THESIS

EVALUATION OF CYTOSOLIC DNA SYNTHESIS RATES AS A POTENTIAL DRIVER OF MUSCULOSKELETAL AGING IN THE HARTLEY GUINEA PIG

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ABSTRACT

EVALUATION OF CYTOSOLIC DNA SYNTHESIS RATES AS A POTENTIAL DRIVER OF MUSCULOSKELETAL AGING IN THE HARTLEY GUINEA PIG

Advancing age is the greatest risk factor for many chronic diseases and, by 2035, the number of U.S. citizens over the age of 65 will exceed the number under the age of 18 for the first time in history. Inflammaging, systemic aging associated low grade chronic inflammation, is a contributing factor to age-related chronic disease in multiple tissue types and organ systems, including the musculoskeletal system. Age-related musculoskeletal decline is characterized by diseases such as sarcopenia and osteoarthritis and has a strong correlation with an increase in all-cause mortality. While markers of inflammation are present in circulation, key drivers of inflammation that propagate age-related musculoskeletal decline are not well established. However, DNA that resides misplaced in the cytosol (cytoDNA) is a potent activator of the cGAS-STING pathway, an innate immune response that drives inflammation and disrupts cell function. While cytoDNA and the cGAS-STING pathway have been linked to increased inflammation in a host of tissues and disease states, very little evidence supports its role in skeletal muscle aging. Hartley guinea pigs (HGPs) are a translational, non-transgenic model of spontaneous musculoskeletal decline known to develop systemic inflammation and progressive age-related comorbidities characteristic of human aging. To begin establishing if cytoDNA contributes to age-related musculoskeletal decline, we assessed age-related differences in rates of newly synthesized cytoDNA in the tibialis anterior (TA), a locomotor muscle susceptible to age-related decline, from male and female 5- and 15-month-old (mo) HGPs. To assess this, we used stable isotope labeling with deuterium oxide, a sensitive approach for measuring rates of DNA synthesis in vivo. Although our primary findings showed that overall rates of cytoDNA synthesis did not increase with age or

differ between sexes, we did identify a species of cytoDNA for which synthesis decreased with age, suggesting that the relationship between cytoDNA synthesis and aging is more complex than initially anticipated.

TABLE OF CONTENTS

CHAPTER I – INTRODUCTION

By 2035 the number of U.S. citizens over the age of 65 will exceed the number under the age of 18 for the first time in history.[1] By 2050, the number of Americans projected to be living with at least one chronic disease will almost double.[2] This places an enormous burden on the healthcare system as age-related diseases such as cardiovascular, renal and neurological diseases have both impetuous and expensive health outcomes. Although the prevalence of age-related diseases increases over the lifespan, various factors – including biological and social determinants – play a crucial role in shaping mortality outcomes in older adults.[3] Regardless of biology or social circumstances, age itself is a potent risk factor for all-cause mortality.[4] The identification of the "hallmarks of aging," including but not limited to inflammation, mitochondrial dysfunction and cellular senescence, have accelerated research aimed at monitoring and targeting cellular aging to extend healthspan; the years spent free of the burden of all age-related chronic diseases. [5]

Inflammaging, the systemic chronic low-grade inflammation associated with aging, is a complex interplay of cellular and molecular mechanisms that disrupt bodily systems including the musculoskeletal system.[6] Inflammaging is a well-established contributor to cellular aging and is a shared etiology to both sarcopenia and osteoarthritis (OA).[7,8,9] The dysregulation of cellular function that accompanies inflammaging occurs systemically and at the level of individual joints and skeletal muscle as well.[10] The detriments of age associated musculoskeletal decline are characterized by frailty, loss of muscle mass and function, and joint pain, each of which share inflammation as a potent contributor towards their development.[11,12] Although inflammaging contributes to age-related musculoskeletal decline in both men and women, hormonal differences — such as estrogen loss during menopause in females and gradual testosterone decline in males — may drive distinct sex-specific differences in the progression of this decline.[13]

The impact of age-related musculoskeletal decline on physical health is not limited to decrements in pain free movement and loss of strength, but also a stark increase in the risk of comorbidities as well.[12] Skeletal muscle is a crucial metabolic organ that plays a key role in blood glucose homeostasis, energy balance, and prevention of insulin resistance, making it a powerful organ system to preserve metabolic health throughout the lifespan. The age associated decrease in skeletal muscle mass and function, called sarcopenia, has severe consequences downstream of muscle loss and impairments in movement. This includes an increased risk of metabolic syndrome, obesity, neurodegenerative disease, cardiovascular disease (CVD) and type two diabetes.[14-17] Furthermore, sarcopenia and its associated comorbidities can contribute to the loss of independence, resulting in negative mental health outcomes.[18] Although the risk of comorbidities of aging increase with sarcopenia, differences in mortality and chronic disease prevalence between women and men underscore the importance of investigating the sex differences of age-related musculoskeletal decline and chronic disease burden. Although the cause-and-effect relationship between age-related musculoskeletal decline and the aforementioned comorbidities has yet to be clearly established, evidence clearly supports that preserving muscle mass and strength with age reduces the risk of all-cause mortality.[16,19]

The individual burden of musculoskeletal aging with co-morbidities is onerous for both health care systems and individuals. Hospitalizations come with significant healthcare costs, posing both public and personal health challenges.[20, 21] These challenges drive the need for effective therapies against sarcopenia and a deeper understanding of the mechanisms by which inflammaging perturbates the musculoskeletal system and impacts overall health[19, 22, 23]

CytoDNA is a marker of cellular aging that has been directly linked to an inflammation pathway promoting biological aging and senescence in several tissues, but has yet to be thoroughly studied in muscle.[24] DNA is typically localized to the nucleus and mitochondria, but can end up in

the cytosol and drive inflammation with aging.[25] Cells sense "misplaced" DNA in the cytosol as a threat, triggering an immune response. The intracellular sensors activate cyclic GMP-AMP synthase (cGAS) and stimulation of interferon genes (STING) as an immune response that prevents foreign pathogens from replicating.[26] Cells sense "misplaced" DNA in the cytosol as a threat, triggering an immune response. The Intracellular sensors activate cyclic GMP-AMP synthase (cGAS) and stimulation of interferon genes (STING) as an immune response which prevents foreign pathogens from replicating.[26] These cellular sensors are a vital first line of defense and a necessary component of the immune system's ability to respond to foreign DNA.[27] However, the cGAS-STING pathway does not differentiate between foreign DNA and DNA originating from the host cells, and can propagate inflammation independent of pathogen derived DNA, hastening cellular aging.[28] The indiscriminatory trait of the cytoDNA/cGAS-STING pathway is a deleterious quality of the sensor which has been identified in multiple tissue types and organ systems, and has been implicated in a number of autoinflammatory, autoimmune, and neurodegenerative diseases.[29] Despite these findings, a connection between cytoDNA and age-related musculoskeletal decline remains unclear. Given the gaps in understanding the specific mechanisms that diminish cellular function in aging muscle, identifying novel biomarkers of inflammation and propagators of senescence in skeletal muscle could offer new insights into the underlying processes driving muscle decline with age.

To investigate the relationship between cytoDNA and muscle aging, an animal model that closely mimics human aging is needed. While yeast, worms, flies and mice have been common models in aging research, there are fundamental gaps in the translational knowledge they provide as they do not replicate the complex musculoskeletal decline or multimorbidity's seen in aging humans.[30] For example, mice are often used due to their short lifespan and biological similarity to humans, but they do not fully recapitulate the natural progression of musculoskeletal aging.[31, 32] An

animal model that better reflects human aging, without relying on genetic modification, is necessary to accurately study the triggers and drivers of age related musculoskeletal degeneration.

The Dunkin Hartley guinea pig (HGP) is a non-transgenic outbred strain of guinea pig that develops primary joint degeneration beginning in early adult life, with progression that culminates in severely impaired mobility.[33] Similar to observations in humans, the HGP exhibits joint degeneration accompanied by a significant decline in muscle density, indicative of greater fat deposition. From 5 to 15 months of age, myofiber remodeling is also observed, characterized by a decrease in type II fibers and an increase in type I fibers.[34] Moreover, skeletal muscle age-related changes include decreased mitochondrial function and protein dyshomeostasis evidenced by age-related decreases in mitochondrial, cytosolic, myofibrillar, and collagen protein synthesis rates.[34,35] In addition to musculoskeletal decline, HGPs also model aging-related multimorbidities, such as metabolic and cardiovascular disease, and exhibit hallmarks of brain aging and Alzheimer's disease seen in humans.[36-38] Lastly, the early onset of knee OA in HGPs have made them a useful model for identifying the relationship between joint degeneration and obesity.[39] Collectively, these changes mirror what is observed from aging humans, making HGPs a valuable model for both discovery and translational research to identify effective interventions to slow or prevent musculoskeletal aging.[40,41]

GAP IN KNOWLEDGE AND STATEMENT OF THE PROBLEM

Cellular triggers of inflammation that drive age-related musculoskeletal decline remain incompletely understood. The presence of DNA in the cytosol is an established driver of inflammation in many tissues but its presence in skeletal muscle and role in muscle aging is undetermined. We will leverage the established HGP multi-morbidity model of age-related musculoskeletal decline to identify age-related differences in the rate of cytoDNA synthesis in skeletal muscle.

HYPOTHESIS

We hypothesize that 15-month-old HGPs will have significantly higher rates of cytoDNA synthesis in skeletal muscle when compared to same sex 5mo HGPs. Further, female 5 and 15mo HGPs will have significantly higher rates of cytoDNA synthesis than males.

CHAPTER II – REVIEW OF THE LITERATURE

UNHEALTHY AGING, A POTENTIAL PUBLIC HEALTH CRISIS

The prevalence of age-related chronic diseases is an escalating public health concern. By 2035, Americans aged 65 and older will outnumber those under 18 for the first time in history.[1] By 2050, the number of Americans living with at least one chronic disease is projected to nearly double.[2] Aging is an inevitable part of life, and remains the strongest risk factor for chronic diseases yielding consequential public health implications for an aging population.[3,42]

The risk of chronic disease with age is heavily influenced by a host of factors, including lifestyle behaviors and genetics.[43,44] It is important to note that, while advancing age is a powerful risk factor for chronic diseases such as CVD, dementia and osteoarthritis, chronic disease is not an absolute guarantee. Healthspan refers to the period of life marked by good health and the absence of chronic disease or disability, and can be promoted by lifestyle factors.[45-48] Food choices and eating behaviors that reduce inflammation, exclude ultra processed foods, and provide essential nutrients, are critical behaviors needed to reduce the risk for age-related chronic diseases and improve quality of life.[49,50] Physical activity is also a robust defender against age-related chronic disease by supporting muscle health and metabolism. [15,16,19,48,51]

Unfortunately, the percentage of middle aged and older adults with obesity, a disease associated with the development of sarcopenia, OA, and other chronic diseases, has continued to grow in the United States.[14,52] The combined impact of biological aging and excess visceral adiposity is an increasing threat to public health for both current and future generations.[53] In addition to the personal health challenges of unhealthy aging, living with more than one chronic disease is expensive. Estimates of health care costs for patients with obesity in 2019 were approximately 173 billion dollars and, although this includes children as well as middle aged and older adults, children with obesity are more likely to age with obesity than without.[54,55] The United

States' aging population's predicted health decline poses a public health crisis that must be prevented.

Conversely, targeting cellular aging by increasing healthspan is a viable and vital strategy to combat obesity and compress the age associated risks of chronic disease into the latest years of life.[44,45,56] Further, maintaining musculoskeletal health into early and late adulthood has profound benefits on preserving functional mobility, reducing joint pain, preventing or mitigating osteoarthritis, and maintaining the capacity to accomplish activities of daily living.[57-59] However, OA and sarcopenia can affect mobility, lower limb joint pain and overall musculoskeletal function with age, making exercise and physical activity more difficult in the later years of life.[60,61] These detriments of musculoskeletal aging can contribute to negative downstream effects on overall health in older adults, as sedentary behavior is a risk factor for age-related chronic disease.[62] Increases in healthspan not only benefit individuals but have a cumulative positive effect on public health. Compressing morbidity to the latest years of life, improving quality of life, and reducing the amount of individuals with obesity, has the potential to reduce the pressure on an already strained healthcare system.[63,64] Although significant advances have been made in the field of aging, such as the popularization of healthspan and the identification of the hallmarks of cellular aging, much remains to be understood about the underlying mechanisms of aging and pathways leading to age-related chronic disease.[5,65,66]

AGE RELATED MUSCULOSKELETAL DECLINE

The musculoskeletal system, which accounts for roughly 40% of total body mass, plays a vital role in supporting movement, regulating energy balance, disposing of blood glucose, and promoting bone mineral density through resistance training. With age, this system undergoes progressive decline—including loss of skeletal muscle mass, bone density, articular cartilage, and

connective tissue integrity.[67] This decline is more than a matter of reduced mobility, as it is linked to broader health consequences. Sarcopenia, and OA, a degenerative joint disease, are both associated with increased risk of CVD and type 2 diabetes.[17,68] In addition, low muscle strength in older adults is strongly associated with higher all-cause mortality.[16]

Whether age-related declines in musculoskeletal health initiate biological stressors that increase the development of chronic diseases or vice versa is yet to be fully understood.[17,68] Regardless, of the lack of an established cause and effect relationship, the link between age-related musculoskeletal decline and other comorbidities is well-established.[16,69,70] A meta-analysis by Pacifico and colleagues found that sarcopenia was more common in individuals with dementia, CVD, and diabetes mellitus compared to their healthy counterparts. Independent of other comorbidities, sarcopenia itself presents many health challenges including increased incidence of falls, fractures, and chronic pain among older adults.[71] The challenges associated with these physical ailments not only impair mobility and functional independence but also contribute to psychological distress, as chronic pain and disability can intensify feelings of isolation and depression.[72]

Although age related musculoskeletal decline affects both females and males, the etiology is not the same.[41] While males experience a gradual and greater loss of strength and total lean muscle mass, females generally experience a much more rapid decline in lean mass and strength.[13] Hormonal changes play a critical role in these differences. Estrogen, which has protective effects on bone density and muscle health, decreases significantly in women after menopause, leading to accelerated bone loss and increased risk of conditions such as sarcopenia, osteoporosis and OA.[73,74] In contrast, males maintain higher levels of testosterone, which supports muscle mass and strength, contributing to a slower decline in these parameters as they age.[75] This hormonal disparity is reflected in the prevalence of musculoskeletal disorders, where females

have higher rates of OA and associated pain compared to men.[76] These sex-based differences reinforce the need to investigate the similarities and disparities in drivers of cellular aging and inflammation between males and females.

INFLAMMATION, SENESCENCE AND MITOCHONDRIAL DYSFUNCTION: MECHANISMS CONTRIBUTING TO BIOLOGICAL AGING

Aging is a well-established risk factor for all-cause mortality and a multitude of chronic diseases. The twice-updated hallmarks of aging offer a framework for understanding 12 interconnected processes that contribute to the progression of biological aging.[5,30,65] Within this framework, inflammation, mitochondrial dysfunction, and cellular senescence are deeply interconnected processes that reinforce one another in a feedforward loop-where chronic inflammation disrupts mitochondrial function, dysfunctional mitochondria promote cellular senescence, and senescent cells further drive inflammation—ultimately accelerating age-related cellular decline.[77-80] Understanding the interplay between these mechanisms is critical for determining how deterioration of cellular function with age contributes to chronic disease.

