparameters are meta-parameters that are not learnable from the training data; examples are regularization parameters like lambda or the value of *K* in K-fold cross validation. We initially use previous knowledge or default settings for the hyperparameters and can subsequently optimize them by using caret's train() function and setting different hyperparameter combinations using preProcOptions(). Tuning alpha may only result in modest boosts in performance, but it still recommended to examine results from setting different alpha values during the model optimization process.

Enrichment Analysis for Evolutionarily Conserved Sequences

To assign conservation scores to windows in rhesus macaque coordinates, we calculated phastCons scores directly using the "57 mammals EPO" multiple species alignment obtained from Ensembl (release 101). Multiple alignment format (MAF) files were processed in the following manner: First, ancestral species were removed, along with blocks not containing rhesus macaque sequences using maffilter v1.3.1 (Dutheil, Gaillard, and Stukenbrock 2014). We then removed species duplicates from each alignment and indexed each block to the rhesus macaque reference genome using mafTools (Mayakonda et al. 2018). Next, we used maf_parse from the PHAST utilities (Hubisz, Pollard, and Siepel 2011) to extract blocks corresponding to the 155,347 windows in this analysis. After extracting each window, we performed a local realignment of each block using MAFFT (v7.402) (Katoh and Standley 2013) and maffilter. Some rhesus sequences that were originally from the same window were split across multiple

blocks or MAF files. We thus rearranged alignment blocks such that each MAF file contained only blocks from the same rhesus macaque window using a custom shell script. We then combined blocks using the Merge() function in maffilter with rhesus macaque set as the reference species. We calculated conservation scores using the phastCons program (v1.5). First, we fit a phylogenetic model using phyloFit, the REV nucleotide substitution model, and the phylogenetic tree provided with the dataset in the Ensembl release. A minority of windows were excluded (7,008, or 4.5%) from this analysis because they were not represented in the multiple species alignment. We ran phastCons using the arguments "--expected-length 45 --target-coverage 0.3 --rho 0.3", which are identical to arguments used in the UCSC Genome Browser pipeline for generating conservation tracks. The resulting phastCons scores represent probabilities of negative selection at the per-site level. We summarized each window by calculating the mean phastCons score across all windows.

Sample Removal in the Test Datasets

For the Yerkes macaques, we removed two samples due to insufficient library size (remaining n = 43). For the baboons, we removed nine samples that failed three attempts at alignment to the bisulfite-converted rhesus genome (*Mmul10*), and six with low library sizes (remaining n = 271).

Supplemental Results

Tests for Associations between Epigenetic Age, Rank and Grooming in the Yerkes Dataset

Previous studies have found clear associations between indicators of physiological health like immune function and dominance rank (Simons and Tung 2019; Snyder-Mackler et al. 2016a). Because we were able to access behavioral data for the Yerkes RRBS dataset (Snyder-Mackler et al. 2016a; 2016b), we also used the RheMacAge blood clock to test if certain social variables were associated with accelerated aging. We found no significant association between age acceleration and dominance rank (a measure of social status) or grooming (a measure of social connectedness). Because rates of aggression are inversely correlated with rank and low

rank is correlated with increased psychosocial stress, we hypothesized that low-ranking individuals who receive more harassment may show accelerated epigenetic aging. The association between age acceleration and received harassment approached but did not reach significance ($p = 0.08$). This trend was largely driven by an individual who experienced more than twice as much aggression as any other individual and exhibited the most extreme age acceleration (predicted age > calendar age + 10 years).

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