Inflammation, particularly inflammaging, is a hallmark of aging that shares a common ground with many age-related diseases.[81] It is characterized by a persistent pro-inflammatory state that arises from factors including the accumulation of senescent cells, decreased mitochondrial function, and dysregulation of the immune system.[9] This inflammatory environment can exacerbate tissue damage and promote the progression of age-related musculoskeletal decline and development of sarcopenia and OA.[82,83]

The inflammatory response results in the release of cytokines that trigger muscle wasting, leading to decreased muscle mass and function.[83,84] Although the relationship between inflammation and skeletal muscle decline has been extensively studied, a specific origin of

intracellular drivers of inflammation have yet to be tied directly to age related musculoskeletal decline.[16,85 86]

For example, NLRP3 is an inflammasome that plays a key role in mediating age-associated inflammation and has been implicated in the development of sarcopenia, OA, as well as other age related chronic diseases.[87-90] Findings from McBride and colleagues show that whole body NLRP3 knockout older mice (24mo) are protected from decreased muscle mass and relative strength with age when compared to mice treated with the NLRP3 inflammasome.[91] Further, NLRP3 knockout mice preserved glycolytic myofibers when compared to mice treated with the NLRP3 inflammasome.[91] Ferrucci and colleagues found that the leucocytes of older individuals show greater release of interleukin-6 (IL-6), resulting in increased circulating concentrations of the inflammatory cytokine. Further, Bian et al. found that among over 400 adults aged 60 and older, the emergence of sarcopenia was accompanied by elevated circulating IL-6 levels.[92] In contrast, da Costa Teixeira et al. observed that in a cohort of older Brazilian women with sarcopenia, IL-6 and NLRP3 were not the predominantly elevated pro-inflammatory cytokine; instead, interleukin-8 (IL-8), sTNFr-1, and sTNFr-2 were the most prevalent pro-inflammatory markers.[93] These contrasting findings underscore the uncertainty surrounding the primary drivers of inflammation in age-related musculoskeletal decline and highlight the need to clearly establish the key drivers involved.

Age-related mitochondrial dysfunction may also play a role in the OA and sarcopenia-inflammaging link, with the overproduction of mitochondrial reactive oxygen species (ROS) potentially mediated by the NLRP3 inflammasome and interleuken-1 (IL-1) secretion.[12] Mitochondrial outer membrane permeability, a characteristic of mitochondrial dysfunction, leads to the release of mitochondrial DNA (mtDNA) and ROS, contributing to NLRP3 recruitment and subsequent maturation of other pro-inflammatory signals such as IL-1, IL-8, and interleukin-33 (IL-33).[91,94] NLRP3 activity has also been linked to pyroptosis, a damaging inflammatory cell death

mechanism implicated in OA, and to the upregulation of muscle atrophy–associated ubiquitin ligases in cultured myotubes.[89,95] Beyond NLRP3, Tyrell et al. reported that age-related mitochondrial dysfunction increases IL-6 production in aortic tissue cultures from aged mice, creating a feedforward loop that promotes inflammation and primes the vasculature for enhanced atherogenesis following a hyperlipidemic challenge, thus underscoring mitochondrial dysfunction as a key contributor to atherosclerosis. [95] Together, these findings support a bidirectional relationship in which mitochondrial dysfunction and inflammation amplify one another, driving the progression of age-related diseases.[96]

In addition to inflammation and mitochondrial dysfunction, senescence contributes to cellular aging.[97,98,99] Cellular senescence is a state of irreversible cell cycle arrest that occurs in response to stressors such as DNA damage, oxidative stress, and inflammation.[100-102] Although senescence is commonly viewed as a detrimental process, it also serves as a crucial defense mechanism that prevents the proliferation of cancerous cells.[103] However, the accumulation of senescent cells leads to a decline in cumulative cellular function, disrupting tissue homeostasis.[100] Senescence associated secretory phenotypes, or SASPs, refer to the array of factors released from senescent cells that not only contribute to local tissue inflammation, but also affect systemic immune responses, leading to a cycle of inflammation that accelerates cell aging and further promotes cellular senescence.[104] The systemic effects of senescence and the accumulation of SASPs is linked to age-related pathologies including CVD and neurodegenerative disease, further promoting a cycle of chronic inflammation.[77,105] However, the role of senescence is not well understood in age-related musculoskeletal decline.[40,106]

Skeletal muscle is comprised of post-mitotic multinucleated myofibers and a mixture of mitotically competent cells, which makes the susceptibility of these cell populations to senescence with advancing age unclear.[99] γH2AX is a marker of DNA damage that accumulates at

sites of double-strand breaks and is commonly used to identify senescent or stressed cells.[107] A study by Dungan et al. found no increase in γH2AX+ cells in muscle from older individuals compared to younger ones; however, individuals with obesity were associated with higher γH2AX+ cell abundance, including elevated γH2AX levels in postmitotic myofiber nuclei from obese individuals compared to lean ones. Thus, this suggests that obesity may exacerbate cellular stress leading to senescence in skeletal muscle.[108] Zhang et al. reported findings that mRNA levels of the senescence-associated genes p53 and p21 were elevated in skeletal muscle of older mice, monkeys, and humans, while others failed to detect senescence-associated genes in skeletal muscle from older adults.[99,108] In contrast, Welle et al. identified elevated levels of several senescence associated genes including p53, p21, and Gadd4 in skeletal muscle of older adults.[109] While strong evidence of senescence markers have been identified in whole skeletal muscle tissue, evidence within individual muscle-resident cell types—such as myofibers, satellite cells, and fibro-adipogenic progenitors—remains limited.[40] This highlights important gaps in understanding which specific cells contribute to the senescent phenotype observed in aged skeletal muscle.

Solidifying primary drivers of inflammation in skeletal muscle with age will elucidate important pathways for therapeutic targets and contribute to the understanding of the cause-and-effect relationship between inflammation, mitochondrial dysfunction, senescence and skeletal muscle decline.

CYTOSOLIC DNA AND DNA SENSORS

CytoDNA is DNA that exists outside of the nucleus or mitochondria and resides, misplaced, in the cytosol. This misplaced DNA may appear inauspicious, but it is detected by cellular defense mechanisms and has been shown to drive inflammation in a host of cells including myocardial cells, microglia, renal carcinoma cells and endothelial cells.[28,79,110] While this defense mechanism

is vital for detecting foreign DNA and subsequently activating an immune response, chronic activation of the innate immune response is a potent driver of inflammaging and age-associated chronic diseases such as Alzheimer's disease and CVD. [29,111]

However, findings in the musculoskeletal system are much more limited. In nucleolus pulpous cells, cells critical to the integrity of intervertebral discs, Zhang et al. showed cytoDNA to be a potent driver of inflammation, promoting cellular aging and senescence.[78] In skeletal muscle, Li et al. discovered myofibers of progeria aged mice feature increased release of mtDNA from damaged mitochondria.[112] Although these novel findings from Li and colleagues are evident of a potential role of a cytoDNA mediated immune response driving muscle aging, similar findings have yet to be published.

Cells are equipped with sensors that have evolved over time to detect foreign DNA and trigger an innate immune response to fight pathogens. Cyclic GMP-AMP synthase (cGAS), a cellular sensor known to detect foreign and self-DNA in the cytoplasm, synthesizes cyclic GMP-AMP (cGAMP), which acts as a messenger to stimulate the production of interferon genes (STING). [25,113]

CGAS remains primarily sequestered and inactivated in the nucleus tightly bound by nucleosomes unless it binds to a DNA backbone.[27] Despite cGAS being a nucleic acid sensor, mechanisms preventing cGAS from binding to DNA within the nucleus remain unclear. Once cGAS binds to DNA, it synthesizes cGAMP and induces type I interferons such as IFN-β, IFN-α), however it also triggers NF-κB–dependent cytokines such as IL-6, TNF-α, and CXCL10,

which are potent mediators of chronic inflammation.[26,78] Although the cGAS-STING pathway is a vital front-line defense against foreign viruses and bacteria, it relies on pattern recognition receptors (PRR) to detect pathogens and its ability to differentiate self-DNA from DNA originating from a foreign pathogen is absent.[113]

In addition to cGAS, other cytosolic DNA sensors contribute to immune surveillance and inflammatory signaling. RNA Polymerase III (Pol III) detects double-stranded DNA and transcribes it into 5'-triphosphate RNA, which activates the RIG-I/MAVS pathway, leading to the production of type I interferons as well as pro-inflammatory cytokines such as TNF-α and IL-6.[114] Another key sensor, absent in melanoma 2 (AIM2), forms an inflammasome complex upon binding to double-stranded DNA in the cytosol.[115] This inflammasome activates caspase-1, which processes and promotes the secretion of IL-1β and IL-18.[116] There are at least four species of self-DNA that intracellular sensors will detect in the cytoplasm and initiate an immune response. They include the following:

Micronuclei

Micronuclei (MN) are small, membrane-bound structures that contain whole chromosomes or chromosome fragments in the cytosol and primarily form due to mitotic defects during cell division.[24] These defects can occur in two main ways: chromosomes that lag and become isolated during anaphase, or DNA double-strand breaks (DSBs) that are not properly repaired and can result in chromosome fragments.[117] When these fragments are fused by their telomeres, they can also become MN.[118] Although MN are enclosed by a nuclear envelope, this envelope is often weakened with age and prone to rupture, exposing the DNA inside to the cytosol.[119]

Cytosolic Chromatin Fragments

Unlike micronuclei, many cytosolic chromatin fragments (CCF) appear in the cytosol through mechanisms not related to mitotic defects.[120,121] CCFs are characterized by a suite of chromatin modifications and are suggested to have been formed from heterochromatin.[118] Cells that have ceased proliferation and show reduced levels of Lamin B1, a protein crucial for maintaining nuclear envelope integrity, are particularly associated with CCF formation. This is

especially common in senescent cells, where degradation of the nuclear envelope is frequently observed.[122]

Mitochondrial DNA

MtDNA resides in the inner mitochondrial matrix and encodes genes essential for oxidative phosphorylation, ribosomal RNA, and transfer RNA.[121] However, mtDNA is found outside the mitochondria in the cytosol under certain conditions including aging.[26] In addition to aging, mtDNA release can occurs during apoptosis/mitophagy, or programmed cell death, when apoptotic signals cause large pores to form in the outer mitochondrial membrane, allowing mtDNA to escape into the cytoplasm.[24] Additionally, sub-lethal stress can also lead to mitochondrial membrane pore formation, which can release mtDNA.[123] Other factors related to mitochondrial dysfunction may also contribute to mtDNA leakage into the cytosol, although these mechanisms are still being studied.[101]

Retrotransposons

Retrotransposons are under the family of transposable elements (TEs), or mobile DNA sequences that make up approximately 50 percent of the human genome and translocate from the nucleus.[124,125] Retrotransposons can be broadly classified into two subclasses: long terminal repeat (LTR) retrotransposons and non-LTR retrotransposons, which include long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs).[126] Both LINEs and SINEs may be exported from the nucleus to the cytosol where they can drive a cGAS-STING response.[127,128]

CGAS- STING: AN EMERGING DRIVER OF INFLAMMAGING

The cGAS-STING pathway has emerged as a key driver of inflammaging and senescence, playing a central role in the propagation of inflammaging upstream of age-related chronic diseases.[28,129,130,111] For example, the nuclear membrane becomes increasingly permeable with

cellular age, compromising the integrity of the nucleus and affecting genomic stability. This rise in permeability is linked to a decline in Lamin B1, a key component of the nuclear lamina that helps maintain nuclear structure and regulate transport between the nucleus and the cytosol.[122] Reduced Lamin B1 levels contribute to increased leakage of CCF and impaired DNA repair mechanisms, driving the cGAS-STING pathway in senescent cells.[119,114] Further, Lamin B1 depletion is a common characteristic of inflammation associated with neurodegenerative disorders and senescent cells.[131] In addition, SASPs released from senescent cells further induce senescence in surrounding cells leading to a larger amount of pro-inflammatory cells instigating the cGAS-STING sterile immune response.[118,132] DNA damage can also induce senescence and has been identified as a contributor to the development of neurodegeneration.[133] DNA damage, specifically double strand breaks, are strongly positive in CCF, a potent activator of the cGAS-STING pathway.[118,134]

Having been identified in the onset of neurodegeneration, cancer, and decreased vascular endothelial function, the deleterious promotion of sterile inflammation and cyclic like promotion of cGAS-STING mediated senescence is particularly relevant in the context of aging.[135,77,110] Self-DNA from tumors and genomic instability by-products can activate the cGAS-STING pathway, influencing tumor progression.[130] Additionally, the presence of cytoDNA originating from damaged mitochondria can activate the cGAS-STING pathway in endothelial cells, leading to inflammation that may compromise healthy vascular aging.[77] In autoimmune diseases, the activation of cGAS by self-DNA has been linked to the pathogenesis of autoimmune conditions by aberrant production of IFN-1.[29] Moreover, in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), the detection of mtDNA released in response to cellular stress can activate the STING pathway, leading to neuroinflammation and disease progression.[136] The cGAS-STING pathway has also been linked to the development of neurodegenerative conditions such as Alzheimer's and Parkinson's

diseases, where inflammation plays a critical role in disease progression.[135] Lastly, Gulen et al. found that the inhibition of STING showed significant improvements in both cognitive function, grip strength and physical endurance in mice, suggesting a bidirectional relationship where inflammation associated with musculoskeletal decline can exacerbate neurodegenerative processes or vice versa.[137]

Although the role of cGAS-STING in age-related diseases and inflammaging is increasingly recognized, its specific involvement in musculoskeletal aging remains underexplored. Given the critical role of the musculoskeletal system in maintaining mobility, metabolic health, and quality of life, identifying whether cytoDNA plays an important role in musculoskeletal decline would be an impactful step toward improving musculoskeletal health and reducing multimorbidities of aging. An ideal approach for addressing this gap in knowledge is to leverage a model that not only mimics the musculoskeletal decline observed in human aging, but also includes the comorbidities frequently observed with advancing age.

THE DUNKIN HARTLEY GUINEA PIG AS A MODEL OF MUSCULOSKELETAL AGING

Identifying the molecular underpinnings of age-related decline in musculoskeletal function is essential for developing effective strategies to slow or prevent the loss of mobility and potentially help abrogate comorbidities of aging. In particular, understanding the processes leading to a "tipping point" when inflammation in muscle may become maladaptive will require use of a model that closely mimics the complexities of human aging. Frequently, models are limited by factors including an unfeasibly long timeline to reach age-related decline, genetic modification that complicates data analysis, and biology that does not closely mimic humans. To effectively model the characteristics important for capturing the decreasing cellular function associated with musculoskeletal decline in humans, it is essential to consider a range of factors that reflect the pathophysiology.

A reliable animal model of human musculoskeletal decline must exhibit aging phenotypes that closely resemble those seen in humans. These include shifts in myofiber size from large to small, joint degeneration that impairs mobility, changes in muscle mass, loss of mitochondrial function and proteostasis, and persistent low-grade inflammation.[138-141] The Dunkin Hartley guinea pig (HGP) is a non-transgenic, outbred strain, and is a well-characterized model of primary OA that shares similar pathophysiological features with humans. One of the key advantages of this model is its early and predictable decline in musculoskeletal function, with pathological signs of OA emerging as early as four months of age.[34] HGPs also display hallmarks of muscle aging, including significant reductions in skeletal muscle protein synthesis across multiple subcellular fractions including mitochondrial, myofibrillar, cytosolic, and collagen. In addition, they exhibit significant declines in mitochondrial function, such as submaximal ADP-stimulated respiration and uncoupled respiration. [35]

Another strength of the HGP model is its development of age-related comorbidities. As they age, HGPs may develop vascular disease, obesity, and neurodegenerative impairments—conditions that frequently co-occur in aging human populations.[36,39,142] This is in contrast to many other rodent models of muscle aging, which fail to spontaneously develop such multimorbidities without genetic modification.[32]

Finally, because HGPs are not genetically modified, they avoid the consequential side effects associated with knockout mouse models. These genetically altered models, while useful for studying specific mechanisms, can produce phenotypes that deviate from the natural progression of human muscle aging, limiting their translational relevance.[32,143] These findings demonstrate that HGPs display key features of musculoskeletal aging and age-related multimorbidities, making them an ideal model to investigate the relationship between musculoskeletal decline and cytoDNA synthesis rates.

SUMMARY

CytoDNA and the cGAS-sting pathway are potent drivers of inflammation and senescence in many tissues, and therefore, a possible propagator of age-related chronic disease. However, the role of cytoDNA as a driver of inflammaging in skeletal muscle has yet to be elucidated. To investigate a relationship between rates of cytoDNA synthesis and age-related musculoskeletal decline, an animal model that mirrors human musculoskeletal aging is needed. The HGP is a novel model of age-related musculoskeletal decline that closely resembles multimorbidities characteristic of aging humans. Therefore, we will leverage this unique animal model to investigate the potential role of cytoDNA in the development of a skeletal muscle aging phenotype.

CHAPTER III-METHODS

HUSBANDRY

All procedures were approved by the Colorado State University Institutional Animal Care and Use Committee and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Samples used in this study were collected from control animals in a previously funded project. Male and female HGPs were obtained from Charles River Laboratories (Wilmington, MA, USA). We also collected samples from a comparison strain that does not develop multimorbidities of aging over the same age range as the HGP strain. Specifically, we obtained samples from female pigmented (PET) guinea pigs (Elm Hill Laboratories; Chelmsford, MA). Animals were acclimated to Colorado State University's Laboratory Animal Resources housing facilities, singly housed in solid bottom cages, maintained on a 12-12-hour light-dark cycle, and provided ad libitum access to food and water.

DEUTERIUM OXIDE LABELING

To assess long-term rates of total and cytosolic skeletal muscle DNA synthesis, all guinea pigs were given a subcutaneous injection of 0.9% saline enriched with 99% deuterium oxide (^2H_2O) equivalent to 3% of their body weight 30 days prior to euthanasia. Drinking water was enriched to 8% ^2H_2O for the purpose of maintaining deuterium enrichment of the body water pool during the 30-day labelling period. At the time of euthanasia and tissue harvest, the guinea pigs were 5mo or 15mo of age.

EUTHANASIA AND TISSUE COLLECTION

Euthanasia was carried out in accordance with the standards of the American Veterinary Medical Association. Following an overnight fast, animals were anesthetized with a mixture of isoflurane and oxygen; thoracic cavities were opened, and blood was collected via direct cardiac

puncture. Whole blood was centrifuged (1200 *g*, 4°C, 15 min) to separate plasma, which was frozen at -80°C until further analysis. After blood collection, the anesthetized animals were transferred to a chamber filled with carbon dioxide for euthanasia. The soleus, gastrocnemius, and tibialis anterior were trimmed of tendons and connective tissue, weighed, and flash frozen in liquid nitrogen. The tibialis anterior (TA) samples were used as part of current studies.

Two females required humane euthanasia prior to final analysis due to underlying issues unrelated to treatment (HGP final: n = 12 5mo males, n=10 15mo males, n=10 5mo females, n=8 15mo females; PET final: n=4 5mo females and n=4 15mo females). Other gross necropsy findings by veterinarians were unremarkable.

TISSUE SAMPLE FRACTIONATION

The TA muscle was homogenized and fractionated following established laboratory protocols and with the experimenter blinded to group. The muscle was first pulverized into a fine powder, using mortar and pestle. Liquid nitrogen was used to keep the muscle frozen and prevent thawing. Powdered TA between 50-75mg was weighed, transferred to 1.5 ml microcentrifuge tubes, and stored at -80 °C. Briefly, tissues (50 – 75 mg) were homogenized at 1:10 ratio in mito 1 lysis buffer (100 mM KCl, 40 mM Tris HCl, 10 mM Tris Base, 5 mM MgCl2, 1 mM EDTA, 1 mM ATP, pH – 7.50) using a tissue homogenizer (Bullet Blender, Next Advance Inc., Averill Park, NY, USA) with zirconium beads (Next Advance Inc., Averill Park, NY, USA). After homogenization, subcellular fractions were isolated via differential centrifugation at 800g for 10 minutes at 4 °C. Supernatant of the previous spin was transferred to a 1.5ml microcentrifuge tube and spun at 9,000g for 10 minutes at 4 °C. 200ul of supernatant, namely cytosol fraction, was transferred to a new 1.5ml centrifuge tube and stored at -80 °C.

DNA EXTRACTION

Two sequential procedures of DNA extraction were used to collect different cytoDNA species:1, in the shorter length range estimated at approximately 10-400 base pairs; 2, and in the longer length range estimated at approximately 400-2000+ base pairs.[144] We categorize these as short cytoDNA and long cytoDNA respectively. The pool of DNA extracted with these procedures contain double stranded DNA (dsDNA), ssDNA (single stranded DNA) and potentially deoxynucleotide triphosphates (dNTPs) (Figure 1).

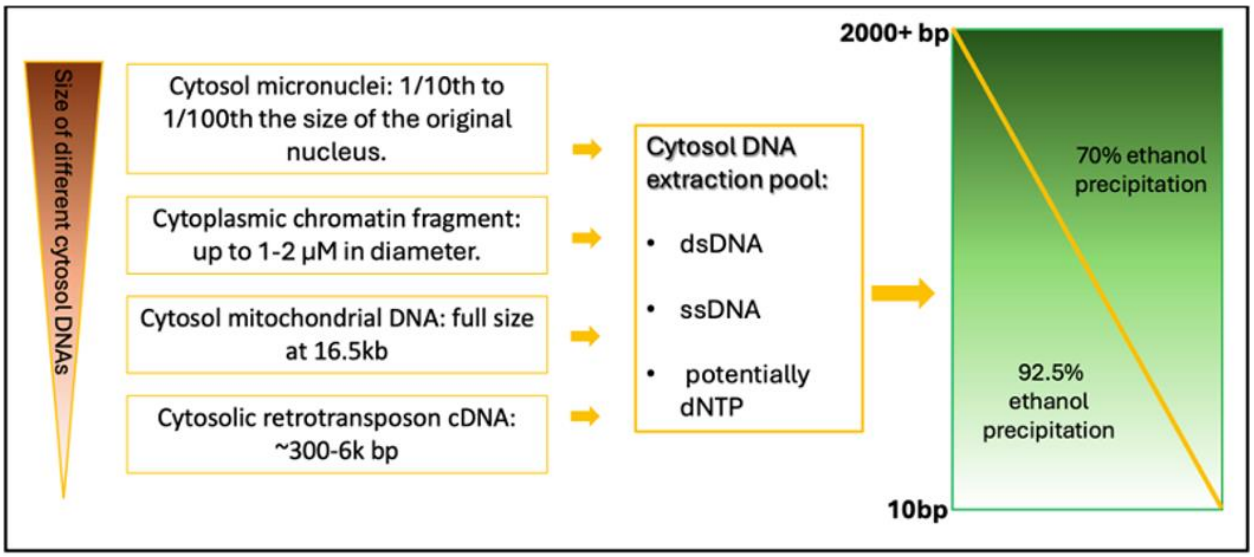

Figure 1. DNA extraction through differential percentages of ethanol precipitation enriches different sizes of cytoDNA. We posit DNA extraction with 70% ethanol precipitation enrich longer DNA species in the range of approximately 400-2,000+ base pairs. In addition, we posit DNA extraction by 92.5% ethanol precipitation enrich shorter cytoDNA species, approximately within the range of 10-400 base pairs.

Long CytoDNA Extraction

200ul cytosol fraction was incubated with 20ul of proteinase K at 55 °C and shaken at 1,500rpm for 20 hours. Samples were then taken off the thermal shaker and cooled at room temperature for 10 minutes. 200ul of 10mM EDTA, 40mM Tris pH 8.0 was added to the proteinase K treated sample, and then 410ul of phenol chloroform (pH 8.0) was added to sample and vortexed for 30 seconds. Then, the samples were spun at 17000g for 15 minutes at 4 °C.

360ul of the top layer of supernatant was carefully removed at 90ul intervals to a new 1.5 ml Eppendorf tube without disturbing the middle and bottom layer. 360ul of chloroform was added to the solution, vortexed for 15 seconds and spun for 10 minutes at 17,000g and 4 °C. 300ul of supernatant was transferred to a new 1.5 ml Eppendorf tube at 100ul intervals three times without disturbing the other layers. 30ul of 3M sodium acetate (pH 7.0) and 4ul of (5mg/ml) glycogen was added to each sample and vortexed for 5 seconds. 780ul of 100% cold ethanol was added and samples were vortexed for 15 seconds. Samples were then incubated at –80 °C for 30 minutes.

Following the 30-minute incubation at -80°C of the fractionation protocol, samples were removed from the freezer and immediately centrifuged for 45 minutes at 17,000g at 4°C. The supernatant was then removed and saved at -80°C for the subsequent precipitation of the short DNA species. 500ul of 70% EtOH was added to the pellet which was carefully loosened with light flicking and tapping. The resuspended pellets were then spun at 17,000g at 4 °C for 10 minutes. Supernatant was then removed, and tubes were lightly spun to bring all residual sample to the bottom of the tube. Then, using a 20ul pipette, the residual supernatant was removed without disturbing the pellet. Under a lab bench light, the pellets were dried for 5-20 minutes, until the pellet was visually dry and transparent. Once all pellets were dried, 100ul of milli Q water was added to dissolve the pellet. DNA concentrations were measured by a NanoDrop 2000 spectrophotometer.

Short CytoDNA Extraction

The supernatant from the 70% EtOH long cytoDNA species precipitation step was used for further precipitation of the short cytoDNA species. Supernatant was removed from the -80 °C freezer and thawed. After thawing and vortexing briefly, 500ul of sample was transferred to a separate tube and 15ul of 3M sodium acetate (pH=7) and 4ul of 5mg/ml glycogen was added. 1,500ul of 100% EtOH was added to reach 92.5% EtOH and samples were incubated at –80°C for

30 minutes. Following the incubation, samples were spun at 17,000 g for 45 minutes at 4°C. The supernatant was removed and 500ul of 92.5% EtOH was added to the sample for washing. Pellet was loosened with light tapping and then spun at 17,000g for 5 minutes. The supernatant was removed and 500ul of 92.5% EtOH was added to the sample followed by centrifuging at 17,000g for 5 minutes once more. The supernatant was removed as well as any remaining liquid with a 20ul pipette. Under a lab bench light, the pellets were dried for between 5 and 20 minutes, until the pellet was visually dry and transparent. Once all pellets were dried, 100ul of milli Q water was added to dissolve the pellet. DNA concentrations were determined by a the same NanoDrop machine.

SAMPLE DERIVATION AND ANALYSIS VIA GC-MS: CYTO DNA

For DNA hydrolysis, 25ul of DNA hydrolysis buffer ((Potato acid phosphatase 1 kU; Calbiochem, cat. no. 80602-592), (S1 nuclease Sigma, cat. no. N5661)) was added to 100ul of DNA sample followed with a quick vortex and incubation on shaker for 20 hours at 37 °C at 150 rpm. The hydrolysates were removed from incubator afterward and cooled at room temperature for 10 minutes before being transferred to 10ml glass tubes. 200ul of pentafluorobenzyl hydroxylamine (1mg/ml) and 150ul of glacial acetic acid were added and vortexed for 5 seconds before incubating samples at 100 °C for 30 min. After incubation, samples were cooled at room temperature for 10 minutes and then acetylated with 2ml of acetic anhydride and 200ul of n-methylimidazole. N-methylimidazole was added to samples one at a time, quickly capped and lightly vortexed. 15 minutes were allotted to allow the reaction to proceed before moving on to the next steps. After the reaction tubes cooled down to room temperature, 3ml of milli Q water was added to each tube and vortexed for 10 seconds. 1.2ml of dichloromethane was added at 600ul intervals and vortexed for 5 seconds. 5 minutes were allotted for the phases to separate and settle before the next steps. The samples were then spun at room temperature briefly to facilitate the layer separation. Once the

speed reached 1,500rcf, the centrifuge was immediately stopped, and samples were removed after deceleration. The bottom layer of the tubes was then transferred to a new set of glass tubes containing anhydrous sulfate. 600ul of dichloromethane was then added to the original sample tubes, vortexed for 5 seconds and given 5 minutes for phases to separate and settle. Samples were centrifuged until the speed reached 1,500rcf, and then immediately stopped. Samples were removed after deceleration. The bottom layer was then transferred into a new set of glass tubes containing anhydrous sulfate. The sample tubes containing anhydrous sulfate were vortexed for 5 seconds before being carefully transferred to a new 2ml glass vial. No anhydrous sulfate was transferred between tubes, only the solution. The extracted sample was then dried *in vacuo at 55* ^{o}C for 24 hours and resuspended in 80ul ethyl acetate. Samples resuspended in ethyl acetate were transferred in vial inserts, and capped and crimped for the analysis by GC/MS. The newly synthesized fraction of cytosolic DNA was calculated from the true precursor enrichment based upon blood plasma analyzed for $^{2}H_2O$ enrichment and adjusted using mass isotopomer distribution analysis.[145]

QUANTIFICATION OF CYTOSOLIC DNA SYNTHESIS RATES

To quantify the fractional synthesis rate (FSR) of deuterium labeled DNA in the cytosol, GC/MS was used to detect the stable isotope and calculate the average FSR across 30 days as well as the daily FSR. In this study, the term *cytosolic DNA synthesis* refers to DNA in the cytosol during the 30-day labeling period. While the word "synthesis" is used throughout for clarity and consistency, it is important to note that this does not represent DNA produced within the cytosol, rather DNA that has appeared or accumulated in the cytosol from nuclear and mitochondrial sources. We use this term acknowledging these limitations, as alternative terminology (e.g., accumulation or appearance) would imply assumptions about regulation of cytoDNA and kinetics that our data do not directly support.

STATISTICAL ANALYSES

Statistical analysis was performed using PRISM GraphPad 10 Software (GraphPad Software, La Jolla, CA) and R studio (version 2024.12.1+563). Two-way ANOVAs with Greenhouse-Geisser corrections were performed to assess the main effects of age, sex, and interaction. When significant main effects were identified, post hoc multiple comparisons were performed using an uncorrected Fisher's LSD test to further assess differences between groups. Variability in the ratio of cytosolic to total DNA synthesis was assessed using two-tailed F-tests for equality of variance. When no main effect of sex was identified in the two-way ANOVAs, data were collapsed across sex and unpaired t-tests with Welch's post hoc correction were used to compare data from the young vs aged guinea pigs. Critical value for significance was set a priori to $P = 0.05$ for both the t-tests and two-way ANOVAs. However, due to the exploratory nature of the experiment, trends approaching significance ($0.05 < P < 0.1$) were also reported and interpreted cautiously. Comparisons were made between age groups within each sex, as well as between young and old animals collapsed across sex. To evaluate the linear relationship between cytosolic DNA synthesis and total DNA synthesis within each group, Pearson correlation coefficients (Pearson's *r*) were calculated. Correlation analyses were conducted separately for males and females at 5 and 15 months of age, and on sex-collapsed data for young and old groups. Two-tailed P-values were reported for all correlation analyses.

SHORT DNA SPECIES RESULTS

Rates Of CytoDNA Synthesis in the TA do not Differ with Age in Male and Female HGPs

A two-way ANOVA was conducted to assess the influence of sex and age on rates of cytoDNA synthesis. There were no significant main effects of age or sex in the rates of short cytoDNA synthesis (Figure 2A). When sex groups were collapsed, there was no significant difference between the rates of newly synthesized short cytoDNA between 5 and 15mo HGPs (Figure 2B).

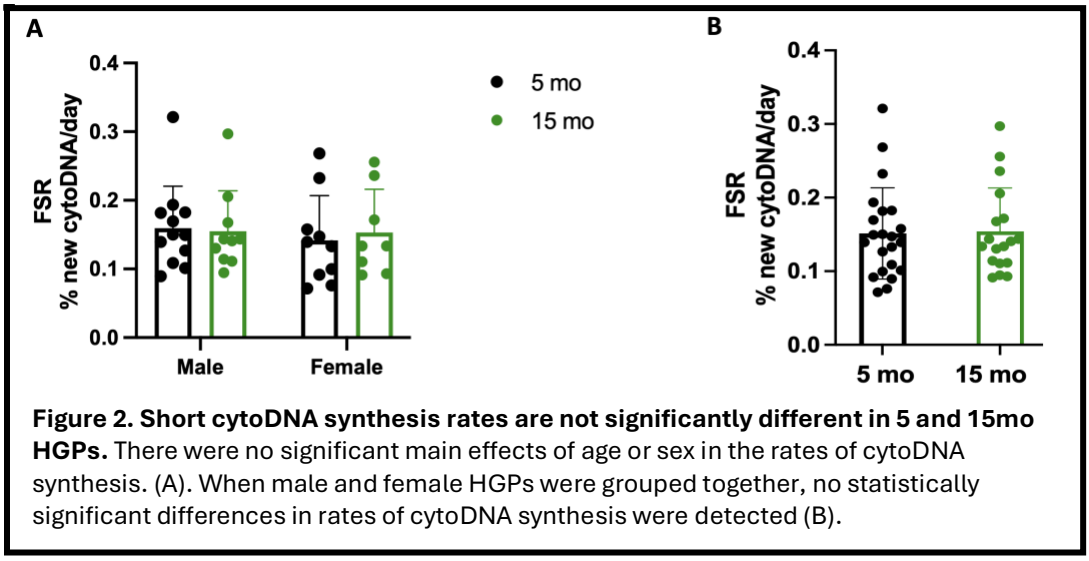

Figure 2. Short cytoDNA synthesis rates are not significantly different in 5 and 15mo HGPs. There were no significant main effects of age or sex in the rates of cytoDNA synthesis. (A). When male and female HGPs were grouped together, no statistically significant differences in rates of cytoDNA synthesis were detected (B).

Cyto: Total DNA Synthesis Rates Trend Towards an Effect of Age, and are more Variable in Older HGPs

F-tests were run to evaluate the variability of cytoDNA: total DNA synthesis rates. F-test results revealed a statistically significant difference in variability between 5mo and 15mo males (F= 0.1147, P= 0.0015), with older males exhibiting greater variability (Figure 3A). Similarly, a highly significant difference in variability was observed between 5mo and 15mo females (F= 0.0572, P= 0.0003), with greater variability also present in the older females (Figure 3B).

A two-way ANOVA was performed to analyze effect of sex and age in cytoDNA: total DNA synthesis rates. In male and female, 5 and 15mo HGPs, a trend toward a main effect of age was observed, but no significant effect of sex (Figure 3C). To further investigate this relationship, male and female groups were collapsed. Results from a Welch's -test showed the trend for an age effect persisted (Figure 3D).

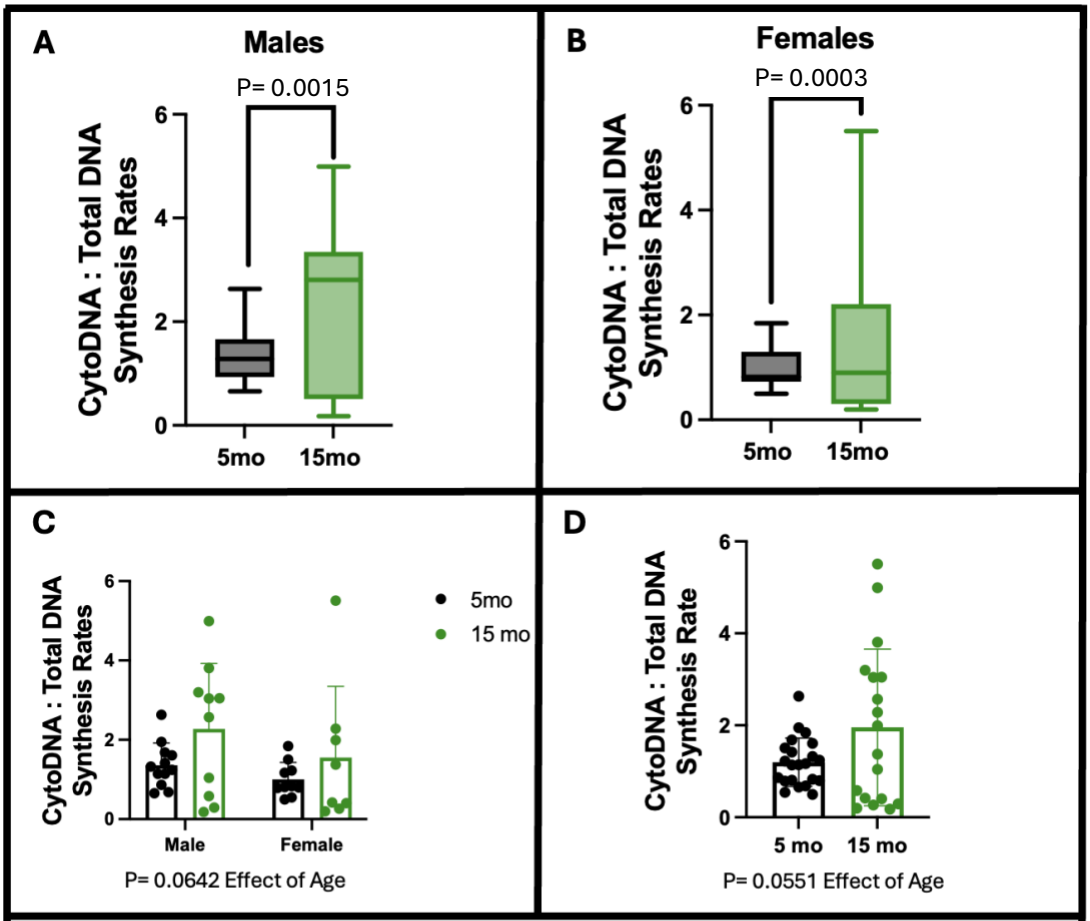

Figure 3. Increased variability and age-related trends in cytoDNA: total DNA synthesis rates. In both males (A) and females (B), the variability of cytoDNA: total DNA synthesis rates is significantly greater in 15mo HGPs compared to 5mo HGPs. Analysis of cytoDNA: total DNA synthesis rates with a two-way ANOVA showed a trend towards a main effect of age (C). When male and female groups were collapsed the trend towards an effect of age in cytoDNA: total DNA synthesis rates strengthened (D).

CytoDNA Synthesis Rates do not Correlate with Total DNA Synthesis Rates

Pearson correlations were conducted to evaluate the relationship between short cytoDNA synthesis and total DNA synthesis rates in the TA of HGPs. No statistically significant correlations were found in any individual group (Figure 4).

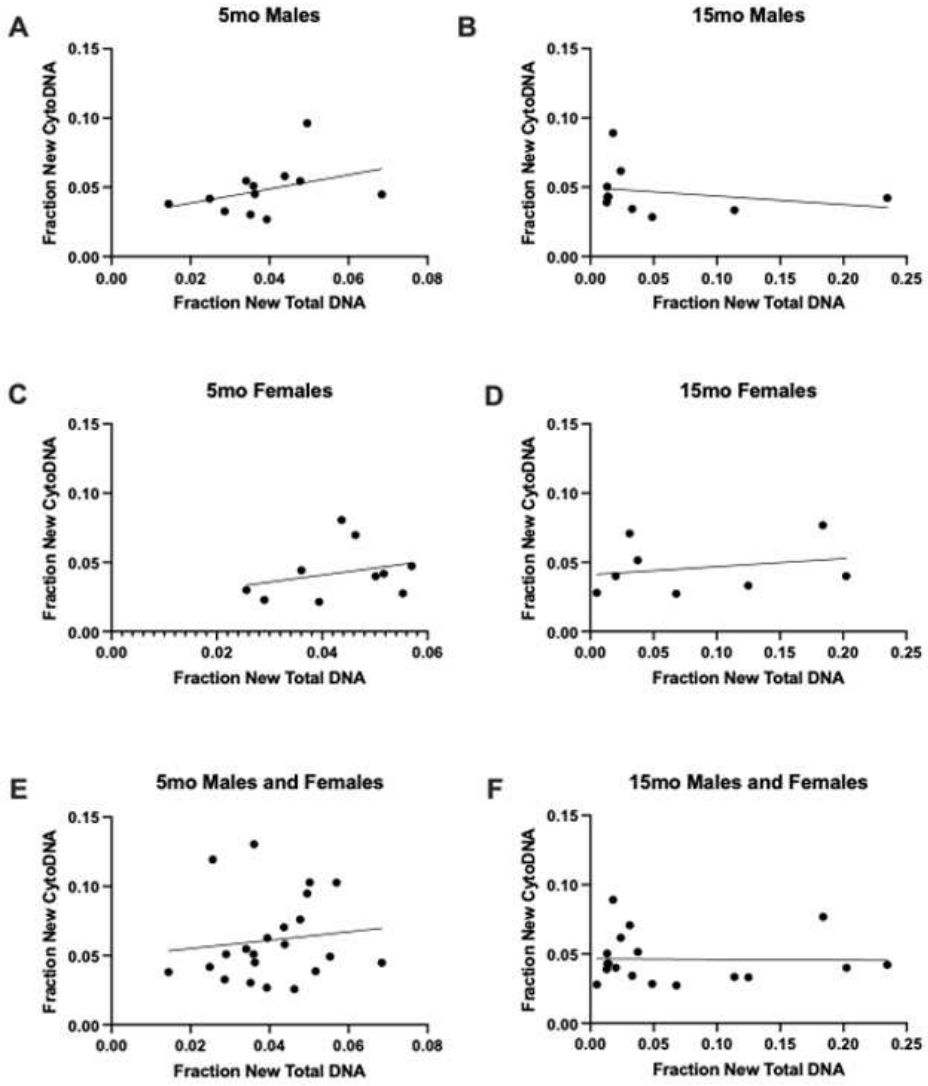

Figure 4. Rates of cytoDNA synthesis do not correlate with total DNA synthesis. No significant correlations between rates of short cytoDNA synthesis (Y-axis) and total DNA synthesis (X-axis) across HGP age and sex groups were observed. Figures A-D show results organized by age and sex. Panels E and F represent data collapsed across sex for each age group.

Compared To PETs, Synthesis Rates of CytoDNA in Female HGPs are Significantly Higher in Both 5mo and 15mo Animals

A two-way ANOVA was used to analyze synthesis rates of newly synthesized short cytoDNA between 5 and 15mo female HGPs, and 5 and 15mo female PET guinea pigs, revealed a main effect of strain (P= 0.001). Post hoc tests showed significantly higher rates of cytoDNA synthesis in 5mo HGPs compared to 5mo PETs (P= 0.0076), and 15mo HGPs compared to 15mo PETs (P= 0.0276) (Figure 5).

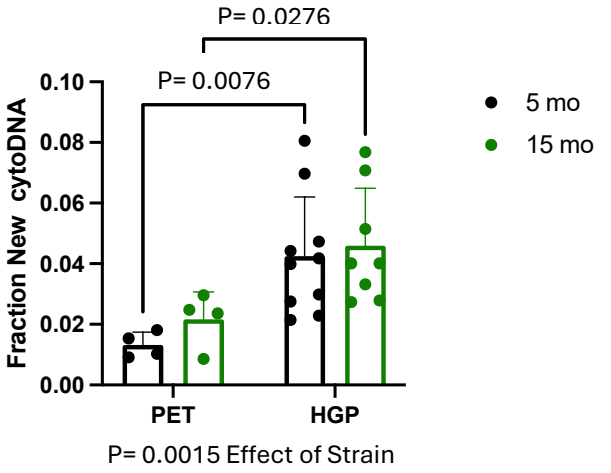

Figure 5. Female HGPs have higher rates of short cytoDNA synthesis than PETs. Rates of cytoDNA synthesis are significantly higher, in both 5 and 15mo female HGPs when compared to same age PET guinea pigs.

LONG DNA SPECIES RESULTS

Rates Of CytoDNA Synthesis are Lower in 15mo Male and Female HGPs

A two-way ANOVA was used to analyze rates of long cytoDNA synthesis between male and female 5 and 15mo HGPs revealing a significant effect of age (P= 0.0014) and a trend towards an effect of sex (P= 0.0532) (Figure 6). Contrary to our hypothesis, cytoDNA synthesis rates were significantly lower in both older male (P= 0.0123) and female (P= 0.0277) HGPs when compared to their younger counterparts (Figure 6).

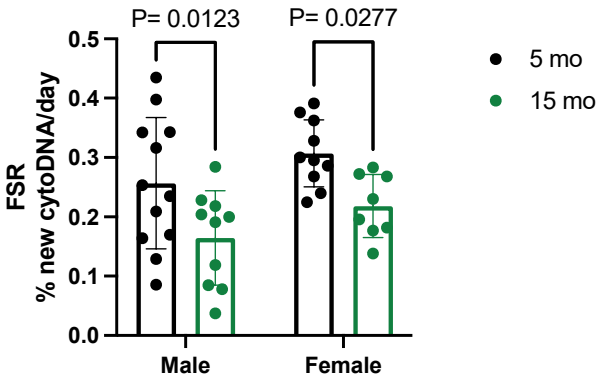

Figure 6. 15mo HGPs have lower rates of cytoDNA synthesis compared to 5mo HGPs. Results from a two-way ANOVA reveal a main effect of age and a trend towards an effect of sex. Synthesis rates of long cytoDNA are significantly lower in 15mo male and female HGPs when compared to their sex matched

CytoDNA: Total DNA Synthesis Rates are Not Different Between Age or Sex, But Variability Increases with Age in Female HGPs

To evaluate age-related differences in the variability of cytoDNA: total DNA synthesis rates, we performed F-test were performed. No significant differences in variability between male 5 and 15mo HGPs (Figure 7A). However, in females, a there was significantly greater variability (F= 0.0373, P= 0.00004925) in 15mo compared to 5mo HGPs (Figure 7B). A two-way ANOVA was used to analyze the difference in the ratio of long cytoDNA: total DNA synthesis rates in HGPs. There were no main effects of sex or age in the ratio of longer cytoDNA synthesis rates to total DNA synthesis rates (Figure 7C). When sex groups were collapsed, no statistical differences in cytoDNA: total DNA synthesis rates were detected (Figure 7D).

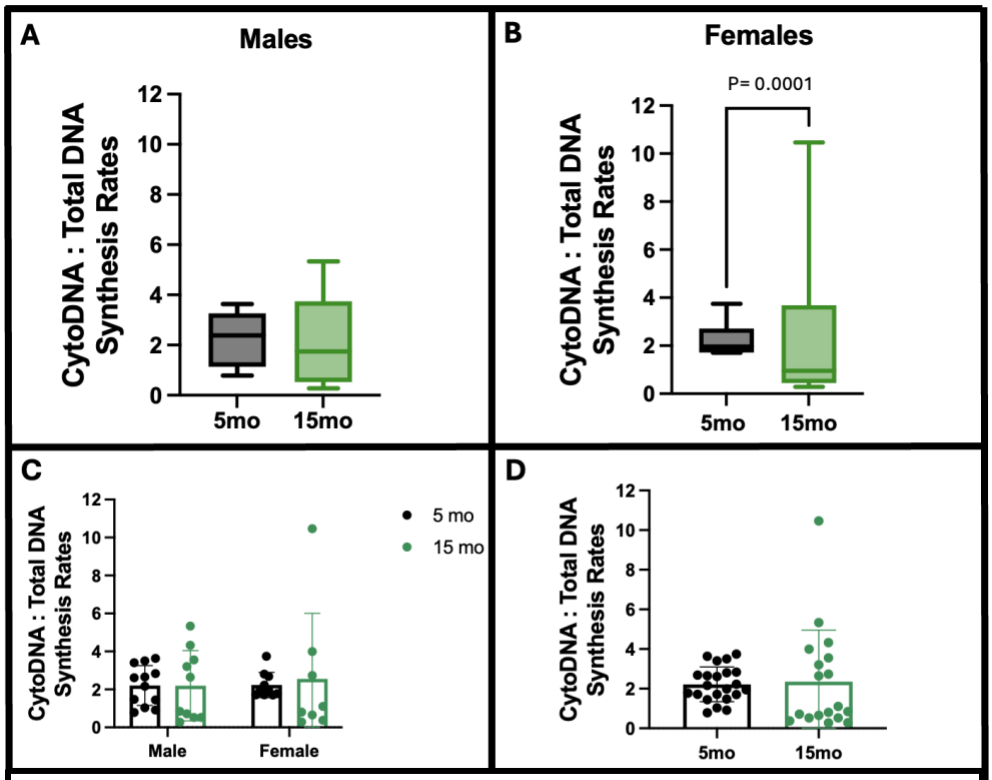

Figure 7. Variability of cytoDNA: total DNA synthesis rates increase with age in females only. No significant differences in variability were observed between 5mo and 15mo male HGPs (A). In contrast, variability of 15mo females was significantly greater compared to their 5mo counterparts (B). CytoDNA: total DNA synthesis rates are not significantly different in 15mo HGPs compared to same sex 5mo HGPs (C). In a comparison of 5mo HGPs to 15mo HGPs with sex groups collapsed, no statistical differences are detected (D).

CytoDNA Synthesis Rates Do Not Correlate with Total DNA Synthesis Rates

Pearson correlations were conducted to assess the relationship between long cytoDNA synthesis and total DNA synthesis rates s in HGPs. No statistically significant correlations were found in any group (Figure 8).

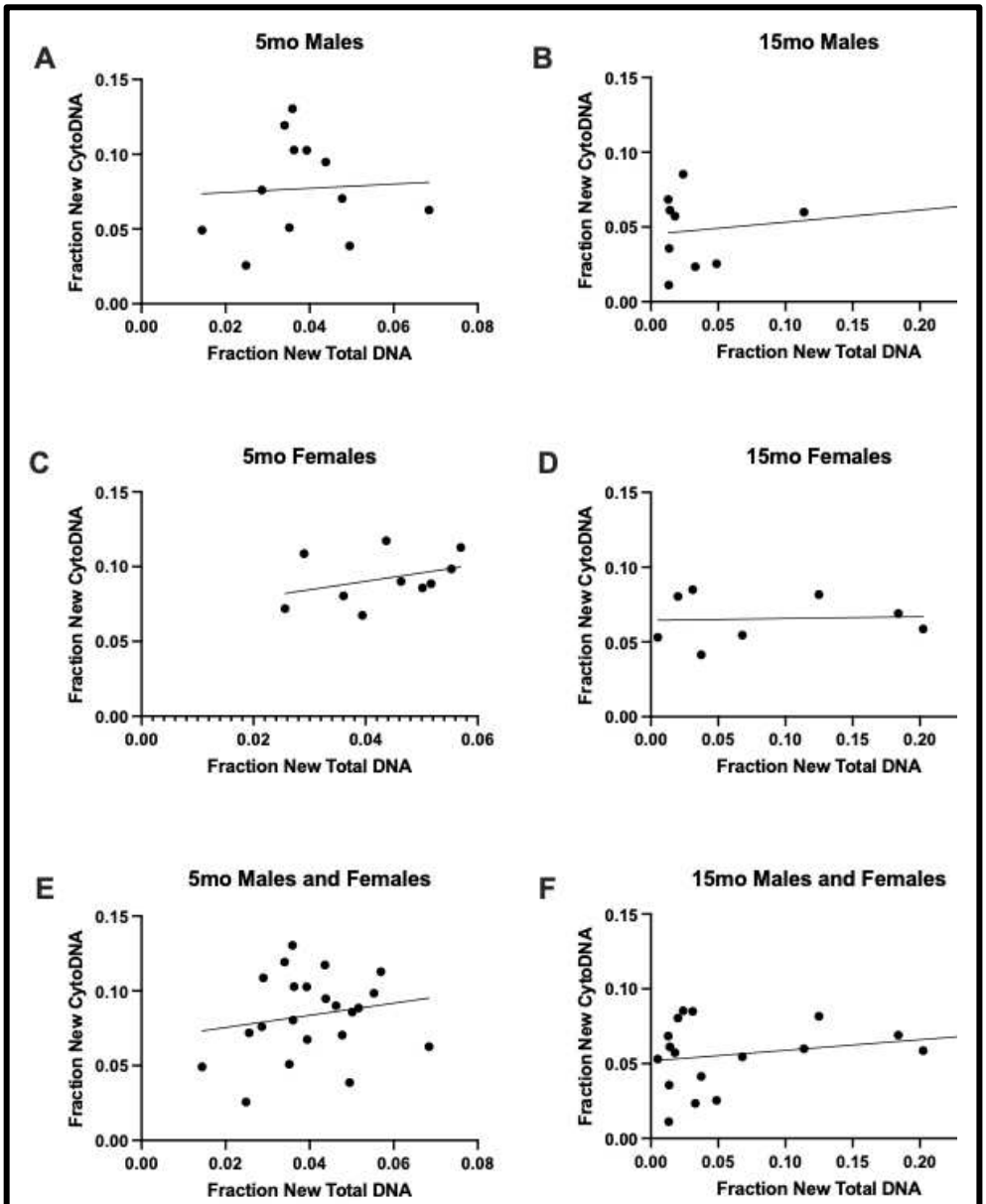

Figure 8. Rates of long cytoDNA synthesis do not correlate with total DNA synthesis. No statistically significant correlations between rates of long cytoDNA synthesis (Y-axis) and total DNA synthesis (X-axis) across HGP age and sex groups were observed. Figures A-D show results organized by age and sex. Panels E and F represent data collapsed across sex for each age group.

Synthesis Rates of CytoDNA Species are Not Significantly Different in Female PET Guinea Pigs When Compared to Female HGPs

In contrast to the findings in rates of synthesis of short cytoDNA species, there were no significant differences in long cytoDNA synthesis rates between PET and HGP strains. There was a main effect of age, with post hoc multiple comparisons showing lower rates of long cytoDNA synthesis in 15mo compared to 5mo HGPs (figure 9).

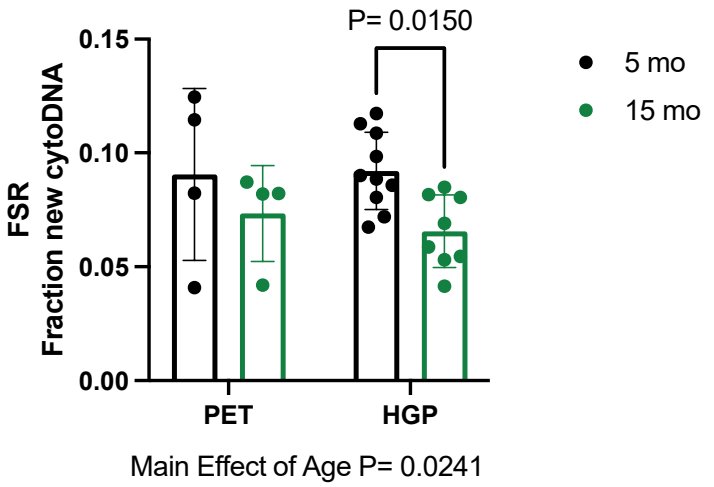

Figure 9. Rates of long cytoDNA synthesis are not statistically different between female PETs and HGPs. There was no main effect of strain in the rates of long cytoDNA synthesis. The main effect of age was explained by post hoc comparisons showing lower rates of long cytoDNA synthesis in the 15mo compared to 5mo HGPs.

CHAPTER V: DISCUSSION

SUMMARY OF RESULTS

The objective of this study was to evaluate the rates of cytoDNA synthesis in the TA of HGPs, an outbred non-transgenic model of progressive age-related musculoskeletal decline also characterized by other co-morbidities mimicking human aging. The primary findings show that rates of cytoDNA synthesis are not greater with age regardless of sex or cytoDNA base pair length. Interestingly, rates of longer cytoDNA synthesis are significantly lower in both male and female 15mo when compared to 5mo HGPs. These findings contrast with our original hypothesis that 15mo HGPs would exhibit higher rates of cytoDNA synthesis when compared to 5mo, and that females would have higher rates of cytoDNA synthesis than males. However, rates of synthesis in a DNA pool enriched with shorter cytoDNA species were significantly higher in both ages of HGPs when compared to PETs, a strain that does not develop age-related musculoskeletal decline or other co-morbidities over this 5-15mo age range. These findings suggest that rates of short cytoDNA synthesis may be more involved than long cytoDNA synthesis rates in the development of age-related musculoskeletal decline in HGPs.

Importantly, this approach measures newly synthesized DNA over a 30-day labeling period, and subcellular fractionation methods were used to isolate and identify newly synthesized DNA specifically within the cytosolic fraction. As such, the term “cytoDNA synthesis” refers to the detection of newly synthesized DNA localized to the cytosol and does not reflect mechanisms which may regulate the presence of DNA in the cytosol.

AGE RELATED DIFFERENCES IN CYTODNA SYNTHESIS RATES ARE ONLY IN LONG DNA SPECIES

A key finding in our study was that rates of cytoDNA synthesis in the TA did not significantly differ between age or sex in shorter cytoDNA synthesis rates (Figure 2A). However, rates of long cytoDNA synthesis were significantly lower in 15mo HGPs compared to 5mo (Figure 6). While precise classification of cytoDNA source origin by base pair length is currently unknown, emerging evidence suggest that shorter and longer cytosolic DNA fragments may arise from distinct cellular sources or processing mechanisms.[146,147] Further, Identifying the source origin of cytoDNA in both long and short pools would allow for more supported conclusions regarding these differences; however, our methods did not include processes to categorize our pools of cytoDNA into potential sources, such as mtDNA, CCF, MN or retrotransposons. Despite this shortcoming, it is possible that the significantly lower rates of synthesis of the longer cytoDNA pool in older HGPs, could be attributed to intracellular clearance of cytoDNA. Findings from Watson et al. revealed that, in macrophages infected with mycobacterium tuberculosis, the recognition of bacterial DNA by the cGAS-STING pathway elicited ubiquitin-mediated autophagy, which then delivered the bacteria to autophagosomes for degradation.[148] In addition, Liang et al. reported direct interaction between cGAS and the Beclin-1 protein to release the Rubicon-mediated inhibition of autophagy, thereby delivering cytosolic microbial DNA to autophagosomes for degradation.[149] Findings in lymphoma cells from Shen and colleagues show the co-localization of cytoDNA with lysosomes and a subset of cytoDNA co-localized with CD63, a late endosomal marker that can recycle cellular material via exocytotic pathways.[150] In addition to these findings, the discovery of DNA in extracellular vesicles of cancer cells (EVs) points to exocytotic mechanisms that can clear DNA from the cytosol.[151] For instance, Yokoi et al. and Takahashi et al. have both shown that EVs play a role in maintaining cellular homeostasis by removing cytoplasmic

chromatin and micronuclei in both cancerous ovarian cells, and senescent diploid cells respectively.[152,153] The combination of these published findings show the regulation of DNA through autophagy and exocytosis in both cancerous cells and senescent cells; however, similar findings in host cytoDNA of healthy cells have yet to be published. These mechanisms of DNA clearance from the cytosol may explain why our 15mo guinea pigs had lower rates of long cytoDNA synthesis than the 5mo animals.

While we hypothesize that these regulatory mechanisms of cytoDNA may account for the lower rates of long cytoDNA synthesis in 15mo HGPs, they do not explain the lack of age-related differences in short cytoDNA synthesis rates. Although we cannot confirm length-based regulation of cytoDNA, our data suggest that aging cells may more effectively limit the accumulation of longer DNA fragments in the cytosol compared to shorter ones. (Figure 1). While we did not directly compare the yields or distributions of DNA fragment sizes between the long and short pools, this remains a plausible explanation that warrants further investigation.

Despite our unexpected results, these findings are important as they suggest that age-related differences in cytoDNA synthesis rates are more pronounced in long DNA species than in short ones. The lack of significantly lower rates of synthesis in the 15mo HGPs in short cytoDNA points to a potential selective mechanism that favors the regulation of larger DNA fragments in the cytosol. We propose that the selective clearance of cytoDNA fragments by autophagy and exocytotic mechanisms may limit cytoDNA accumulation in a base pair length dependent manner. To test this hypothesis, future studies should compare autophagic activity in cells harboring long versus short cytoDNA fragments, with a focus on potential length-dependent targeting for degradation.

THE RATIO OF CYTODNA TO TOTAL DNA SYNTHESIS RATES TREND TOWARDS AGE-RELATED DIFFERENCES IN SHORT CYTODNA SPECIES BUT NOT LONG

Aging cells have increased nuclear and mitochondrial membrane permeability.[41,122] Lamin B1, a vital protein for the integrity of the nuclear membrane decreases with senescence and leads to the blebbing of chromatin from the nucleus forming CCF, thus propagating inflammation.[122] Further, as mitochondrial dysfunction in cells increases with age, extrusion of the inner mitochondrial membrane leading to a release of mitochondrial DNA in the cytoplasm can drive inflammation.[154] In a previous study in HGPs from our lab, markers of cellular senescence, including p21 and phospho-H2AX, were detected in TA muscle, indicating that this tissue undergoes senescence-associated changes with age (Walsh et al. Unpublished). Because senescent cells exhibit altered proliferation dynamics, are a marker of aging, and produce CCF, we investigated the potential of a relationship between cytoDNA synthesis rates and nuclear DNA replication.[118] To assess this, we compared the rate of cytoDNA synthesis relative to total DNA synthesis, which reflects overall proliferation, by analyzing the cytoDNA: total DNA ratio in the TA.

We found no significant differences between the ratio of cytoDNA synthesis to total DNA synthesis in either short or long DNA species. However, there was a trend ($P= 0.0642$) for a main effect of age in the short cytoDNA: total DNA synthesis rates (Figure 3C). Further, when sex groups were collapsed, this trend for a higher ratio in the 15mo compared to 5mo was stronger ($P= 0.055$; Figure 3D). We posit that high variability in the older animals prevented significant findings in both species.

DISENTANGLING THE RELATIONSHIP OF CYTODNA AND TOTAL DNA SYNTHESIS RATES IN HGPS

To evaluate age-related differences in the variability of cytoDNA: total DNA synthesis rates, F-tests were performed. These analyses revealed significantly greater variability with age in all

groups except for long cytoDNA synthesis rates in males (Figure 7A). This increased variability may reflect biological heterogeneity, a feature that becomes more pronounced with aging humans.[155] While inbred lab rodents generally show lower variability in data related to cell aging, HGPs are outbred and are comparatively more genetically heterogenous than inbred rodent models. Therefore, the greater variability with age may reflect more pronounced genetic heterogeneity expected in aging.

Although higher variability contributed to the lack of significant differences between 5 and 15mo HGPs, we propose that cytoDNA: total DNA synthesis rates hold promise as a biomarker for cellular aging. To further confirm the relationship of cytoDNA: total DNA synthesis rates as a marker of cellular aging, future studies should investigate whether higher cytoDNA: total DNA synthesis rates predict the onset and progression of musculoskeletal decline and hallmarks of cell aging in HGPs.

In addition to our analyses of variability, we evaluated the correlation of cytoDNA synthesis and total DNA synthesis rates to further investigate the relationship between a driver of inflammation with cell proliferation. We did not identify any significant correlations of cytoDNA synthesis and total DNA synthesis in any groups in either short or long cytoDNA pools (Figure 4, Figure 8). Further, in an analysis of cytoDNA and total DNA in 5mo HGPs with sex collapsed, and an analysis in 15mo HGPs with sex collapsed, no significant correlations were identified (Figure 4E-F, Figure 8E-F). This suggests that rates of cytoDNA synthesis vary independently from total DNA synthesis within HGPs of both ages, and that high or low rates of total DNA synthesis do not reliably predict cytoDNA synthesis rates or vice versa.

CYTODNA SYNTHESIS IS HIGHER IN A MODEL OF AGE-RELATED MUSCULOSKELETAL DECLINE

Our primary hypothesis that rates of cytoDNA synthesis would be higher in older animals characterized by a phenotype of age-related musculoskeletal decline was rejected, suggesting that cytoDNA synthesis does not contribute to the development of musculoskeletal decline with age. However, we posited that the early onset of age-related musculoskeletal decline in HGPs may be associated with rates of cytoDNA synthesis that are already high at 5mo. PET guinea pigs are not characterized by early development and marked progression of musculoskeletal decline or other comorbidities over this age range, making them a good comparison strain. However, our access to samples of TA from PET guinea pigs was limited to n=8 females (n=4, 5mo and n=4, 15mo). In our comparison of short cytoDNA synthesis rates between female guinea pigs, we detected significantly higher rates of cytoDNA synthesis in both 5mo (P= 0.0076) and 15mo female HGPs (P= 0.0276) when compared to PETs (Figure 6). Although these findings are in a small sample size of females, they suggest rates of short cytoDNA synthesis are higher in the TA of animals characterized by age-related musculoskeletal decline beginning early in life and progressing relatively rapidly. However, there were no significant differences in rates of long cytoDNA synthesis between female PETs and HGPs (Figure 9). In addition, the lack of significant differences in cytoDNA synthesis rates between strains for long cytoDNA species supports our speculation that longer cytoDNA fragments may be more selectively targeted by mechanisms that regulate cytosolic DNA accumulation compared to shorter species. Further analyses of long cytoDNA with a larger sample size, in male guinea pigs and in other skeletal muscle should be conducted to firmly establish a relationship with cytoDNA and muscle aging between PETs and HGPs. Lastly, the finding of greater short cytoDNA synthesis rates in both ages of HGPs compared to PETs suggests that the 5mo HGPs may not be representative of a “young biological age”. Future study designs

may require an even younger chronological age comparison group to represent a young vs old analysis in HGPs.

IMPLICATIONS FOR MUSCULOSKELETAL AGING

Overall, these findings support the idea that cytoDNA synthesis is relevant in the musculoskeletal aging process. However, the degree to which cytoDNA drives sterile inflammation in skeletal muscle was not determined. Identifying the contribution of a cGAS-STING mediated response to cytoDNA with age is a necessary next step to firmly establish cytoDNA as a key driver of age-related musculoskeletal decline. Further investigation into the role of length specific cytoDNA species, and the activation of cGAS-STING in muscle from aging humans will provide clarity into how cytoDNA and sterile inflammation contribute to musculoskeletal aging. Although our hypotheses were not supported, the detected variability in rates of cytoDNA relative to total DNA synthesis may be reflective of dysregulation of cellular mechanisms responsible for preserving skeletal muscle health with age. While cytoDNA: total DNA synthesis could prove to be a novel marker of healthy cellular aging in the skeletal muscle, the relationship of cytoDNA to total DNA synthesis rates should be established in highly replicative tissue types susceptible to aging to better understand its biological relevance and validate its utility across different tissues.

LIMITATIONS

A primary limitation of this study is the lack of confirmation of cGAS-STING activation. Although cytoDNA is a known driver of this signaling pathway, without determining activation of cGAS-STING, no conclusions can be made regarding the influence of this inflammatory pathway on age-related musculoskeletal decline. Further, not being able to identify the origin source of the cytoDNA extracted prevents us from assessing the contribution of different cytoDNA species in skeletal muscle. Similarly, we did not assess the average base pair length of the DNA extracted in

our short and long cytoDNA pools. In addition, confirmation of the base pair length in our two pools of extracted cytoDNA would give useful insight into which cytoDNA species are more prevalent in skeletal muscle in these models, as well as into any useful modifications of this newly established method. Lastly, the lack of samples from male PET guinea pigs and a small sample size of female PETs prevents us from making concrete conclusions about the relationship of cytoDNA synthesis rates with HGPs.

CONCLUSION

This study highlights the potential role of cytoDNA synthesis in musculoskeletal aging, with evidence pointing toward age-dependent differences in synthesis rates and variability of cytoDNA synthesis relative to cell proliferation. While our findings did not fully support our initial hypotheses, they underscore the complexity of cytoDNA synthesis with aging in this HGP model. Further research is needed to better understand the mechanisms driving age related changes in cytoDNA synthesis, and to explore the role of cytoDNA and cGAS-STING in muscle dysfunction and other age-related chronic diseases associated with muscle decline.

REFERENCES

1. Bureau USNC. Bureau USNC. 2017 National Population Projections Tables: Main Series. https://www.census.gov/data/tables/2017/demo/popproj/2017-summary-tables.html.
2. Ansah JP, Chiu CT. Projecting the chronic disease burden among the adult population in the United States using a multi-state population model. *Front Public Health*. 2022;10:1082183. doi:10.3389/fpubh.2022.1082183
3. Niccoli T, Partridge L. Ageing as a risk factor for disease. *Curr Biol*. Sep 11 2012;22(17):R741-52. doi:10.1016/j.cub.2012.07.024
4. Brutto OHD. The Impact of Biological and Social Factors on Mortality in Older Adults Living in Rural Communities. *Journal of Primary Care & Community Health*. 2024;15doi:10.1177/21501319241228123
5. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. *Cell*. Jan 19 2023;186(2):243-278. doi:10.1016/j.cell.2022.11.001
6. Fulop T, Larbi A, Pawelec G, et al. Immunology of Aging: the Birth of Inflammaging. *Clin Rev Allergy Immunol*. Apr 2023;64(2):109-122. doi:10.1007/s12016-021-08899-6
7. Xia S, Zhang X, Zheng S, et al. An Update on Inflamm-Aging: Mechanisms, Prevention, and Treatment. *Journal of Immunology Research*. 2016;2016:1-12. doi:10.1155/2016/8426874
8. Marzetti E, Picca A, Marini F, et al. Inflammatory signatures in older persons with physical frailty and sarcopenia: The frailty "cytokinome" at its core. *Exp Gerontol*. Jul 15 2019;122:129-138. doi:10.1016/j.exger.2019.04.019
9. Franceschi C, Campisi J. Chronic inflammation (inflammaging) and its potential contribution to age-associated diseases. *J Gerontol A Biol Sci Med Sci*. Jun 2014;69 Suppl 1:S4-9. doi:10.1093/gerona/glu057
10. Wilson D, Jackson T, Sapey E, Lord JM. Frailty and Sarcopenia: The Potential Role of an Aged Immune System. *Ageing Research Reviews*. 2017;36:1-10. doi:10.1016/j.arr.2017.01.006
11. Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *J Am Geriatr Soc*. May 2002;50(5):889-96. doi:10.1046/j.1532-5415.2002.50216.x
12. Livshits G, Kalinkovich A. Inflammaging as a common ground for the development and maintenance of sarcopenia, obesity, cardiomyopathy and dysbiosis. *Ageing Res Rev*. Dec 2019;56:100980. doi:10.1016/j.arr.2019.100980
13. de Jong J, Attema BJ, van der Hoek MD, et al. Sex differences in skeletal muscle-aging trajectory: same processes, but with a different ranking. *Geroscience*. Aug 2023;45(4):2367-2386. doi:10.1007/s11357-023-00750-4
14. Bilski J, Pierzchalski P, Szczepanik M, Bonior J, Zoladz JA. Multifactorial Mechanism of Sarcopenia and Sarcopenic Obesity. Role of Physical Exercise, Microbiota and Myokines. *Cells*. Jan 4 2022;11(1)doi:10.3390/cells11010160
15. El Assar M, Álvarez-Bustos A, Sosa P, Angulo J, Rodríguez-Mañas L. Effect of Physical Activity/Exercise on Oxidative Stress and Inflammation in Muscle and Vascular Aging. *Int J Mol Sci*. Aug 5 2022;23(15)doi:10.3390/ijms23158713
16. Li R, Xia J, Zhang XI, et al. Associations of Muscle Mass and Strength with All-Cause Mortality among US Older Adults. *Med Sci Sports Exerc*. Mar 2018;50(3):458-467. doi:10.1249/mss.0000000000001448
17. Mesinovic J, Fyfe JJ, Talevski J, et al. Type 2 Diabetes Mellitus and Sarcopenia as Comorbid Chronic Diseases in Older Adults: Established and Emerging Treatments and Therapies. *Diabetes Metab J*. Nov 2023;47(6):719-742. doi:10.4093/dmj.2023.0112

18. Holt-Lunstad J, Smith TB, Baker MD, Harris T, Stephenson D. Loneliness and Social Isolation as Risk Factors for Mortality. *Perspectives on Psychological Science*. 2015;10(2):227-237. doi:10.1177/1745691614568352
19. Distéfano G, Goodpaster BH. Effects of Exercise and Aging on Skeletal Muscle. *Cold Spring Harbor Perspectives in Medicine*. 2017;8(3):a029785. doi:10.1101/cshperspect.a029785
20. Goates S, Du K, Arensberg MB, Gaillard T, Guralnik J, Pereira SL. Economic Impact of Hospitalizations in US Adults with Sarcopenia. *J Frailty Aging*. 2019;8(2):93-99. doi:10.14283/jfa.2019.10
21. Janssen I, Shepard DS, Katzmarzyk PT, Roubenoff R. The healthcare costs of sarcopenia in the United States. *J Am Geriatr Soc*. Jan 2004;52(1):80-5. doi:10.1111/j.1532-5415.2004.52014.x
22. Miljkovic N, Lim JY, Miljkovic I, Frontera WR. Aging of skeletal muscle fibers. *Ann Rehabil Med*. Apr 2015;39(2):155-62. doi:10.5535/arm.2015.39.2.155
23. Gorza L, Sorge M, Seclì L, Brancaccio M. Master Regulators of Muscle Atrophy: Role of Costamere Components. *Cells*. Jan 3 2021;10(1)doi:10.3390/cells10010061
24. Miller KN, Victorelli SG, Salmonowicz H, et al. Cytoplasmic DNA: sources, sensing, and role in aging and disease. *Cell*. Oct 28 2021;184(22):5506-5526. doi:10.1016/j.cell.2021.09.034
25. Akbari M, Shanley DP, Bohr VA, Rasmussen LJ. Cytosolic Self-DNA-A Potential Source of Chronic Inflammation in Aging. *Cells*. Dec 15 2021;10(12)doi:10.3390/cells10123544
26. Decout A, Katz JD, Venkatraman S, Ablasser A. The cGAS-STING pathway as a therapeutic target in inflammatory diseases. *Nat Rev Immunol*. Sep 2021;21(9):548-569. doi:10.1038/s41577-021-00524-z
27. Hopfner KP, Hornung V. Molecular Mechanisms and Cellular Functions of cGAS–STING Signalling. *Nature Reviews Molecular Cell Biology*. 2020;21(9):501-521. doi:10.1038/s41580-020-0244-x
28. Zheng W, Feng D, Xiong X, et al. The Role of cGAS-STING in Age-Related Diseases from Mechanisms to Therapies. *Aging Dis*. Aug 1 2023;14(4):1145-1165. doi:10.14336/ad.2023.0117
29. Skopelja-Gardner S, An J, Elkon KB. Role of the cGAS-STING pathway in systemic and organ-specific diseases. *Nat Rev Nephrol*. Sep 2022;18(9):558-572. doi:10.1038/s41581-022-00589-6
30. Kennedy BK, Berger SL, Brunet A, et al. Geroscience: linking aging to chronic disease. *Cell*. Nov 6 2014;159(4):709-13. doi:10.1016/j.cell.2014.10.039
31. Kerr HL, Krumm K, Anderson B, et al. Mouse sarcopenia model reveals sex- and age-specific differences in phenotypic and molecular characteristics. *J Clin Invest*. Jun 11 2024;134(16)doi:10.1172/jci172890
32. Yılmaz D, Mathavan N, Wehrle E, Kuhn GA, Müller R. Mouse models of accelerated aging in musculoskeletal research for assessing frailty, sarcopenia, and osteoporosis - A review. *Ageing Res Rev*. Jan 2024;93:102118. doi:10.1016/j.arr.2023.102118
33. Santangelo KS, Kaeding AC, Baker SA, Bertone AL. Quantitative Gait Analysis Detects Significant Differences in Movement between Osteoarthritic and Nonosteoarthritic Guinea Pig Strains before and after Treatment with Flunixin Meglumine. *Arthritis*. 2014;2014:503519. doi:10.1155/2014/503519
34. Musci RV, Walsh MA, Konopka AR, et al. The Dunkin Hartley Guinea Pig Is a Model of Primary Osteoarthritis That Also Exhibits Early Onset Myofiber Remodeling That Resembles Human Musculoskeletal Aging. *Front Physiol*. 2020;11:571372. doi:10.3389/fphys.2020.571372
35. Musci RV, Andrie KM, Walsh MA, et al. Phytochemical compound PB125 attenuates skeletal muscle mitochondrial dysfunction and impaired proteostasis in a model of musculoskeletal decline. *J Physiol*. Jun 2023;601(11):2189-2216. doi:10.1113/jp282273

36. Wahl D, Moreno JA, Santangelo KS, et al. Nontransgenic Guinea Pig Strains Exhibit Hallmarks of Human Brain Aging and Alzheimer's Disease. *J Gerontol A Biol Sci Med Sci*. Sep 1 2022;77(9):1766-1774. doi:10.1093/gerona/glac073
37. West KL, Fernandez ML. Guinea pigs as models to study the hypocholesterolemic effects of drugs. *Cardiovasc Drug Rev*. Spring 2004;22(1):55-70. doi:10.1111/j.1527-3466.2004.tb00131.x
38. Podell BK, Ackart DF, Richardson MA, DiLisio JE, Pulford B, Basaraba RJ. A model of type 2 diabetes in the guinea pig using sequential diet-induced glucose intolerance and streptozotocin treatment. *Dis Model Mech*. Feb 1 2017;10(2):151-162. doi:10.1242/dmm.025593
39. Radakovich LB, Marolf AJ, Culver LA, Santangelo KS. Calorie restriction with regular chow, but not a high-fat diet, delays onset of spontaneous osteoarthritis in the Hartley guinea pig model. *Arthritis Res Ther*. Jun 13 2019;21(1):145. doi:10.1186/s13075-019-1925-8
40. Englund DA, Zhang X, Aversa Z, LeBrasseur NK. Skeletal muscle aging, cellular senescence, and senotherapeutics: Current knowledge and future directions. *Mech Ageing Dev*. Dec 2021;200:111595. doi:10.1016/j.mad.2021.111595
41. Granic A, Suetterlin K, Shavlakadze T, Grounds MD, Sayer AA. Hallmarks of ageing in human skeletal muscle and implications for understanding the pathophysiology of sarcopenia in women and men. *Clin Sci (Lond)*. Nov 29 2023;137(22):1721-1751. doi:10.1042/cs20230319
42. Meijer Cd, Wouterse B, Polder JJ, Koopmanschap M. The Effect of Population Aging on Health Expenditure Growth: A Critical Review. *European Journal of Ageing*. 2013;10(4):353-361. doi:10.1007/s10433-013-0280-x
43. Dominguez LJ, Veronese N, Barbagallo M. Dietary Patterns and Healthy or Unhealthy Aging. *Gerontology*. 2024;70(1):15-36. doi:10.1159/000534679
44. Guan Y, Yan Z. Molecular Mechanisms of Exercise and Healthspan. *Cells*. Mar 3 2022;11(5)doi:10.3390/cells11050872
45. Seals DR, Justice JN, LaRocca TJ. Physiological geroscience: targeting function to increase healthspan and achieve optimal longevity. *J Physiol*. Apr 15 2016;594(8):2001-24. doi:10.1113/jphysiol.2014.282665
46. Fischer F, Grigolon G, Benner C, Ristow M. Evolutionarily conserved transcription factors as regulators of longevity and targets for geroprotection. *Physiol Rev*. Jul 1 2022;102(3):1449-1494. doi:10.1152/physrev.00017.2021
47. Rattan SIS, Kaur G. Nutrition, Food and Diet in Health and Longevity: We Eat What We Are. *Nutrients*. Dec 18 2022;14(24)doi:10.3390/nu14245376
48. Bianchi A, Marchetti L, Hall Z, et al. Moderate Exercise Inhibits Age-Related Inflammation, Liver Steatosis, Senescence, and Tumorigenesis. *The Journal of Immunology*. 2021;206(4):904-916. doi:10.4049/jimmunol.2001022
49. Poggioli R, Hirani K, Jogani VG, Ricordi C. Modulation of inflammation and immunity by omega-3 fatty acids: a possible role for prevention and to halt disease progression in autoimmune, viral, and age-related disorders. *Eur Rev Med Pharmacol Sci*. Aug 2023;27(15):7380-7400. doi:10.26355/eurrev_202308_33310
50. Ivancovsky-Wajcman D, Fliss-Isakov N, Webb M, et al. Ultra-processed food is associated with features of metabolic syndrome and non-alcoholic fatty liver disease. *Liver Int*. Nov 2021;41(11):2635-2645. doi:10.1111/liv.14996
51. Batsis JA, Villareal DT. Sarcopenic obesity in older adults: aetiology, epidemiology and treatment strategies. *Nat Rev Endocrinol*. Sep 2018;14(9):513-537. doi:10.1038/s41574-018-0062-9
52. Jura M, Kozak LP. Obesity and related consequences to ageing. *Age (Dordr)*. Feb 2016;38(1):23. doi:10.1007/s11357-016-9884-3

53. Tiwari A, Balasundaram P. Public Health Considerations Regarding Obesity. *StatPearls*. StatPearls Publishing

54. Control CfD. Adult Obesity Facts. Accessed February 10th 2025, 2024. https://www.cdc.gov/obesity/adult-obesity-facts/index.html
55. Simmonds M, Llewellyn A, Owen CG, Woolacott N. Predicting adult obesity from childhood obesity: a systematic review and meta-analysis. *Obes Rev*. Feb 2016;17(2):95-107. doi:10.1111/obr.12334
56. Cesari M, Sumi Y, Han ZA, et al. Implementing care for healthy ageing. *BMJ Glob Health*. Feb 2022;7(2)doi:10.1136/bmjgh-2021-007778
57. Raposo F, Ramos M, Lúcia Cruz A. Effects of exercise on knee osteoarthritis: A systematic review. *Musculoskeletal Care*. Dec 2021;19(4):399-435. doi:10.1002/msc.1538
58. Saleem N, Zahid S, Mahmood T, Ahmed N, Maqsood U, Chaudhary MA. Effect of Pilates based exercises on symptomatic knee osteoarthritis: A Randomized Controlled Trial. *J Pak Med Assoc*. Jan 2022;72(1):8-12. doi:10.47391/jpma.495
59. Sasaki R, Honda Y, Oga S, et al. Effect of exercise and/or educational interventions on physical activity and pain in patients with hip/knee osteoarthritis: A systematic review with meta-analysis. *PLoS One*. 2022;17(11):e0275591. doi:10.1371/journal.pone.0275591
60. Demura T, Demura S, Uchiyama M, Sugiura H. Examination of factors affecting gait properties in healthy older adults: focusing on knee extension strength, visual acuity, and knee joint pain. *J Geriatr Phys Ther*. Apr-Jun 2014;37(2):52-7. doi:10.1519/JPT.0b013e318295daba
61. Alghadir AH, Anwer S, Sarkar B, Paul AK, Anwar D. Effect of 6-week retro or forward walking program on pain, functional disability, quadriceps muscle strength, and performance in individuals with knee osteoarthritis: a randomized controlled trial (retro-walking trial). *BMC Musculoskelet Disord*. Apr 9 2019;20(1):159. doi:10.1186/s12891-019-2537-9
62. Song J, Lindquist LA, Chang RW, et al. Sedentary Behavior as a Risk Factor for Physical Frailty Independent of Moderate Activity: Results From the Osteoarthritis Initiative. *Am J Public Health*. Jul 2015;105(7):1439-45. doi:10.2105/ajph.2014.302540
63. Rahman MS. Health Care in Aged Population: A Global Public Health Challenge. *Journal of Medicine*. 2019;20(2):95-97. doi:10.3329/jom.v20i2.42010
64. Briggs AM, Cross M, Hoy D, et al. Musculoskeletal Health Conditions Represent a Global Threat to Healthy Aging: A Report for the 2015 World Health Organization World Report on Ageing and Health. *The Gerontologist*. 2016;56(Suppl 2):S243-S255. doi:10.1093/geront/gnw002
65. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell*. Jun 6 2013;153(6):1194-217. doi:10.1016/j.cell.2013.05.039
66. Chakravarti D, LaBella KA, DePinho RA. Telomeres: history, health, and hallmarks of aging. *Cell*. Jan 21 2021;184(2):306-322. doi:10.1016/j.cell.2020.12.028
67. Frontera WR. Physiologic Changes of the Musculoskeletal System with Aging: A Brief Review. *Phys Med Rehabil Clin N Am*. Nov 2017;28(4):705-711. doi:10.1016/j.pmr.2017.06.004
68. Damluji AA, Alfaraidhy M, AlHajri N, et al. Sarcopenia and Cardiovascular Diseases. *Circulation*. May 16 2023;147(20):1534-1553. doi:10.1161/circulationaha.123.064071
69. Pacifico J, Geerlings MAJ, Reijnierse EM, Phassouliotis C, Lim WK, Maier AB. Prevalence of sarcopenia as a comorbid disease: A systematic review and meta-analysis. *Exp Gerontol*. Mar 2020;131:110801. doi:10.1016/j.exger.2019.110801
70. Sartori R, Romanello V, Sandri M. Mechanisms of muscle atrophy and hypertrophy: implications in health and disease. *Nat Commun*. Jan 12 2021;12(1):330. doi:10.1038/s41467-020-20123-1

71. Dennison E, Sayer AA, Cooper C. Epidemiology of Sarcopenia and Insight Into Possible Therapeutic Targets. *Nature Reviews Rheumatology*. 2017;13(6):340-347. doi:10.1038/nrrheum.2017.60
72. Patel KV, Guralnik JM, Dansie EJ, Turk DC. Prevalence and Impact of Pain Among Older Adults in the United States: Findings From the 2011 National Health and Aging Trends Study. *Pain*. 2013;154(12):2649-2657. doi:10.1016/j.pain.2013.07.029
73. Shamsi M, Safari A, Samadzadeh S, Yoosefpour N. The prevalence of musculoskeletal pain among above 50-year-old population referred to the Kermanshah-Iran health bus in 2016. *BMC Res Notes*. Feb 12 2020;13(1):72. doi:10.1186/s13104-020-4940-6
74. Jia S, Zhao W, Hu F, et al. Sex differences in the association of physical activity levels and vitamin D with obesity, sarcopenia, and sarcopenic obesity: a cross-sectional study. *BMC Geriatr*. Nov 24 2022;22(1):898. doi:10.1186/s12877-022-03577-4
75. Wang YXJ. Menopause as a potential cause for higher prevalence of low back pain in women than in age-matched men. *J Orthop Translat*. Jan 2017;8:1-4. doi:10.1016/j.jot.2016.05.012
76. Segal NA, Nilges JM, Oo WM. Sex differences in osteoarthritis prevalence, pain perception, physical function and therapeutics. *Osteoarthritis Cartilage*. Sep 2024;32(9):1045-1053. doi:10.1016/j.joca.2024.04.002
77. Zheng ZH, Wang JJ, Lin JG, et al. Cytosolic DNA initiates a vicious circle of aging-related endothelial inflammation and mitochondrial dysfunction via STING: the inhibitory effect of Cilostazol. *Acta Pharmacol Sin*. Sep 2024;45(9):1879-1897. doi:10.1038/s41401-024-01281-0
78. Zhang W, Li G, Luo R, et al. Cytosolic escape of mitochondrial DNA triggers cGAS-STING-NLRP3 axis-dependent nucleus pulposus cell pyroptosis. *Exp Mol Med*. Feb 2022;54(2):129-142. doi:10.1038/s12276-022-00729-9
79. Guo Y, Gu R, Gan D, Hu F, Li G, Xu G. Mitochondrial DNA drives noncanonical inflammation activation via cGAS-STING signaling pathway in retinal microvascular endothelial cells. *Cell Commun Signal*. Oct 28 2020;18(1):172. doi:10.1186/s12964-020-00637-3
80. Holloway GP, Holwerda AM, Miotto PM, Dirks ML, Verdijk LB, van Loon LJC. Age-Associated Impairments in Mitochondrial ADP Sensitivity Contribute to Redox Stress in Senescent Human Skeletal Muscle. *Cell Rep*. Mar 13 2018;22(11):2837-2848. doi:10.1016/j.celrep.2018.02.069
81. Franceschi C, Garagnani P, Parini P, Giuliani C, Santoro A. Inflammaging: A New Immune–metabolic Viewpoint for Age-Related Diseases. *Nature Reviews Endocrinology*. 2018;14(10):576-590. doi:10.1038/s41574-018-0059-4
82. Liberale L, Montecucco F, Tardif JC, Libby P, Camici GG. Inflamm-Ageing: The Role of Inflammation in Age-Dependent Cardiovascular Disease. *European Heart Journal*. 2020;41(31):2974-2982. doi:10.1093/eurheartj/ehz961
83. Rezuş E, Burlui A, Cardoneanu A, et al. Inactivity and Skeletal Muscle Metabolism: A Vicious Cycle in Old Age. *Int J Mol Sci*. Jan 16 2020;21(2)doi:10.3390/ijms21020592
84. Deschenes MR. Effects of aging on muscle fibre type and size. *Sports Med*. 2004;34(12):809-24. doi:10.2165/00007256-200434120-00002
85. Spendiff S, Vuda M, Gouspillou G, et al. Denervation drives mitochondrial dysfunction in skeletal muscle of octogenarians. *J Physiol*. Dec 15 2016;594(24):7361-7379. doi:10.1113/JP272487
86. Jimenez-Gutierrez GE, Martínez-Gómez LE, Martínez-Armenta C, Pineda C, Martínez-Nava GA, Lopez-Reyes A. Molecular Mechanisms of Inflammation in Sarcopenia: Diagnosis and Therapeutic Update. *Cells*. Aug 1 2022;11(15)doi:10.3390/cells11152359
87. Duewell P, Kono H, Rayner KJ, et al. NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals. *Nature*. Apr 29 2010;464(7293):1357-61. doi:10.1038/nature08938

88. Chen Y, Ye X, Escames G, et al. The NLRP3 inflammasome: contributions to inflammation-related diseases. *Cell Mol Biol Lett*. Jun 27 2023;28(1):51. doi:10.1186/s11658-023-00462-9
89. Liu Y, Wang Y, Yan P, et al. NLRP3 Inflammasome-Mediated Osteoarthritis: The Role of Epigenetics. *Biology (Basel)*. Jan 14 2025;14(1)doi:10.3390/biology14010071
90. Liu Y, Bi X, Zhang Y, Wang Y, Ding W. Mitochondrial dysfunction/NLRP3 inflammasome axis contributes to angiotensin II-induced skeletal muscle wasting via PPAR-γ. *Lab Invest*. May 2020;100(5):712-726. doi:10.1038/s41374-019-0355-1
91. McBride MJ, Foley KP, D'Souza DM, et al. The NLRP3 inflammasome contributes to sarcopenia and lower muscle glycolytic potential in old mice. *Am J Physiol Endocrinol Metab*. Aug 1 2017;313(2):E222-e232. doi:10.1152/ajpendo.00060.2017
92. Bian AL, Hu HY, Rong YD, Wang J, Wang JX, Zhou XZ. A study on relationship between elderly sarcopenia and inflammatory factors IL-6 and TNF-α. *Eur J Med Res*. Jul 12 2017;22(1):25. doi:10.1186/s40001-017-0266-9
93. da Costa Teixeira LA, Avelar NCP, Peixoto MFD, et al. Inflammatory biomarkers at different stages of Sarcopenia in older women. *Sci Rep*. Jun 26 2023;13(1):10367. doi:10.1038/s41598-023-37229-3
94. Kufer TA, Nigro G, Sansonetti PJ. Multifaceted Functions of NOD-Like Receptor Proteins in Myeloid Cells at the Intersection of Innate and Adaptive Immunity. *Microbiol Spectr*. Aug 2016;4(4)doi:10.1128/microbiolspec.MCHD-0021-2015
95. Tyrrell DJ, Blin MG, Song J, et al. Age-Associated Mitochondrial Dysfunction Accelerates Atherogenesis. *Circ Res*. Jan 31 2020;126(3):298-314. doi:10.1161/circresaha.119.315644
96. Shorter E, Sannicandro AJ, Poulet B, Goljanek-Whysall K. Skeletal Muscle Wasting and Its Relationship With Osteoarthritis: a Mini-Review of Mechanisms and Current Interventions. *Curr Rheumatol Rep*. Jun 15 2019;21(8):40. doi:10.1007/s11926-019-0839-4
97. Sharma R. Exploring the Emerging Bidirectional Association Between Inflamm-aging and Cellular Senescence in Organismal Aging and Disease. *Cell Biochemistry and Function*. 2024;42(2)doi:10.1002/cbf.3970
98. Mobasheri A, Matta C, Zákány R, Musumeci G. Chondrosenescence: Definition, Hallmarks and Potential Role in the Pathogenesis of Osteoarthritis. *Maturitas*. 2015;80(3):237-244. doi:10.1016/j.maturitas.2014.12.003
99. Zhang X, Habiballa L, Aversa Z, et al. Characterization of cellular senescence in aging skeletal muscle. *Nat Aging*. Jul 2022;2(7):601-615. doi:10.1038/s43587-022-00250-8
100. Ogrodnik M. Cellular aging beyond cellular senescence: Markers of senescence prior to cell cycle arrest in vitro and in vivo. *Aging Cell*. Apr 2021;20(4):e13338. doi:10.1111/acel.13338
101. Yu Q, Katlinskaya Y, Carbone CJ, et al. DNA-Damage-Induced Type I Interferon Promotes Senescence and Inhibits Stem Cell Function. *Cell Reports*. 2015;11(5):785-797. doi:10.1016/j.celrep.2015.03.069
102. Increase of Cytosolic Double-Stranded DNA in Nucleus Pulposus Cell Accelerates Intervertebral Disc Degeneration via Activation of cGAS-STING Signaling. 2023;doi:10.21203/rs.3.rs-81111/v1
103. Childs BG, Gluscevic M, Baker DJ, et al. Senescent Cells: An Emerging Target for Diseases of Ageing. *Nature Reviews Cancer*. 2017;16(10):718-735. doi:10.1038/nrd.2017.116
104. Liu Y, Schwam J, Chen Q. Senescence-Associated Cell Transition and Interaction (SACTAI): A Proposed Mechanism for Tissue Aging, Repair, and Degeneration. *Cells*. 2022;11(7):1089. doi:10.3390/cells11071089
105. Gerdes EOW, Zhu Y, Weigand BM, et al. Cellular Senescence in Aging and Age-Related Diseases: Implications for Neurodegenerative Diseases. 2020:203-234. doi:10.1016/bs.irn.2020.03.019

106. Baar MP, Perdiguero E, Muñoz-Cánoves P, de Keizer PL. Musculoskeletal senescence: a moving target ready to be eliminated. *Curr Opin Pharmacol*. Jun 2018;40:147-155. doi:10.1016/j.coph.2018.05.007
107. Siddiqui MS, François M, Fenech MF, Leifert WR. Persistent γH2AX: A promising molecular marker of DNA damage and aging. *Mutat Res Rev Mutat Res*. Oct-Dec 2015;766:1-19. doi:10.1016/j.mrrev.2015.07.001
108. Dungan CM, Peck BD, Walton RG, et al. In vivo analysis of γH2AX+ cells in skeletal muscle from aged and obese humans. *Faseb j*. May 2020;34(5):7018-7035. doi:10.1096/fj.202000111RR
109. Welle S, Brooks AI, Delehanty JM, et al. Skeletal muscle gene expression profiles in 20-29 year old and 65-71 year old women. *Exp Gerontol*. Mar 2004;39(3):369-77. doi:10.1016/j.exger.2003.11.011
110. Wu Z, Lin Y, Liu L, et al. Identification of Cytosolic DNA Sensor cGAS-STING as Immune-Related Risk Factor in Renal Carcinoma Following Pan-Cancer Analysis. *Journal of Immunology Research*. 2022;2022:1-11. doi:10.1155/2022/7978042
111. Oduro PK, Zheng X, Wei J, et al. The cGAS-STING signaling in cardiovascular and metabolic diseases: Future novel target option for pharmacotherapy. *Acta Pharm Sin B*. Jan 2022;12(1):50-75. doi:10.1016/j.apsb.2021.05.011
112. Li Y, Cui J, Liu L, et al. mtDNA release promotes cGAS-STING activation and accelerated aging of postmitotic muscle cells. *Cell Death Dis*. Jul 23 2024;15(7):523. doi:10.1038/s41419-024-06863-8
113. Zheng W, Xia N, Zhang J, et al. How the Innate Immune DNA Sensing cGAS-STING Pathway Is Involved in Autophagy. *Int J Mol Sci*. Dec 8 2021;22(24)doi:10.3390/ijms222413232
114. Kong LZ, Kim SM, Wang C, et al. Understanding nucleic acid sensing and its therapeutic applications. *Exp Mol Med*. Nov 2023;55(11):2320-2331. doi:10.1038/s12276-023-01118-6
115. Antiochos B, Trejo-Zambrano D, Fenaroli P, et al. The DNA sensors AIM2 and IFI16 are SLE autoantigens that bind neutrophil extracellular traps. *Elife*. May 24 2022;11doi:10.7554/eLife.72103
116. Hornung V, Ablasser A, Charrel-Dennis M, et al. AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature*. Mar 26 2009;458(7237):514-8. doi:10.1038/nature07725
117. Basit A, Cho M-G, Kim EY, Kwon D-H, Kang S-W, Lee JH. The cGAS/STING/TBK1/IRF3 Innate Immunity Pathway Maintains Chromosomal Stability Through Regulation of P21 Levels. *Experimental & Molecular Medicine*. 2020;52(4):643-657. doi:10.1038/s12276-020-0416-y
118. Dou Z, Ghosh K, Vizioli MG, et al. Cytoplasmic chromatin triggers inflammation in senescence and cancer. *Nature*. Oct 19 2017;550(7676):402-406. doi:10.1038/nature24050
119. Shah PP, Donahue G, Otte GL, et al. Lamin B1 depletion in senescent cells triggers large-scale changes in gene expression and the chromatin landscape. *Genes Dev*. Aug 15 2013;27(16):1787-99. doi:10.1101/gad.223834.113
120. Vizioli MG, Liu T, Miller K, et al. Mitochondria-to-Nucleus Retrograde Signaling Drives Formation of Cytoplasmic Chromatin and Inflammation in Senescence. *Genes & Development*. 2020;34(5-6):428-445. doi:10.1101/gad.331272.119
121. Hela F. Interaction Between Autophagy and Senescence in Pancreatic Beta Cells. *Biology*. 2023;12(9):1205. doi:10.3390/biology12091205
122. Freund A, Laberge RM, Demaria M, Campisi J. Lamin B1 loss is a senescence-associated biomarker. *Mol Biol Cell*. Jun 2012;23(11):2066-75. doi:10.1091/mbc.E11-10-0884
123. Rodríguez-Nuevo A, Díaz-Ramos A, Noguera E, et al. Mitochondrial DNA and TLR9 drive muscle inflammation upon Opa1 deficiency. *Embo j*. May 15 2018;37(10)doi:10.15252/embj.201796553

124. Hayward A, Gilbert C. Transposable elements. *Curr Biol*. Sep 12 2022;32(17):R904-r909. doi:10.1016/j.cub.2022.07.044
125. Hancks DC, Kazazian HH, Jr. Roles for retrotransposon insertions in human disease. *Mob DNA*. 2016;7:9. doi:10.1186/s13100-016-0065-9
126. De Cecco M, Ito T, Petrashen AP, et al. L1 drives IFN in senescent cells and promotes age-associated inflammation. *Nature*. Feb 2019;566(7742):73-78. doi:10.1038/s41586-018-0784-9
127. De Cecco M, Criscione SW, Peckham EJ, et al. Genomes of replicatively senescent cells undergo global epigenetic changes leading to gene silencing and activation of transposable elements. *Aging Cell*. Apr 2013;12(2):247-56. doi:10.1111/acel.12047
128. Shah S, Yu S, Zhang C, et al. Retrotransposon SINEs in age-related diseases: Mechanisms and therapeutic implications. *Ageing Res Rev*. Nov 2024;101:102539. doi:10.1016/j.arr.2024.102539
129. Sakai C. A Possible Role for Proinflammatory Activation via cGAS-STING Pathway in Atherosclerosis Induced by Accumulation of DNA Double-Strand Breaks. *Scientific Reports*. 2023;13(1)doi:10.1038/s41598-023-43848-7
130. Loo TM, Miyata K, Tanaka Y, Takahashi A. Cellular Senescence and Senescence-associated Secretory Phenotype via the cGAS-STING Signaling Pathway in Cancer. *Cancer Science*. 2019;111(2):304-311. doi:10.1111/cas.14266
131. Bedrosian TA, Houtman J, Eguiguren JS, et al. Lamin B1 decline underlies age-related loss of adult hippocampal neurogenesis. *Embo j*. Feb 1 2021;40(3):e105819. doi:10.15252/embj.2020105819
132. Glück S, Guey B, Gulen MF, et al. Innate Immune Sensing of Cytosolic Chromatin Fragments Through cGAS Promotes Senescence. *Nature Cell Biology*. 2017;19(9):1061-1070. doi:10.1038/ncb3586
133. Shreeya T, Ansari MS, Kumar P, et al. Senescence: A DNA damage response and its role in aging and Neurodegenerative Diseases. *Front Aging*. 2023;4:1292053. doi:10.3389/fragi.2023.1292053
134. White RR, Vijg J. Do DNA Double-Strand Breaks Drive Aging? *Mol Cell*. Sep 1 2016;63(5):729-38. doi:10.1016/j.molcel.2016.08.004
135. Paul BD, Snyder SH, Bohr VA. Signaling by cGAS-STING in Neurodegeneration, Neuroinflammation, and Aging. *Trends Neurosci*. Feb 2021;44(2):83-96. doi:10.1016/j.tins.2020.10.008
136. Yu CH, Davidson S, Harapas CR, et al. TDP-43 Triggers Mitochondrial DNA Release via mPTP to Activate cGAS/STING in ALS. *Cell*. Oct 29 2020;183(3):636-649.e18. doi:10.1016/j.cell.2020.09.020
137. Gulen MF, Samson N, Keller A, et al. cGAS–STING Drives Ageing-Related Inflammation and Neurodegeneration. *Nature*. 2023;620(7973):374-380. doi:10.1038/s41586-023-06373-1
138. Nilwik R, Snijders T, Leenders M, et al. The decline in skeletal muscle mass with aging is mainly attributed to a reduction in type II muscle fiber size. *Exp Gerontol*. May 2013;48(5):492-8. doi:10.1016/j.exger.2013.02.012
139. Drake JC, Yan Z. Mitophagy in maintaining skeletal muscle mitochondrial proteostasis and metabolic health with ageing. *J Physiol*. Oct 15 2017;595(20):6391-6399. doi:10.1113/jp274337
140. Kedlian VR, Wang Y, Liu T, et al. Human skeletal muscle aging atlas. *Nat Aging*. May 2024;4(5):727-744. doi:10.1038/s43587-024-00613-3
141. Caramés B, Taniguchi N, Otsuki S, Blanco FJ, Lotz M. Autophagy Is a Protective Mechanism in Normal Cartilage, and Its Aging-related Loss Is Linked With Cell Death and Osteoarthritis. *Arthritis & Rheumatism*. 2010;62(3):791-801. doi:10.1002/art.27305

142. Krieglstein J, Kewitz T, Kirchhefer U, et al. Damage of guinea pig heart and arteries by a trioleate-enriched diet and of cultured cardiomyocytes by oleic acid. *PLoS One*. Mar 8 2010;5(3):e9561. doi:10.1371/journal.pone.0009561
143. Eisener-Dorman AF, Lawrence DA, Bolivar VJ. Cautionary insights on knockout mouse studies: the gene or not the gene? *Brain Behav Immun*. Mar 2009;23(3):318-24. doi:10.1016/j.bbi.2008.09.001
144. Dai Z, Ji H, Zheng A, Huang AL, Tang KF. Protocol for isolating small cytosolic dsDNA from cultured murine cells. *STAR Protoc*. Jun 21 2024;5(2):102998. doi:10.1016/j.xpro.2024.102998
145. Drake JC, Peelor FF, Biela LM, Miller RA, Hamilton KL, Miller BF. Assessment of protein synthesis and cellular proliferation in long-lived crowded litter mice. *The FASEB Journal*. 2013;27(S1):1202.25-1202.25. doi:https://doi.org/10.1096/fasebj.27.1_supplement.1202.25
146. Platt RN, 2nd, Vandewege MW, Ray DA. Mammalian transposable elements and their impacts on genome evolution. *Chromosome Res*. Mar 2018;26(1-2):25-43. doi:10.1007/s10577-017-9570-z
147. Liu H, Zhuang H, Zeng L, Xie J, Qiu K, Feng D. A protocol for detecting the cytoplasm-exposed mitochondrial DNA. *Mitochondrial Communications*. 2024/01/01/ 2024;2:100-106. doi:https://doi.org/10.1016/j.mitoco.2024.09.001
148. Watson RO, Manzanillo PS, Cox JS. Extracellular M. tuberculosis DNA targets bacteria for autophagy by activating the host DNA-sensing pathway. *Cell*. Aug 17 2012;150(4):803-15. doi:10.1016/j.cell.2012.06.040
149. Liang Q, Seo GJ, Choi YJ, et al. Crosstalk between the cGAS DNA sensor and Beclin-1 autophagy protein shapes innate antimicrobial immune responses. *Cell Host Microbe*. Feb 12 2014;15(2):228-38. doi:10.1016/j.chom.2014.01.009
150. Shen YJ, Le Bert N, Chitre AA, et al. Genome-derived cytosolic DNA mediates type I interferon-dependent rejection of B cell lymphoma cells. *Cell Rep*. Apr 21 2015;11(3):460-73. doi:10.1016/j.celrep.2015.03.041
151. Tsering T, Nadeau A, Wu T, Dickinson K, Burnier JV. Extracellular vesicle-associated DNA: ten years since its discovery in human blood. *Cell Death Dis*. Sep 12 2024;15(9):668. doi:10.1038/s41419-024-07003-y
152. Yokoi A, Villar-Prados A, Oliphint PA, et al. Mechanisms of nuclear content loading to exosomes. *Sci Adv*. Nov 2019;5(11):eaax8849. doi:10.1126/sciadv.aax8849
153. Takahashi A, Okada R, Nagao K, et al. Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun*. May 16 2017;8:15287. doi:10.1038/ncomms15287
154. Rottenberg H, Hoek JB. The Mitochondrial Permeability Transition: Nexus of Aging, Disease and Longevity. *Cells*. Jan 6 2021;10(1)doi:10.3390/cells10010079
155. Ferrucci L, Kuchel GA. Heterogeneity of Aging: Individual Risk Factors, Mechanisms, Patient Priorities, and Outcomes. *J Am Geriatr Soc*. Mar 2021;69(3):610-612. doi:10.1111/jgs.17011